MOLECULAR CHARACTERIZATION AND FUNCTIONAL EVALUATION OF RECOMBINANT CONGLUTININ OF BUFFALO (*Bubalus bubalis*) AND NILGAI (*Boselaphus tragocamelus*)

THESIS

Submitted in partial fulfilment of the requirements for the degree
of
Doctor of Philosophy
in
ANIMAL BIOCHEMISTRY

By
Dr. Ramesh D.
Roll No. 1229

To
DEEMED UNIVERSITY
INDIAN VETERINARY RESEARCH INSTITUTE
IZATNAGAR - 243 122 (U.P.)

2012

Dedicated to....

My Beloved Parents

&

Guide

भारतीय पशु चिकित्सा अनुसंधान संस्थान
(सम विश्वविद्यालय)

DIVISION OF ANIMAL BIOCHEMISTRY
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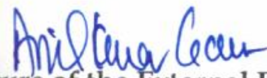

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Date : 6/8/2012
Place : IZATNAGAR

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Abbreviations

%	-	Percentage
A	-	Absorbance
aa	-	Amino acid
BuCGN	-	Buffalo conglutinin
bp	-	Base pair
Ca^{+2}	-	Calcium
CD	-	Cluster of Differentiation
cDNA	-	Complementary Deoxy ribonucleic acid
CGN	-	Conglutinin
CL	-	Collectin
CTLDs	-	C-type lectin-like domains
DNA	-	Deoxy ribonucleic acid
dNTP	-	Deoxy nucleotide triphosphate
ds RNA	-	Double stranded ribonucleic acid
DW	-	Distilled Water.
EDTA	-	Ethylene diamine tetra acetic acid
ELISA	-	Enzyme Linked Immuno Sorbent Assay
Etbr	-	Ethidium bromide
Fig	-	Figure
h/hr	-	Hour(s)
HI	-	Haemagglutination inhibition
HIV	-	Human Immuno Deficiency Virus
HRPO	-	Horse Radish Peroxidase
HSV	-	Haemorrhagic Septicaemia Virus
IFN	-	Interferon
IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
IVRI	-	Indian Veterinary Research Institute
Kb	-	Kilo base
kDa	-	Kilo Dalton
Kg	-	Kilo gram
L.B	-	Luria Bertini

LPS	-	Lipopolysachharide
M	-	Molar
MBL	-	Mannan Binding Lectin
MBP	-	Mannose Binding Protein
$MgCl_2$	-	Magnesium Chloride
Ml	-	Millilitre
Mol.Wt.	-	Molecular weight
mRNA	-	Messenger Ribonucleic acid
NAGA	-	N acetyl glucosamine
NCBI	-	National Centre for Biological Information
NCDV	-	Nebraska calf diarrhoea virus
NCRD	-	Neck and Carbohydrate Recognition Domain
ng	-	Nanogram
NCGN	-	Nilgai conglutinin
NOD	-	Nucleotide binding Oligomerisation Domain
OD	-	Optical Density
PAMP	-	Pathogen associated Molecular Pattern
PBS	-	Phosphate Buffered Saline
Pc-Lec	-	*Procambarus clarkia* Lectin
PCR	-	Polymerase Chain Reaction
pmol	-	Picomole
PRRs	-	Pattern Recognition Receptors
RE	-	Restriction Enzyme
ROI	-	Reactive Oxygen Intermediates
Rpm	-	Revolutions per minute
rSCGN	-	Recombinant Sheep Conglutinin
RT	-	Room Temperature
RT-PCR	-	Reverse Transcription Polymerase Reaction.
rBuCGN	-	Recombinant buffalo conglutinin
rNCGN	-	Recombinant nilgai conglutinin
SDS-PAGE	-	Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis
sec	-	Second(s)
SP	-	Surfactant Protein

TAE	-	Tris Acetate EDTA buffer
Taq Pol.	-	*Thermus aquaticus* Polymerase
TBS	-	Tris buffered saline
TLR	-	Toll-like Receptor
UV	-	Ultra violet
V	-	Volts
µg	-	Microgram
µl	-	Microliter
µm	-	Micrometre
µM	-	Micro Molar

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Introduction

Introduction

An efficient defence mechanism is necessary for the successful survival of an animal and so the immune system is equipped with a variety of molecules and cells at different levels to protect the animal. The mammalian innate immune system provides the first line defence against potential pathogens, bridging the interval between exposure to the pathogen and the specific response of the adaptive immune system. Components of the innate immune system include epithelial barrier, phagocytic barrier, complement proteins etc. Adaptive immune system consists of B cells and T cells. Both systems rely on the ability to distinguish self from non-self and this is affected through specific receptors. Antibodies and T-cell receptors are the receptors of adaptive immunity. They recognize details of molecular structure of complex and relatively large molecules and can discriminate with high specificity between antigens possessing only slight structural differences. On the other hand, the receptors of innate immunity recognize broad structural motifs that are highly conserved within microbial species but are generally absent from the host. These receptors are called the pattern recognition receptors.

Pattern recognition receptors can be membrane bound or soluble. The TLRs, NOD like receptors, scavenger receptors etc. are membrane bound PRRs (Myeong and Young-Joon, 2007). Soluble PRRs include collectins, which are secreted into the extracellular space. To date, several different types of collectins have been identified

such as mannan binding lectin (MBL), conglutinin, collectin-43 (CL-43) and collectin-46 (CL-46). They are all serum proteins, which are synthesized mainly in the liver (Liou *et al.*, 1994). However, the latter three proteins have only been identified in animals of *Bovidae* family. It is interesting to note that no reasons are known for the absence of conglutinin in the sera of herbivorous animals other than ruminants, like the horses that also live in symbiosis with enormous numbers of bacteria present in their caecum but recently the presence of conglutinin with same antigenic specificity and functional similarity but with minor differences as that of bovine conglutinin has been confirmed in animals not coming under bovidae like horse, pig and rabbit, some wild animals and fishes (Kania *et al.*, 2010).

Bovine conglutinin which is a member of the collectin family of proteins is composed of C-type lectin domains connected to collagen-like regions. Conglutinin binds microorganisms either directly or via the complement degradation product iC3b through the lectin domains. This binding leads to agglutination of the microorganisms which in turn promotes phagocytosis, and thus conglutinin plays an important role in the bovine innate immune defence system (Holmskov *et al.* 1998). Functional aspects of conglutinin isolated from bovine serum have been studied as early as 1959 (Ingram, 1959) and the protein has been characterized in 1967 (Lachmann, 1967). The protein was shown to possess antimicrobial, antiviral, antiparasitic and antifungal activities (Brummer and Stevens, 2010). In fact, serum conglutinin level was found to be a heritable trait which could be used for selection for disease resistance (Holmskov *et al.* 1998). Bovine conglutinin in cattle (*Bos taurus*) is well-characterized known to play a pivotal role in bovine immune system (Œliwa-Dominiak *et al.*, 2010). Recent finding suggests bovine conglutinin has been found to stimulate respiratory burst in bovine peripheral granulocytes to produce reactive

oxygen intermediates having microbicidal activity in the presence of sugar like yeast mannan and also show down regulation of ROI production in the absence of sugar (Dec *et al.*, 2012).

Buffalo is distributed in India, Bangladesh, Pakistan, Nepal, Bhutan, and Thailand. All the domestic varieties and breeds descend from one common ancestor, the wild water buffalo (*Bubalus arnee*), which is now an endangered species (IUCN 2012). Buffaloes contribute about 15% of the total world milk supply. In India, *Bubalus bubalis*, the Indian domestic buffalo is used for draft, meat and dairy purpose. Nilgai is found in the north Indian plains from the base of the Himalayas in the north, down to the state of Karnataka in the South, and all along the entire eastern length of Pakistan and over across the border of Rajasthan in the West to the states of Assam and West Bengal in the East. In Nepal, they occur patchily in the southern lowlands. Nilgai with stable population has been mentioned as Least Concern in IUCN Red List of Threatened Species (Mallon, 2008, IUCN, 2012). It has been reported that serum level of conglutinin is positively correlated to disease resistance in exotic cattle. But no reports on conglutinin are available in indigenous ruminant like Indian buffalo or wild ruminant like nilgai which are thought to possess better resistance.

Comparative evaluation of the membrane bound TLR2, TLR3 and TLR4 expression in buffalo and nilgai (Das *et al.*, 2008; Dhara *et al.*, 2007) and TLR 9 in goat and blackbuck (Singh, 2008; Doreswamy *et al.*, 2010) indicated stronger innate immunity in wild ruminants. An investigation of conglutinin as soluble PRR in buffalo and nilgai is likely to provide further insight into hypothesis. A sandwich ELISA using native conglutinin purified from serum as antigen has been developed to assess serum conglutinin level in *Bos taurus* (Akiyama

et al., 1992). Recombinant partial conglutinin composed of the neck and carbohydrate binding domain (NCRD) has been expressed in prokaryotic system and shown to possess activities similar to native conglutinin *in vitro* (Wakamiya, 2005). The possibility of standardizing sandwich ELISA using recombinant protein for assessing serum conglutinin level has also to be explored. In this background, this work was undertaken with the following objectives

Objectives

1. Cloning and characterization of cDNA encoding conglutinin (NCRD) of buffalo and nilgai
2. Production of recombinant conglutinin in prokaryotic system
3. Functional characterization of recombinant conglutinin
4. Standardization of ELISA using recombinant antigen to assess the serum level of conglutinin.

Review of Literature

Review of Literature

2.1 Innate immunity markers

The vertebrate innate immune system recognizes and responds to variety of ligands containing molecular patterns known as pathogen-associated molecular patterns (PAMPs) that are indicative of pathogenic microorganisms. These PAMPs are unique to the type of microorganism that are being encountered and includes bacterial lipopolysaccharides (LPS), peptidoglycan, lypopeptides, flagellin, unmethylated CpG DNA sequence etc. Numerous PRRs that are distributed either in cellular compartments of immune cells like membrane and cytoplasm or those present in the extracellular milieu, are commonly referred to as innate immune markers. These markers include Toll-like receptors, C-type Lectin receptors, Scavenger receptors, Complement receptors, IFN-inducible proteins, CARD helicases, NOD-like receptors, Complements, Pentraxins and Collectins (Myeong and Young-Joon, 2007). The innate immune system is known to respond to the recognition of PAMPs by the appropriate PRRs resulting in the induction of downstream signals aimed at warding off the microbes (Medzhitov and Janeway 2000).

2.2 C-type lectins

C-type lectins were among the first animal lectins to be discovered. C-type lectins are extracellular, Ca^{2+} dependent

carbohydrate binding proteins containing a conserved carbohydrate recognition domain (Zelensky and Gready, 2005). The C-type lectin superfamily is a large group of proteins that are characterized by the presence of one or more C-type lectin-like domains (CTLDs). The latest addition to the C type lectin superfamily is the Pc-Lec1 reported in freshwater crayfish *Procambarus clarkia* (Zhang *et al.*, 2011). Bovine conglutinin, which belongs to the collectin group of C-type lectins, has been known since 1906. Till date, nine different collectins have been identified: mannose-binding protein (MBP), conglutinin, surfactant proteins SP-A and SP-D, and collectins CL-43, CL-46, CL-P1, CL-L1, and CL-K1. The collectins MBP, conglutinin, CL-43, CL-46, CL-K1, SP-A, and SP-D are soluble, whereas CL-L1 and CL-P1 are membrane proteins (Kawai *et al.*, 2002). Despite the presence of a highly conserved domain, C-type lectins are functionally diverse and have been implicated in various processes like they promote chemotaxis, stimulate the production of cytokines and reactive oxygen species by immune cells, in addition cell adhesion, tissue integration and remodelling, platelet activation, complement activation, pathogen recognition, endocytosis and phagocytosis.

2.3 Bovine Conglutinin

Ehrlich and Sachs in 1902 reported for the first time the heat-stable protein which was present in normal bovine serum for its activity in aggregating erythrocytes (Ehrlich and Sachs, 1902). The name conglutinin was given by Bordet and Streng in 1909 as it depends on complement for its action in aggregating erythrocytes. The presence of conglutinin was intially detected in only in ruminants, such as cattle, dromedary, *Kobus ellipsiprymnus*, kobus from Uganda, kobus from Kenia, dikdik antelope (*Madoqua saltiana*), *Damaliscus lunatus* and the

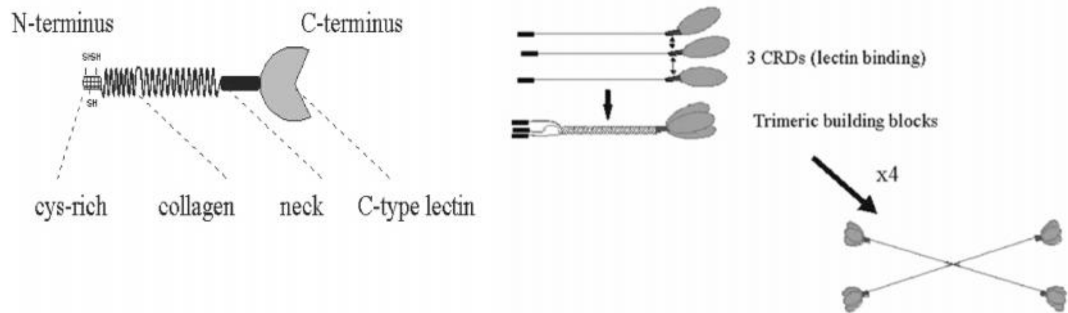
hartebeest (*Alcelaphus buselaphus*). In hartebeest the serum conglutinin level was high (Kakoma and Kinyanjuni, 1974; Dec and Wernicki, 2006).

The gene encoding conglutinin is located on the bovine chromosome 28, and phylogenetic analysis indicates its common origin-from the lung surfactant protein D gene. The 1519 bp cDNA encoding bovine conglutinin from liver has been cloned and characterized. The open reading frame is 1116bp and it encodes a protein of 371 amino acids, of which the first twenty constitute the signal sequence, the mature peptide has 351 amino acids (Liou *et al.*, 1994). The chromosomal localization of conglutinin gene in river buffalo has been done using sequential RBH-banding (R-banding by late incorporation of BrdU and Hoechst 33258 followed by Hoechst fluorochrome staining) and Fluorescent *in situ* hybridization (FISH) by Iannuzzi *et al.*, 1994. Wakamiya (2005) obtained a patent on the production of recombinant bovine conglutinin in prokaryotic system (US patent 6979727 B2).

2.3.1 Structure of bovine conglutinin

Serum bovine conglutinin is a multimeric molecule composed of four structural units each composed of three identical disulphide-linked polypeptides. The relative molecular mass (Mr) of serum bovine conglutinin is more than 10^6 Da. On SDS-PAGE, the single peptide shows Mr of 43,000 Da under reducing conditions. The monomer consists of an N-terminal cysteine-rich domain, a collagen domain, a coiled-coil neck domain and finally a C-type lectin domain, also known as carbohydrate recognition domain (CRD). The neck region of the conglutinin appears to participate in stabilizing the trimer through formation of a three-stranded coiled coil structure. Both collagenous domain and carbohydrate

recognition domain (CRD) possess sequence homology to other C-type lectins (Hoppe *et al.*, 1994).



(Hickling *et al.* 2004)

2.3.2 Recognition of PAMPs by bovine conglutinin

The monosaccharide or disaccharide moieties of specific surface glycol-conjugates involved in recognition and binding to innate immune receptors varies among the pathogenic microorganisms. The selection of its ligands is based mainly on the positioning of two vicinal hydroxyl groups, which form two coordination bonds with ligated calcium, four hydrogen bonds with calcium ligands and a single apolar van der waals contact. C-type lectins, to which the collectins belong, can be divided into mannose/ glucose-type or galactose-type, based on relative monosaccharide specificity, and the bovine conglutinin is categorized under mannose type lectins. The molecular basis on which CRDs discriminate between mannose- and galactose-type ligands lies in the presentation of two vicinal hydroxyl groups on the 3- and 4-position of the sugar ring of hexoses. For ligand binding in mannose-type CRDs, these hydroxyl groups need to have an equatorial position, whereas for high affinity binding by galactose-type CRDs, they have to be placed axially. The fucose is bound by mannose-type CRDs in a slightly different manner, as this molecule has equatorial hydroxyl groups on its 2- and 3- positions of the sugar ring which, in molecular models, superimpose on the hydroxyl groups on the 3- and 4- position

of the sugar ring of mannose (Van de wetering *et al.*, 2004). The sugar binding activity of the bovine conglutinin in their increasing order of preference is N-acetyl-D-glucosamine >>>> D-mannose> L-fucose > glucose > maltose D-glucosamine > galactosamine > D-galactose > D-fucose. Analysis of multivalent binding interactions of bovine conglutinin revealed that the dissociation constant of interaction of bovine conglutinin with maltosyl albumin or mannan, is 1.6×10^{-8} to 3×10^{-8} M and this binding strength is comparable to the binding of an antibody to an antigen (Holmskov *et al.*, 1996).

2.3.3 Receptor for bovine conglutinin on immune cells

Putative binding site for C1qR (surface variant of calreticulin) was known to be located within the N-terminal portion of the collagenous domains of the bovine conglutinin. Studies of binding of intact and truncated bovine conglutinin to cC1qR identified the receptor-binding site within the N-terminal 54 amino acids of bovine conglutinin. Truncated form of bovine conglutinin in which these residues are missing does not bind to C1qR (Malhotra *et al.*, 1993). This site is composed of five collagen repeats (Gly-Xaa-Yaa triplets), containing many charged residues in the Xaa and Yaa positions. cC1qR is found on a wide variety of cells, including most leukocytes, monocytes, lymphocytes, dendritic cells, endothelial cells, platelets, and fibroblasts (Sim *et al.*, 1998; Ghebrehiwet and Peerschke 2004). The C1q-mediated, cellular responses includes the enhancement of monocyte-phagocytic activity, stimulation of fibroblast adhesion, stimulation of oxidative burst in neutrophils, and enhancement of phagocytosis by pulmonary endothelial cells. Different collectins binding to the same cell types may produce the same cellular responses. It has been shown that, C1q and MBL (mannose binding lectin) can serve as opsonins that stimulate phagocytosis of apoptotic host cells by *E. histolytica* (Teixeira *et al.*, 2008).

2.3.4 Physiological roles of bovine conglutinin

In genetically conditioned adult animals (studies conducted mainly on Holstein cows), the conglutinin concentration in serum is 1.25-35 μg/ml (Akiyama *et al.*, 1992). It has been reported that the level of conglutinin in the serum of dairy cows depends on many factors such as breeding, season of the year, the stage of the reproductive cycle and infection (Dec and Wernicki, 2006). In cows, decrease in serum conglutinin developed shortly before calving, started at 4 weeks before term and persisted up to 2 weeks after delivery. Later, the level rapidly increased reaching high concentrations in approximately the 21st week after delivery (Ingram and Mitchell, 1970). On the contrary, Krogh-Meibom *et al.* (2004) reported that the serum concentration of bovine conglutinin is not affected by physiological rhythms. It is assumed that high inheritance of serum conglutinin concentrations provides a valuable and significant index, which can be taken into account in cattle husbandry since a positive correlation between the serum conglutinin concentration and higher resistance to diseases has been noticed (Holmskov *et al.,* 1998).

Conglutinin binds specifically the carbohydrate moiety of iC3b and agglutinate cells coated with this complement product. Conglutinin concentration decreases in the course of acute infections mainly due to the absorption of conglutinin from the serum by the activated complement component iC3b or a direct effect of microbial activity (Dec and Wernicki. 2006).

2.3.4.1 Haemagglutination Inhibition and Virus neutralization activity of Bovine conglutinin

Bovine conglutinin exhibits haemagglutination inhibition (HI) activity by binding through their lectin domains to the several viral glycoproteins. It exhibits both HI and neutralizing activity against

rotavirus strain Nebraska calf diarrhoea virus (NCDV). Neutralization of rotavirus was dependent upon glycosylation of VP7. It was the first report depicting the antiviral activity of bovine conglutinin against a non-enveloped virus and suggested a potential role for collectins in host defense against rotavirus infection in bovines (Reading *et al.*, 1998). Though influenza virus does not naturally infect cattle, antiviral activities of conglutinin are well characterized against influenza. Bovine conglutinin functions as β-inhibitors of influenza virus, binding to viral carbohydrate and mediating HI and virus neutralization as well as causing virus aggregation. In addition, it also mediates interactions with neutrophils and its activation. Strong virus neutralizing activity, viral growth inhibition and inhibition of viral spreading to adjacent cells against Influenza-A by bovine conglutinin has also been reported by Kawal *et al.* (2007). Chimeric full-length collectin containing the NH2 terminus and collagen domain of SP-D (Surfactant protein-D), coupled to the neck and carbohydrate recognition domain (NCRD) of bovine conglutinin was shown to have increased IAV inhibiting /neutralizing activity (Crouch *et al.*, 2005). Antiviral activity of NCRDs could be increased by two distinct, complementary strategies like cross-linking of NCRDs through various means and mutagenesis of CRD residues to increase viral binding (White *et al.*, 2010). A recent study reports that introduction of specific mutations around the lectin site of (human surfactant protein-D) hSP-D-NCRD increases the apparent affinity for IAV and neutralizing activity (Kevan *et al.*, 2010). Bovine conglutinin potentiates viral uptake and virus-induced respiratory burst responses in neutrophils (Hartshorn *et al.*, 1997). Both MBL and conglutinin bind to immobilized HSV-II antigens in a calcium-dependent manner, which is inhibited by carbohydrates *in vitro*. Bovine conglutinin has been shown to bind to viral envelope glycoprotein gp160 of HIV-1, and exert dose-dependent inhibition of gp160 binding to CD^{4+} T- cells *in vitro* there by preventing

the viral attachment and infection (Andersen *et al.*, 1991). However, in one of the reports, bovine conglutinin has been shown to enhance herpes simplex virus type 2 infection in mice (Fischer *et al.*, 1994).

2.3.4.2 Inhibition of microbial growth by Bovine conglutinin

Bovine conglutinin showed higher protection in mice infected with virulent strains of *Salmonella typhimurium, Pasteurella septica, Klebsiella pneumoniae, Listeria monocytogenes* and *Streptococcus pyogenes* (Ingram, 1959). The protein protected BALB/c mice against infections with *S. typhimurium* I. It has also been demonstrated to possess antibacterial activity against *E. coli* and *S. typhimurium in vitro* (Friis-Christiansen *et al.*, 1990). Conglutinin has been found to opsonize *S. typhimurium* and *E. coli* (Holmskov *et al.,* 1998).

2.3.4.3 Activity of collectins against fungal pathogens

Collectins like lung surfactant proteins (SP) SP-A and SP-D, and the serum collectins- mannose binding lectins have an important role as fungal opsonins, aiding in the recognition and control of fungal pathogens, and play pivotal role in modulating the subsequent immune responses (Willment and Brown, 2008). The binding of collectins to ligands on fungal pathogens and host defense cells, can result in enhancement or inhibition of resistance. The antifungal effect of collectins is mainly mediated through complement fixation, phagocytosis, and stimulation of cytokine/chemokine production. Collectins mediated antifungal effect was found to exist against some of the opportunistic fungal pathogens like *Aspergillus fumigatus, Candida albicans, Cryptococcus neoformans, Pneumocystis* also on primary fungal pathogens like *Blastomyces dermatitidis, Coccidioides, Histoplasma capsulatum, Paracoccidioides brasiliensis* (Brummer and Stevens, 2010).

2.3.5 Conglutinin in other species

Leon (1957) had reported the dependence of bovine conglutinin on calcium for its functional activity but the conglutinating substance in sheep serum was inactivated by the action of 2-mercaptoethanol, did not require calcium ions for its activity, was not inhibited by N-acetyl-D-glucosamine or L-fucose sugars and did not react directly with zymosan (Ingram, 1969 and Mittal, 1973). However, recombinant sheep serum conglutinin expressed in our laboratory has been shown to require calcium for its functional activities like sugar binding activity (Chandra Mohan, 2012)

Other species like goats, gnu antelopes and deer, as well as some non-ruminant species such as llamas, horses, boars, pigs and humans, contain proteins which have similar antigenicity to that of bovine conglutinin. These reacted with monoclonal and polyclonal antibodies specific for bovine conglutinin under reducing and non-reducing conditions in Western blotting (Dec *et al.*, 2011).

Materials and Methods

Materials and Methods

3.1 Materials

3.1 Biological material

Foetal liver tissue of nilgai was collected from the post-mortem of a pregnant nilgai from field conducted at postmortem house, IVRI whereas buffalo liver was collected from the slaughter house. Blood samples were collected from eight buffaloes from the animal sheds of IVRI and serum was separated from them.

BHV-1 virus used in the study was kindly provided by Dr P.K. Gupta, Veterinary Biotechnology Division and NDV vaccine was kidly provided by Dr V.K. Chaturvedi, Head, BP Division, IVRI, Izatnagar.

3.1.1 Vectors

pJET-blunt end cloning vector (MBI, Fermentas, USA) and pRSET-A prokaryotic expression system (Invitrogen, USA) were used in the present study.

3.1.2 Chemicals and Reagents

All the chemicals, buffers, media and reagents used were of molecular biology and analytical grade obtained from standard manufacturer's viz. Sigma (USA), Invitrogen (USA), Promega (USA), Fermentas (USA), Qiagen (USA), SRL (India), Merck (India/Germany), Bangalore Genei (India) and Hi-media (India). Polyclonal sera against buffalo recombinant conglutinin rose in rabbit and poultry were used

in the present study. Details and composition of different reagents, solutions, media and buffers used in the present study are given in the appendix and/or at appropriate places.

3.1.3 Glasswares

All the glasswares used for study were procured from Borosil (India), Schott Duran (Germany). All these glasswares were thoroughly washed and sterilized in hot air oven before being used as per the standard procedure.

3.1.4 Plastic wares

All the plastic wares like petri-plates, centrifuge tubes, micropipette tips including filter tips, microtubes, PCR tubes, 0.22µm and 0.45µm syringe filters were procured from various reputed firms viz., Nunc (USA), Axygen (USA), Nalgene (USA) and Tarsons (India). All the plastic wares used were sterile and DNase/RNase free. Plastic wares used for RNA work were thoroughly treated with 0.1% Diethyl pyrocarbbonate (DEPC) overnight to make them RNase free and then sterilized by autoclaving.

3.1.5 Equipments

The major equipments used in our study are Electrical analytical weighing balance (Model GR202, A&D Co. Ltd, Japan), Agarose gel electrophoresis apparatus (Genei, Bangalore), Air displacement pipettes viz. P10, P100, P200, P1000 (Eppendorf research, Germany), Gel documentation analysis system (AlphaImager TM1220, Alpha Innotech Corporation, USA), PCR work station (Medox, Mx-1239-01), Hot air oven (Yorco instrument, Bombay, India), Ice flaking machine (Model–IM-70, Harrison Scientific Instrument Co. Delhi, India), Refrigerated Microcentrifuge (MPW-350R), Non-refrigerated centrifuge (Sigma 1-13), Refrigerator (BPL India Ltd, India), -20°C Deep freezer (Blue star),

-80°C Deep freezer (Sanyo), Nanodrop (Model, ND 100, Thermo Scientific, USA), Spectrophotometer (Model-5415D, Eppendorf GmbH, Germany), Thermal cycler (TECHNE,TC-312), microwave oven (LG, India), tripple distillation apparatus (Scientronic, Australia), Dry bath (Bangalore Genei), Laminar Flow (Klenzaids), Magetic stirrer (REMI).

3.2 Methods

3.2.1 Cloning of buffalo and nilgai conglutinin cDNA from liver sample

3.2.1.1 Isolation of total RNA from the respective liver samples

Prior to RNA isolation, working area, pipettes, glass wares and gloves swabbed with RNase Zap (Ambion) to minimize the effects of RNase activity and sterilized nuclease free micro tubes were procured from Labware Scientific Inc. Sterile gloves were worn during the whole procedure of RNA isolation. About 50-100 mg of liver tissue sample of either species were taken and sliced using a sterile scalpel blade and transferred to 1.5 ml micro tubes separately and to that 1ml of Trizol reagent were added. After complete homogenization of the tissue, the homogenate in trizol was incubated at room temperature for 5 min. About 0.2 ml of chloroform per ml of trizol was added and mixed well by inverting, incubated at room temperature for 5 min and centrifuged at 12000 g for 15 min at 4°C. Aqueous phase were taken into fresh micro tube and added 0.5 ml of 100% isopropanol mixed and incubated at room temperature for 10 min then centrifuged at 12000 g for 10 min at 4°C. Discarded the supernatant, added 1 ml of 75% ethanol to wash the pellet and centrifuged at 7500 g for 5 min and resuspended the pellet in DEPC water and stored at -80°C for future use.

The absorbances of RNA were measured at 260nm/280nm in Nanodrop spectrophotometer and the OD260/OD280 ratios were calculated to check the purity of RNA from both the species.

3.2.1.2 Reverse transcription to synthesize the complimentary DNA (cDNA)

Isolated mRNA of both the species were used individually to synthesize complementary DNA (cDNA) in separate reactions consisting of the following reaction mixture which contained 5 µl (1-5 µg/µl) of total RNA of respective species, 1 µl of oligo-dT primer (0.5µg/µl), 6 µl of DEPC treated water that were heated in a thermocycler to 70°C for 5 min to remove secondary structures. Then 1µl of Rnasin (20 units/µl), 2µl of 10 mM dNTP mix (dATP, dCTP, dGTP, dTTP), 4 µl of 5X Reverse transcriptase buffer were added and incubated for 37°C for 5 min and finally 1 µl M-MuLV Reverse transcriptase enzyme (20unit/µl) was added and synthesis of cDNA was carried out at 37°C for one hour in thermocycler. The reaction was stopped by heating the mixture at 70°C for 10 min to inactivate the reverse transcriptase. The synthesized cDNA was quantified using spectrophotometer (Nanodrop®, USA) and was stored at -20°C till use.

3.2.1.3 Polymerase chain reaction (PCR) to amplify buffalo and nilgai conglutinin (CGN) gene from their respective cDNA

PCR were performed using proof reading enzyme KAPA HiFi-hot start as per the reaction conditions following the kit. PCR reactions were carried out with forward (5'-GGCTCGAGGGGGAGAGTGGGCTTGCAGA-3') and reverse (5'-GGGAATTCTCAAAACTCGCAGATCACAA-3') primers established for bovine conglutinin (Wakamiya, United States Patent Dec. 27, 2005). The PCR reaction mixture of final volume 25 µl were made which consisted of CGN forward and reverse primers (50 pmol) 0.5 µl each; Template cDNA of respective species 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 conditions employed initial denaturation at 95°C for 5 min, followed by 35 repeated cycles of denaturation at 95°C for 45

sec, annealing at 58°C for 1 min for buffalo CGN and 60°C for 1 min for nilgai CGN, extension at 72°C for 1 min. Final extention at 72°C for 5 min was followed by final hold at 10°C. The PCR amplicons of the respective species along with the 100 bp DNA ladder were resolved by 1% agarose gel electrophoresis and the ethidium bromide stained gel were visualized in Gel documentation system.

3.2.1.4 Purification of respective PCR product and ligation into cloning vector

The PCR products of both the species were run in preparative agarose gel along with DNA ladder and eluted with gel extraction kit (Qiagen, USA) as per manufacturer's instructions. The concentration and purity of the eluted PCR amplicon were checked by running in 1% agarose gel. The ligation reaction mixture of total volume 20 µl was made in an ice-cold 0.5 ml microfuge tube with the following components: 2X Ligation buffer (10.0 µl), Insert (Purified PCR product of buffalo and nilgai separately) 3-5 µl depending upon the concentration; (insert: vector molar ratio 3:1 was found to give 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 reactions were carried out at 22°C for 30 min.

3.2.1.5 Preparation of *Escherichia coli DH5*r competent cells

The competent cells were prepared by the method of Chung *et al.* (1989). A single colony of *E. coli* DH5α was picked from a LB agar plate and grown overnight in 5 ml of SOB without any antibiotic at 37°C with shaking at 180-200 rpm in an orbital shaker. This overnight grown culture was diluted 1:100 in freshly prepared LB broth without antibiotic and incubated at 37°C with shaking at 180-200 rpm till the OD600 of the culture reached 0.25-0.35. The bacteria were pelleted by centrifugation at 2,500 rpm for 10 min. The supernatant was

discarded and kept on ice. 1/10th volume of chilled TSS was added to the pellet and the bacterial pellet was carefully resuspended by slow pipetting for 3 to 4 times. The tubes were incubated in ice for 1 h, at the end of incubation the cells were gently mixed and 200 µl of the competent cell suspension was dispensed using a cut tip in pre-chilled, sterile labelled 1.5 ml microfuge tubes.

3.2.1.6 Transformation of ligated pJET to *DH5*ᴦ competent cells

The transformations were carried out in DH5α competent cells prepared as above. Ligation product of respective species were directly added to individual 200 µl competent cells and mixed without touching the pipette at the bottom and was kept on ice for 60 min. Heat shock were given at 42°C for 45 sec in a water bath and the cells were immediately transferred on chilled ice for 5 min. 900 µl of SOC (ice cold) were added to the tubes. The cells were then incubated at 37°C with slow shaking for 1 h to allow the expression of Ampicillin resistance genes and recover from stress caused by the heat shock. The bacterial cultures were pelleted by centrifugation at 4000 rpm for 5 min at room temperature. Approximately 800 µl of supernatant were discarded and the pellet were resuspended in rest of the supernatant by slow pipetting and plated on separate individual LB agar plate containing Ampicillin (100µg/ml). The plates were allowed to dry under laminar flow and incubated at 37°C for 12-14 hr with constant shaking.

3.2.1.7 Plasmid Isolation

Alkaline lysis method (Sambrook *et al.*, 2001) was used for plasmid isolation from the transformed cells. From the overnight culture, 3 ml were transferred to a microfuge tube and the cells were pelleted by centrifuging at 5000 rpm for 5 min at 4°C. The media were drained off completely and the pellet were resuspended in 300 µl of buffer P1 (refer appendix). These resuspended cells were lysed gently after adding 300

µl of buffer P2 (refer appendix), by inverting the tube several times till the lysate becomes clear viscous solution. To this 300 µl of ice cold buffer P3 (refer appendix) were added and incubated on ice for 10 min. The entire suspensions were centrifuged at 12000 rpm for 10 min at 4°C. The supernatant were then transferred to a fresh tube carefully without disturbing the pellet and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed gently by vortexing. The tubes were centrifuged at 12000 rpm for 10 minutes at 4°C and the upper aqueous phases were transferred to a fresh tube. The phenol: chloroform: isoamyl alcohol extraction step was repeated twice. The DNA were precipitated by addition of 0.7 volume of isopropanol and incubated at room temperature for 20 min. The precipitated DNA was pelleted by centrifugation at 12000 rpm for 15 min after which the pellet was washed with 70% alcohol. The pellet was then air-dried and finally dissolved in 50 µl of nuclease free water and stored at -20°C.

3.2.1.8 Screening of recombinant plasmids pJET-BuCGN and pJET-NCGN

Based on the pJET-easy vector restriction map information and the sequence of NCRD partial gene sequence of bovine conglutinin, restriction enzyme *Pst*1 were selected to release two insert fragments of 566 bp and 256 bp from the recombinant plasmids. The plasmid DNA of both the species were subjected to digestion for 4 h at 37°C in digestion mixture consisting of respective plasmid DNA (2-5 µl depending on its concentration), 10 X RE buffer (2µl), *Pst1* 1µl (10U/µl) and volume made to 20µl with nuclease-free water. Release of the inserts were analysed in 1.5% agarose gel containing ethidium bromide along with the 100 bp plus ladder.

3.2.1.9 Sequencing of the recombinant plasmid

Positive colonies of both the species were selected and kept for culturing overnight in 5 ml of LB broth containing ampicillin (100µg/

ml) at 37°C in an orbital shaker. Plasmids were isolated from the cultures with MDI plasmid miniprep kit as per manufacturers' instructions and were sequenced using T7 universal primer at Xcelris Labs Ltd., Anand, Gujarat.

3.2.1.10 Sequence analysis

The sequences of cDNA encoding neck and CRD region of buffalo conglutinin and nilgai conglutinin retrived from sequence analysis were subjected to blast at NCBI public database. Then these sequences were aligned with the available sequences of conglutinin of ruminants using MegAlign (DNA star). Phylogenetic tree based on evolutionary distances, nucleotide substitution and identity with respect to other species were constructed from nucleotide sequences of these species using Mega 4.0.2 software (www.megasoftware.net/mega4). Secondary structure of BuCGN and NCGN were predicted from the amino acid sequences using Swiss Prot (http://web.expasy.org/docs/swiss-prot_guideline.html).

3.2.2 Expression of the recombinant(r) BuCGN and NCGN protein

3.3.2.1 Subcloning of insert into prokaryotic expression vector and transformation into the expression host

Recombinant plasmids pJET-BuCGN and pJET-NCGN containing the insert were characterized by digesting the vector with enzymes *EcoR1* and *Xho1* to release the insert. The digestion were performed in reaction mix consisting of 5 µl of 10X buffer, 35 µl of respective recombinant plasmid and 4 µl of restriction enzymes and the volume made upto 50 µl using nuclease free water. Simultaneously prokaryotic expression vector pRSET-A vector frame were also digested with same set of enzymes to aid in directional cloning. Both the inserts of the respective species and digested pRSET-A vector were ligated individually by ligation mixture consists of 2 µl of the respective insert,

6 µl of pRSET-A, 1 µl of 10X ligation buffer and 1 µl T4 DNA ligase, these ligated products were transformed to expression host E *coli* BL21 competent cells, plated and plasmid isolation done as per the protocol mentioned above. The recombinant plasmids (pRSET-BuCGN and pRSET-NCGN) were characterized by restriction digestion using *Rsa* I. The reaction mixture consisted of 2 µl of recombinant plasmid, 2 µl of 10X restriction buffer, 1 µl of enzyme and the volume made upto 20 µl using nuclease free water. Thus characterized plasmids of the respective species were used for studying expression of protein. Positive colonies of each, containing the desired BuCGN and NCGN gene, were chosen from the master plate for induction as a pilot study to check the expression. The colonies were picked up into 5 ml of LB broth and cultured overnight at 37°C with constant shaking at 200 rpm. 10 ml of fresh LB broth were then inoculated with overnight grown culture at the rate of 100µl/10ml of LB broth and further incubated at 37°C with constant shaking until mid-log phase (OD600-0.5 to 0.6). One ml of the respective cultures was collected as an un-induced control. To the rest of the culture, IPTG was added at a final concentration of 1mM, and kept at 37°C with constant shaking at 200 rpm. One ml of the induced culture was collected at every 1 hour interval upto 4 hr. All the cultures collected were pelleted by centrifugation at 4000 rpm and kept at -20°C till further use.

3.3.2.2 Sodium Dodecyl Sulfate–Polyacrylamide gel electrophoresis (SDS-PAGE) to check the expression profile

The uninduced (control) and induced *E. coli* BL21 cell pellets of respective species collected at 1 hr intervals were re-suspended in 20 µl of SDS-PAGE sample buffer (5X). The volume was made up to 100 µl by addition of autoclaved distilled water to the samples. The pellets were boiled for 5 min in a water bath in order to lyse and denature the bacterial proteins. Samples were then centrifuged at 12000 rpm to

pellet the cellular debris following which 40 µl of the supernatant were subjected to 12% SDS-PAGE (Laemmli, 1970) under denaturing reducing conditions at 100V for 2-3 hr. The gel stained using Coomassie Brilliant Blue R-250 were analysed for the presence of expressed protein. The compositions of different buffers used for SDS-PAGE are given in the annexure.

3.3.2.3 Purification of recombinant buffalo conglutinin (rBuCGN) and recombinant nilgai conglutinin (rNCGN) proteins

The colonies expressing the recombinant protein of the respective species were selected for bulk culture. Overnight grown culture was added into 500 ml of LB broth and kept for incubation in an orbital shaker at 37°C; 200 rpm till the OD600 reached to 0.5 to 0.6 and the cultures were induced with 1 mM IPTG and kept for incubation at 37°C in an orbital shaker at 200 rpm. After 4 hrs, the cultures were centrifuged at 3500 rpm for 25 min and the pellets were stored at -20°C. The pellets of the respective species were thawed and processed individually by resuspending in 4 volumes of the lysis buffer (50mM sodium phosphate (pH 8.0), 300mM NaCl, 10mM imidazole and 1 mg/ml Lysozyme), mixed and kept in ice and finally sonicated at 40 Hz for 6 cycles with 1 min interval. The mixture was centrifuged at 4000 rpm for 25 min at 4°C to separate soluble proteins. To the pellet, 4 volumes of urea lysis buffer (8M urea, 0.1M sodium phosphate, 0.01M Tris-Cl, adjusted pH to 8.0 with hydrochloric acid) was added in order to release the proteins from inclusion bodies. Contents were mixed until pellet dissolved totally, centrifuged at 12000 rpm and supernatants were passed through the Histidine bound Ni-NTA Resin separately using individual columns for both the species (as per the manufacturer's instructions given by Invitrogen ProBond™ Purification System). The columns were washed with excess of urea wash buffer (8M urea, 0.1M sodium phosphate, 0.01M Tris-Cl, adjusted pH to 6.3

with hydrochloric acid). The bound protein of respective species were eluted by using elution buffer (8M urea, 0.1M sodium phosphate, 0.01M Tris-Cl, adjusted pH to 4.5 with hydrochloric acid) and collecting fractions of 1 ml each. The purity of the eluted protein of both the species was checked in 12% SDS-PAGE as described earlier.

3.3.2.4 Dialysis to renature the rBuCGN and rNCGN proteins

The purified proteins in urea elution buffer were dialysed as per standard protocol (Sambrook *et al.*, 2001). Appropriate size of the dialysis membranes (Sigma with molecular weight cut off of 12kDa) were cut with sterile blade and it were processed by boiling in 2% sodium bicarbonate and 0.05% EDTA for 10 min and then boiled in distilled water, cooled and stored in 20% Ethanol/0.1% sodium azide at 4°C (Stable for 3 months). Care were taken not to dry the membranes. The membranes were tied at one end and appropriate volume of eluted proteins of respective species were added individually into the dialysis bag and then tied from the other end too. Slow dialysis were allowed at 4°C with constant stirring at different molarities of urea in PBS viz 6M, 4M, 3M, 2M, 1 M, 0.5M and finally kept in PBS overnight to equilibrate. Finally the proteins of the respective species were aliquoted and stored at -80°C. The purity of the protein was checked by SDS-PAGE as described earlier and the proteins of the both the species were quantified using Quanti-IT protein assay kit in fluorometer as per manufacturer's guidelines.

3.3.2.5 Immunization to produce Polyclonal Antibodies in Rabbit and Poultry using rBuCGN

3.3.2.5.1 Immunization of rabbit using rBuCGN

Immunization of the rabbit was carried out as per the standard protocol adult rabbits were used for immunization. Blood was collected from the animal prior to immunization; serum was harvested and

stored at -20°C. Two ml of fruend's incomplete adjuvant was put into 5ml tube and then added two ml of rBuCGN into that in such a way that the concentration of the recombinant protein was 200 µg/ml and then mixed the contents by repeated aspiration and dispense using 3-ml glass syringe with a 19-G needle. When the mixture was homogeneous and white, it was tested whether the emulsion is stable or not by extruding a small drop onto the surface of cold water in a beaker. A good oil-in-water emulsion holding together as a droplet on the surface of the water was obtained. Once the emulsion is prepared in sterile aseptic condition, immunization was done using 1ml of the emulsion per rabbit by two deep intramuscular injections (0.5 ml each) into thigh muscles, followed by booster using 100 µg/ml rBuCGN protein in fruend's incomplete adjuvant on 14th day and 21st day. Blood of the animal was tested on 25th day to detect anti-rBuCGN antibodies in direct ELISA. The cross reactivity of the rNCGN was also determined.

3.3.2.5.2 Immunization of chicken using rBuCGN

Immunization of adult broiler chicken by using rBuCGN protein was also performed as described above.

3.3.3 Characterization of recombinant conglutinin of buffalo and nilgai

3.3.3.1 Checking the reactivity of rBuCGN with polyclonal serum raised against recombinant buffalo conglutinin by Direct ELISA

Direct ELISA was performed to check the reactivity of recombinant protein of buffalo with antiserum raised against buffalo conglutinin in poultry. The flat bottom 96 wells ELISA plate (Nunc) were coated with purified rBuCGN protein (4µg/well in coating buffer pH 9.6) overnight at 4°C along with PBS as control. After each treatment, plate was washed three times with PBS-Tween (PBS, pH 7.2; Tween-20, 0.05%). The unbound sites were blocked with 1%

casein in PBS at 37°C for 1hr. Antiserum raised against buffalo conglutinin in poultry were used as primary antibody at 1:6400 dilution and incubated at 37°C for 1hr. Anti poultry IgG conjugated with HRPO (Biorad) was used as secondary antibody at 1:5000 dilution followed by incubation for 1 h at 37°C and finally 100µl of TMB substrate was added. Upon development of blue colour, the reaction was stopped by adding 50 µl of 5% sulphuric acid per well and the plate was read at 450 nm in ELISA reader.

The reactivity of recombinant protein of buffalo with antiserum raised against buffalo conglutinin in rabbit was also checked by ELISA as briefed above with the exception anti-rabbit IgG conjugate (Pierce) at 1:8000 dilution was used as secondary antibody.

3.3.3.2 Characterization of rBuCGN and rNCGN by using Western blotting analysis

The antigen specificity of both rBuCGN and rNCGN were checked in Western blotting (Towbin *et al.,* 1979). About 3 µg of the purified recombinant proteins of both the species along with bacterial lysate, eluted fraction and prestained protein marker was run in 12% SDS-PAGE. The resolved proteins of respective species were subsequently transferred to a PVDF membrane using a semi-dry blotting apparatus (Bio-Rad) in 50 mM Tris base, 380 mM glycine, 0.1% SDS, 20% methanol at 0.8 mA per cm^2 of PVDF membrane for 2hr. Transfers of the protein to the membranes were confirmed by pre-stained marker. The unbound surfaces of the membranes were blocked overnight with 2% skimmed milk in PBS-Tween at 4°C. Following three washing of ten min each with PBS-Tween (PBS, pH 7.2; Tween-20, 0.05%), poultry sera raised against buffalo conglutinin in PBS-Tween (1:400) were added and incubated at 37°C for 1 h. The membranes were washed three times with PBS-Tween and subsequently anti-chicken rabbit IgG HRPO conjugate (1:400) were added and incubated at 37°C for

1h. The membranes were washed 3 times with PBS-Tween and developed by using 4-Chloronapthol (Sigma USA) in substrate buffer (4-Chloronapthol 6 mg, 2ml methanol, H_2O_2 10 µl, added PBS to make volume to 10 ml). Thus developed PVDF membranes of respective species were documented.

3.3.3.3 Characterization of recombinant protein of both buffalo and nilgai by Mass spectrometry

Expressed protein visualized as 27kDa band in SDS-PAGE were sliced. The protein samples were trypsin digested and peptides extracted according to standard techniques (Bringans *et al.*, 2008). Peptides were analysed by MALDITOF-TOF mass spectrometer using a 4800 Proteomics Analyzer (AB Sciex) at Proteomics International Pty Ltd (Australia). Spectra were analysed to identify protein of interest using Mascot sequence matching software (Matrix Science) with Ludwig NR Database. Conserved peptide regions identified in mass spectrometry were subjected to Blast analysis in NCBI database to confirm that the recombinant protein is conglutinin of respective species.

3.3.3.4 Sugar binding and inhibition assay

Evaluations of functional activity of recombinant conglutinin of both the species were performed according to the ELISA system employed for bovine conglutinin by Lu *et al.* (1992) with necessary minor modifications. Briefly, microtiter Plates were coated with 100 µl coating buffer (15mM sodium carbonate, 35 mM sodium hydrogen carbonate, 0.05% sodium azide, pH 9.6) containing yeast mannan (10µg/ml) at 4°C overnight. After each treatment step, the plates were washed three times with TBS/NTC solution (20 mM Tris-HCI, 140 mM sodium chloride, 0.05% sodium azide, 0.05% Tween 20), pH 7.4, 5 mM calcium chloride). After coating, the plates were treated and blocked with TBS/NTC solution containing 1% bovine serum

albumin at room temperature for one hour. Individual dilution (0, 0.1, 1.0, 10, 100 and 1,000 ng/ml) of the buffalo and nilgai recombinant conglutinin in TBS/NTC or TBS/NTC containing 20 mM N-acetyl-D-glucosamine or 10mM EDTA were added in quadruplicate wells respectively. In subsequent two steps, poultry anti-recombinant buffalo conglutinin serum (1: 5,000) and antipoultry-rabbit IgG HRPO conjugate (1:6,000) respectively were added. The plates were incubated at 37° C for 1h in both the steps. Finally, 100 µl of TMB substrate were added to each well and development of colour was monitored. After the addition of 50 µ1 of 5% sulphuric acid, absorbance at 450 nm was measured in ELISA reader.

3.3.3.5 Hemagglutination inhibition assay using NDV-virus

Hemagglutination inhibition assay was performed in 96 well U-bottomed microtitre plates as per the standard protocol using the recombinant proteins of both the species at different concentrations like 2.5µg, 5.0µg, 10µg, and 20µg/well. The assay was performed with the prior incubation of 4HA units of NDV-virus with different concentrations of the proteins for 1hr at 37^{0}C and finally added of the 1% chicken RBCs. NDV-virus mediated hemagglutination inhibition of the recombinant protein of the both the species were visually observed after 30minutes of incubation at 37^{0}C and documented .

3.3.3.6 LPS binding assay

LPS binding property of the both rBuCGN and rNCGN were assessed by using ELISA method with necessary modifications. Briefly, 96 well microtiter Plates were coated with 100 µl coating buffer (15mM sodium carbonate, 35 mM sodium hydrogen carbonate, 0.05% sodium azide, pH 9.6) containing LPS (Sigma USA) (10µg/ml) at 4°C overnight, further test was performed as described above for sugar binding inhibition assay and the absorbance at 450 nm was measured in ELISA reader.

3.3.3.7 BHV-1 Virus neutralization test

3.3.3.7.1 BHV-1 Virus Titration

For titration the BHV-1 infected cell culture supernatant was freeze-thawed about three times and centrifuged at 4000 rpm for 10 min. The supernatant was collected and kept at -70°C. An aliquot was thawed and 10 fold dilutions (10^{-1} to 10^{-10}) of the virus were prepared in DMEM medium (maintenance medium). 100 µl of aliquot from each dilution was dispensed in respective well of 96 well cell culture plate and mixed with 100 µl MDBK cell suspension (1.5×10^5 cells/ml) in six replicates. Six wells without virus were kept as cell control. The plate was incubated at 37°C in CO2 incubator for 3 days and monitored by microscopic observation for development of BHV-1 specific CPE. The titre was calculated following the method of Reed and Muench (1938).

3.3.3.7.2 Virus neutralization test

Virus neutralization test was performed in MDBK cell line as per OIE protocol with necessary modifications (OIE Terrestrial Manual 2010). Briefly, instead of test sera for neutralization recombinant buffalo and nilgai conglutinin were used [in dilutions like 1:10; 1:20; 1:40; 1:80]. Prior to exact experiment, 50 µl of the BHV-1 virus having titre of 4×10^{-3} were incubated for 24hrs at 37°C in an ependroff using the above said dilutions of the recombinant protein of the respective species individually. Next day the actual neutralization experiment was performed by using the previous day incubated virus. Each of the virus dilutions of individual species were diluted 10 fold [10^{-1} to 10^{-8}] in 100 µl of growth medium [DMEM with out HEPES and 2% FCS] and finally added MDBK cell suspension providing 3×10^4 cells per well. The plates were incubated for 3–5 days at 37°C and read microscopically for CPEs; results are expressed as the reciprocal of the dilution of serum that neutralised the virus in at least 50% of the wells.

3.3.4 Standardization of Sandwitch ELISA

Sandwitch ELISA was standardized by using the purified recombinant buffalo conglutinin as standard antigen to screen the different buffalo serum samples to elucidate the normal serum concentration of the conglutinin in this species. ELISA was standardized initially by using checker board analysis at different concentrations of the capturing antibody, antigen, detecting antibody and conjugate concentrations. Finally by determining the appropriate detectable concentrations of the capturing antibody, antigen, detecting antibody and conjugate, the test was performed by coating the 96 well flat bottomed plate @ of 50 µl/well by using capturing antibody [rabbit anti buffalo conglutinin] at the concentrations in 1:200 dilution in coating buffer and kept 4°C overnight. Followed by washing thrice using [PBS –Tween], blocked the wells by using 200 µl/well 5% BSA and kept at 37°C for 1hr. Washed thrice and subsequently recombinant antigen [at different concentrations like 0.325 0.625, 1.25, 2.5, 5 µg/ml] was added @ 50 µl/well diluting in 2% BSA. Similarly nine buffalo sera were also diluted at the rate of 1:5 in 2% BSA and added 50 µl/well kept at 37°C for 1hr. In addition positive control [with Ag and detecting Ab], negative control (without Ag, detecting Ab), conjugate control [with Ag and detecting Ab without conjugate] and Antigen blank [without Ag] were also kept in the plate. Washed thrice and then added detecting antibody [chicken-anti rBuCGN] at the rate of 1:1600 diluted in 2% BSA 50 µl/well followed by addition of rabbit anti-chicken HRPO – conjugate @ 1:5000 in 2% BSA and incubated at 37°C for 1hr. Washed thrice and added TMB substrate @ 50 µl/well and monitored the colour development till a gradation of colour in standard and stoped the reaction by using 5% H_2SO_4. OD value at 450 nm keeping reference reduction at 630nm was recorded.

Thus obtained OD values of the test serum and standard samples were analysed and linear regression curve was plotted between OD values against concentration of the rBuCGN. Thus plotted curve was used to retrieve the quadratic equation, directing to found the normal serum concentrations of the conglutinin in the reacted test serum samples.

Results

4 Results

This section describes the outcome of the present investigation which covers the cloning and characterization of the cDNA encoding buffalo and nilgai conglutinin, expression of the recombinant protein of the respective species in prokaryotic expression system, purification of recombinant proteins followed by functional evaluation fulfilling the biological activity like sugar binding and inhibition assay, virus (BHV-1) neutralization test *in vitro*, LPS binding assay, NDV viral haemagglutination inhibition assay and also development of sandwitch ELISA to detect the normal concentration of this novel protein in the serum sample.

4.1 Cloning of BuCGN and NCGN amplicon

4.1.1 Amplification of the cDNA encoding BuCGN and NCGN

The total RNA isolated from liver tissue of buffalo and foetal liver of nilgai were found to be of good quality as evidenced from OD260/OD280 ratio of >1.8. Complimentary DNA prepared from these RNA of respective species were used as template to amplify BuCGN and NCGN gene respectively using proof reading polymerase and thus obtained 497 bp PCR product of both the species were well resolved in agarose gel electrophoresis incorporated with EtBr (Fig: 1).

4.1.2 Cloning of the BuCGN and NCGN amplicon into pJET cloning vector

The gel-purified purified BuCGN and NCGN amplicons were cloned into pJET blunt end cloning vector. Recombinant bacteria

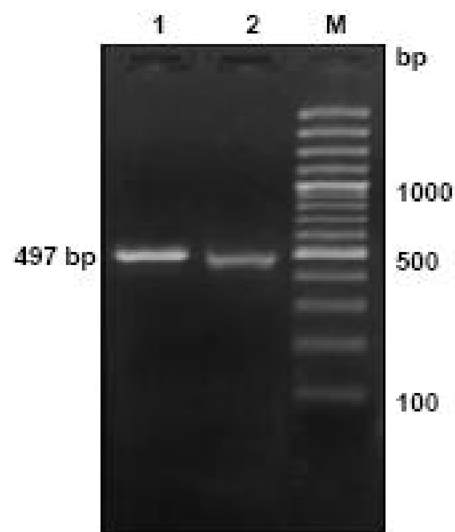


Fig. 1 : **PCR products of cDNA encoding buffalo and nilgai conglutinin NCR domain**

Lane M : 100 bp DNA ladder

Lane 1 : Buffalo amplicon (497 bp)

Lane 2 : Nilgai amplicon (497 bp)

appeared as discrete white colonies when screened on the basis of ampicillin resistance on LB agar plate. Selected colonies of respective species cultures were grown overnight in LB containing ampicillin and plasmids (pJET-BuCGN and pJET-NCGN) were isolated from overnight grown cultures. Restriction digestion of the both the recombinant plasmids with *Pst*I generated three fragments as 261bp, 566bp and 2.6kb bands also confirmed the presence of desired gene (Fig: 2 and Fig: 3).

4.1.3 Sequence analysis of buffalo and nilgai conglutinin NCRD (neck and carbohydrate recognition domain) partial gene

Recombinant plasmids of both the species containing respective NCRD gene (pJET-BuCGN and pJET-NCGN) were sequenced using commercially available automated DNA sequencer, and the sequences so obtained were aligned and analyzed with the existing sequences in the NCBI to retrieve the 488bp of buffalo conglutinin and nilgai conglutinin partial cds respectively. Thus obtained sequences submitted to NCBI Genbank have accession **HQ330990** for buffalo CGN and **HQ330991** for nilgai, respectively. Using DNAstar software, these sequences were aligned (Fig: 4) with sequences of conglutinin of other ruminants available in NCBI database. Nucleotide sequence alignment of the coding region revealed that the BuCGN is 96.5 % similar to bovine conglutinin, 93.9 % to sheep conglutinin, 99.9% to nilgai conglutinin on the other hand NCGN is 96.5% similar to bovine conglutinin and 93.4% similar to sheep conglutinin respectively (Fig: 6). Predicted amino acid sequences of both BuCGN and NCGN were aligned (Fig: 5) and at amino acid level BuCGN is 96.3 % similar to bovine conglutinin, 88.2 % to sheep conglutinin, and 100% to nilgai conglutinin whereas NCGN is 96.3% similar to bovine conglutinin and 88.8% similar to sheep conglutinin (Fig: 7). The phylogenetic information derived on the basis of nucleic acid sequences revealed

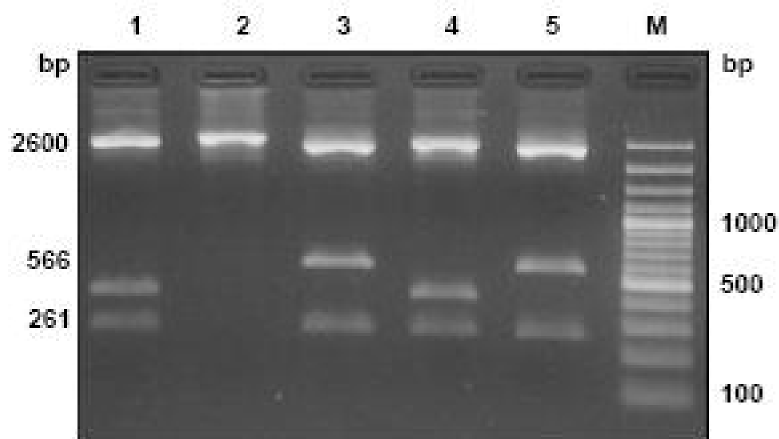


Fig. 2 : **Characterization of buffalo recombinant plasmids (pJET-BuCGN) using *Pst* I restriction digestion**

Lane M	:	100 bp DNA ladder
Lane 2	:	Non-recombinant plasmid
Lane 3&5	:	Recombinant plasmid
Lane 1&4	:	Recombinant plasmid with alternate orientation of insert

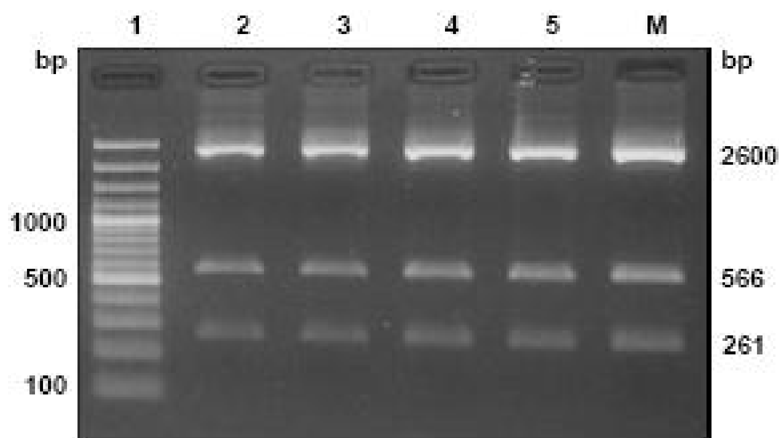


Fig. 3 : **Characterization of nilgai recombinant plasmid (pJET-NCGN) using *Pst* I restriction digestion**

Lane M	:	100 bp DNA ladder
Lane 1-5	:	Recombinant plasmids

Fig. 4 : **Alignment of nucleotide sequences of buffalo and nilgai conglutinin NCRD with other ruminants species**
Identity of the sequences with *B. taurus* is indicated by dots

```
                 10        20        30        40        50        60        70        80
    GSSGLAEVNALKQRVTILDGHLRRFQNAFSQYKKAVLFPDGQAVGEKIFKTAGAVKSYSDAEQLCREAKGQLASPRSSAE Majority
1   GSSGLAEVNALKQRVTILDGHLRRFQNAFSQYKKAVLFPDGQAVGEKIFKTAGAVKSYSDAEQLCREAKGQLASPRSSAE B.taurus CGN
1   ...................................................T.........................K.. B.tragocamelus CGN
1   ...................................................T.........................K.. B.bubalus CGN
1   .............Q....N..............N...................V.......Q...T...........A.. O.aeries CGN

                 90       100       110       120       130       140       150       160
    NEAVTQMVRAQKENAYLSMNDISTEGRFTYPTGEILVYSNWAKGEPNNSDEGQPENCVEIHPEGKWNDVPCSKQLLVICE Majority
81  NEAVTQMVRAQEENAYLSMNDISTEGRFTYPTGEILVYSNWADGEPNNSDEGQPENCVEIFPDGKWNDVPCSKQLLVICE B.taurus CGN
81  ...........K..............................K.................H.E................. B.tragocamelus CGN
81  ...........K..............................K.................H.E................. B.bubalus CGN
81  .E....L...HEE..........................T..T.......D.........S.E.Q...I........... O.aeries CGN

    F   Majority
161 F   B.taurus CGN
161 .   B.tragocamelus CGN
161 .   B.bubalus CGN
161 .   O.aeries CGN

Decoration 'Decoration #1': Hide (as '.') residues that match B.taurus CGN exactly.
```

Fig. 5 : **Alignment of predicted amino acid sequences of buffalo and nilgai conglutinin NCRD with other ruminants species**
Identity of the sequences with *B. taurus* is indicated by dots and differences by corresponding one letter symbol of the amino acids

Percent Identity

Divergence	1	2	3	4		
1		96.5	96.5	93.4	1	B. taurus CGN
2	3.6		99.6	93.9	2	B. bubalis CGN
3	3.6	0.4		93.4	3	B. tragocamelus CGN
4	6.9	6.4	6.9		4	O. aries CGN
	1	2	3	4		

Fig. 6 : **Percent identity and divergence at nucleotide level of BuCGN and NCGN with CGN sequences of other ruminant species**

Percent Identity

Divergence	1	2	3	4		
1		96.3	96.3	88.2	**1**	B.taurus CGN
2	3.8		100.0	88.8	**2**	B.tragocamelus CGN
3	3.8	0.0		88.8	**3**	B.bubalus CGN
4	12.9	12.1	12.1		**4**	O.aeries CGN
	1	2	3	4		

Fig. 7 : **Percent identity and divergence at predicted amino acid level of BuCGN and NCGN with CGN sequences of other ruminant species**

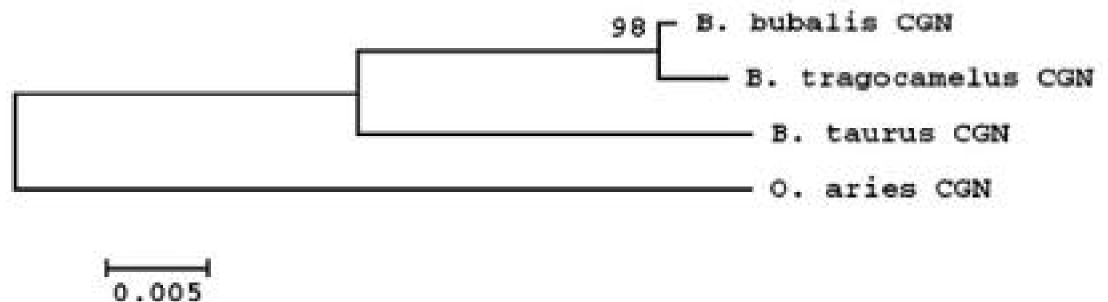


Fig. 8 : **Phylogenetic analysis of BuCGN and NCGN using CGN nucleotide sequences of other ruminant species**

Table 1 : Positions of the amino acid changes which was noticed in the rBuCGN and rNCGN upon alignment with the *B. taurus* conglutinin.

Amino acids in *B.taurus* with their positions in 162 amino acids	Changed amino acids in rBuCGN and rNCGN with similar positions in 162 amino acids
A at 52	T
S at 78	E
E at 92	K
D at 123	K
F at 141	H
D at 143	E

independent cluster for large ruminants like cattle, buffalo and nilgai and separate cluster for sheep among ruminant species (Fig: 8). By aligning buffalo and nilgai conglutinin with bovine conglutinin sequences, number and position of non-synonymous amino acid changes were analyzed (Table: 1) that revealed six amino acids at the positions like 52nd , 78th ,92nd , 123rd ,141st and 143rd in both the species respectively.

4.1.4 Protein structure prediction

The secondary structure of these proteins predicted using nucleotide sequences by online software like SWISS MODEL ProtParam revealed that the both buffalo and nilgai conglutinin neck and carbohydrate recognition domain (NCRD) contained five â-sheets.

4.2 Expression of recombinant buffalo (rBuCGN) and nilgai (rNCGN) conglutinin

4.2.1 Sub cloning of the BuCGN and NCGN fragment from pJET cloning vector into the pRSET-A prokaryotic expression vector

The 497bp conglutinin gene fragment of buffalo and nilgai released from pJET-BuCGN and pJET-NCGN (Fig: 9) were sub cloned into expression vector pRSET-A. The recombinant colonies containing pRSET-BuCGN and pRSET-NCGN were characterized by using *Rsa*I restriction analysis (Fig: 10 and 11) with clear cut demarcation of recombinant plasmid yields 487bp, 1153bp and 1700bp and non recombinant plasmid yielding 1700 and 1153bp of both the species.

4.2.2 SDS- PAGE analysis and study of expression

The recombinant plasmids pRSET-BuCGN and pRSET-NCGN were transformed into the *E. coli* BL-21 (p-Lys) bacterial host. Positive colonies of both the species were grown individually in LB and subsequently induced by using 1mM IPTG for different time intervals

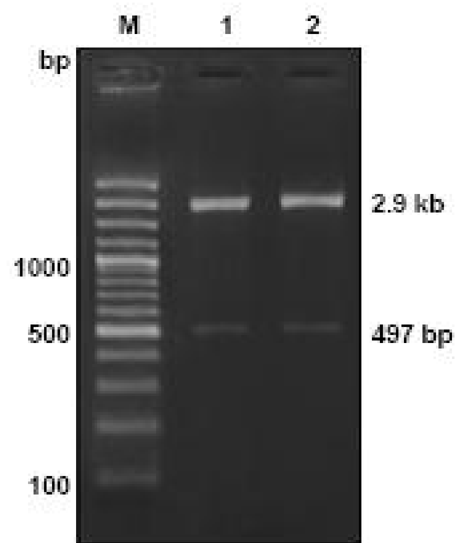


Fig. 9 : **Insert release of from pJET-BuCGN and pJET-NCGN plasmids using *Xho*I and *Eco*RI restriction enzyme**

Lane M : 100 bp DNA ladder

Lane 1 : Insert release of (497 bp) from recombinant BuCGN plasmid

Lane 2 : Insert release of (497 bp) from recombinant NCGN plasmid

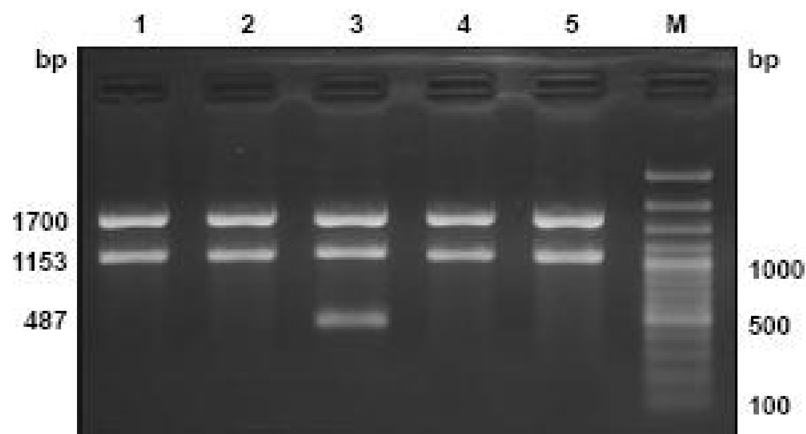


Fig. 10 : **Characterization of buffalo recombinant plasmids (pRSET-BuCGN) using *Rsa*I restriction digestion**

Lane M	: 100 bp DNA ladder
Lane 1-2&4-5	: Non-recombinant plasmid
Lane 3	: Recombinant plasmid

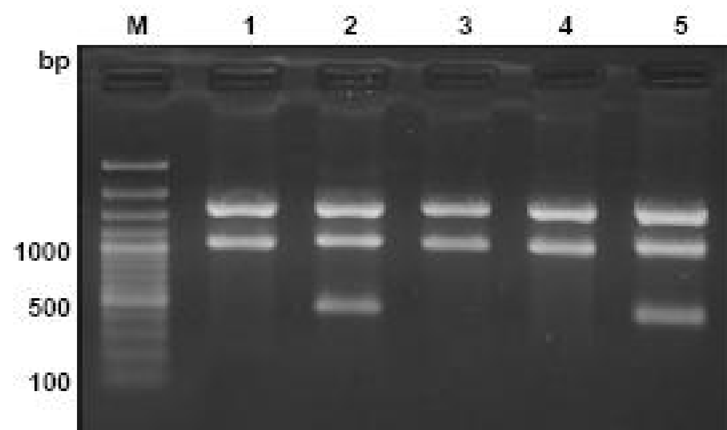


Fig. 11 : **Characterization of nilgai recombinant plasmids (pRSET-NCGN) using *Rsa*I restriction digestion**

Lane M	: 100 bp DNA ladder
Lane 1,3&4	: Non-recombinant plasmid
Lane 2&5	: Recombinant plasmid

(0-4 hr) and then pelleted. The bacterial pellets thus obtained, when resolved in 12% SDS-PAGE under reducing conditions clearly demonstrated the expression of 27kDa rBuCGN and rNCGN fusion proteins in inclusion bodies of induced culture as compared to un-induced cultures (Fig: 12 and 13) .

4.2.3 Affinity purification of the rBuCGN and rNCGN

Using Ni-NTA affinity column chromatography the expressed recombinant conglutinin of both the species (rBuCGN and rNCGN) were purified using urea elution buffer of different 1ml aliquots were also resolved in 12% SDS-PAGE with coomassie blue staining was finely depicted the purified 27kDa recombinant conglutinin of both the species (Fig: 14 and 15).

4.2.4 Developing hyperimmune serum against rBuCGN in rabbit and chicken

The hyper immune sera raised against rBuCGN in rabbit and chicken were analysed using direct ELISA which revealed that there was a clear cut significant difference in the titre value of 0 day sera and 28th day sera of both rabbit and chicken (Fig No.16). The titre of rabbit sera which was found to be effective titre for the antibody related assay was not less than 1:3200 and that of the chicken sera was 1:6400.

4.2.5 Characterization of rBuCGN and rNCGN

4.2.5.1 Mass Spectrometry analysis

In order to confirm the presence of putative conserved regions of the rBuCGN and rNCGN with that of the other collectins existing in the bovines, MALDI-TOF analysis was carried out. The analysis revealed that the mass of the recombinant conglutinin of both the species were approximately 17kDa. Also there were putative peptides

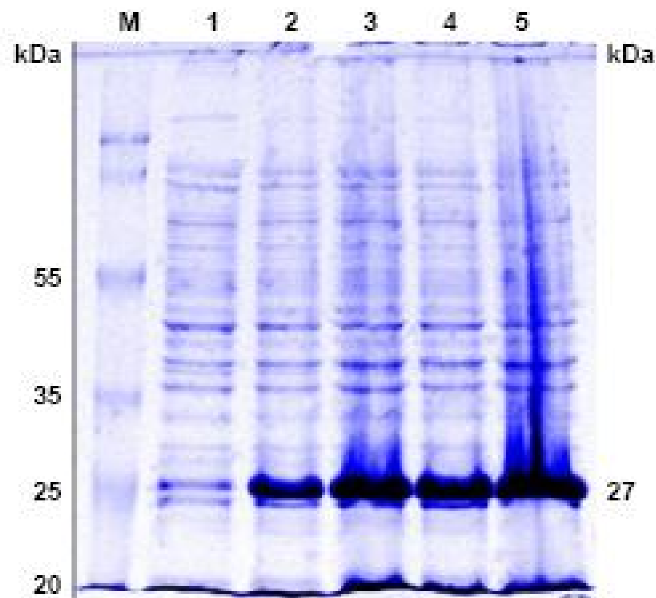


Fig. 12 : **Expression kinetics of buffalo recombinant conglutinin (rBuCGN) upon 1mM IPTG induction in *E. coli* BL21 cells as analyzed in 12% SDS-PAGE**

Lane M : Protein molecular weight marker
Lane 1 : 0 h
Lane 2 : 1 h
Lane 3 : 2 h
Lane 4 : 3 h
Lane 5 : 4 h

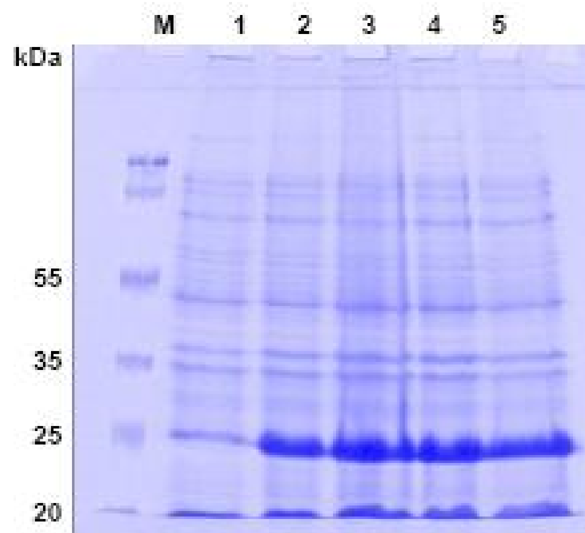


Fig. 13 : **Expression kinetics of nilgai recombinant conglutinin (rNCGN) upon 1mM IPTG induction in *E. coli* BL21 cells as analyzed in 12% SDS-PAGE**

Lane M : Protein molecular weight marker
Lane 1 : 0 h
Lane 2 : 1 h
Lane 3 : 2 h
Lane 4 : 3 h
Lane 5 : 4 h

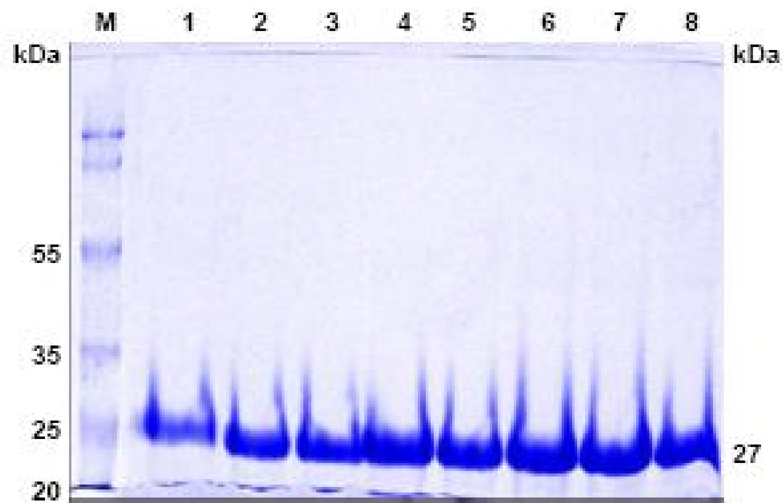


Fig. 14 : **Purification of buffalo recombinant conglutinin (rBuCGN) using Ni-NTA affinity column chromatography as analyzed in 12% SDS-PAGE**

Lane M : Protein molecular weight marker

Lane 1-8 : Eluted fractions

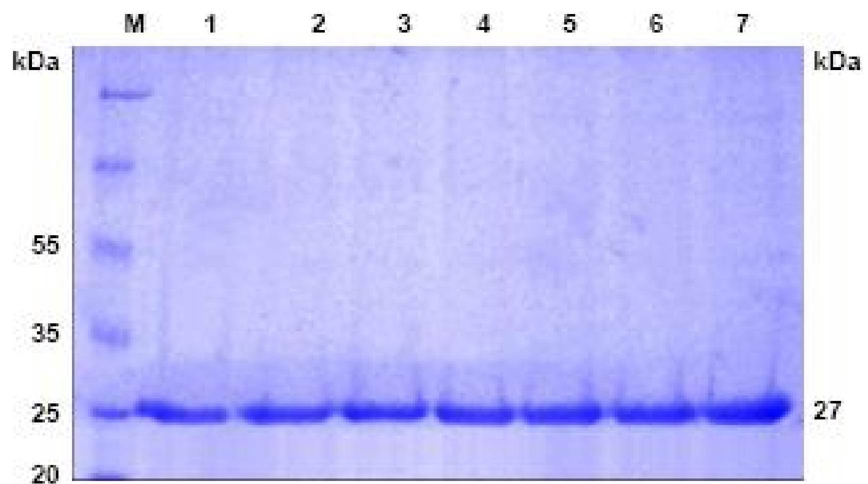


Fig. 15 : **Purification of nilgai recombinant conglutinin (rNCGN) using Ni-NTA affinity column chromatography as analyzed in 12% SDS-PAGE**

Lane M : Protein molecular weight marker

Lane 1-7 : Eluted fractions

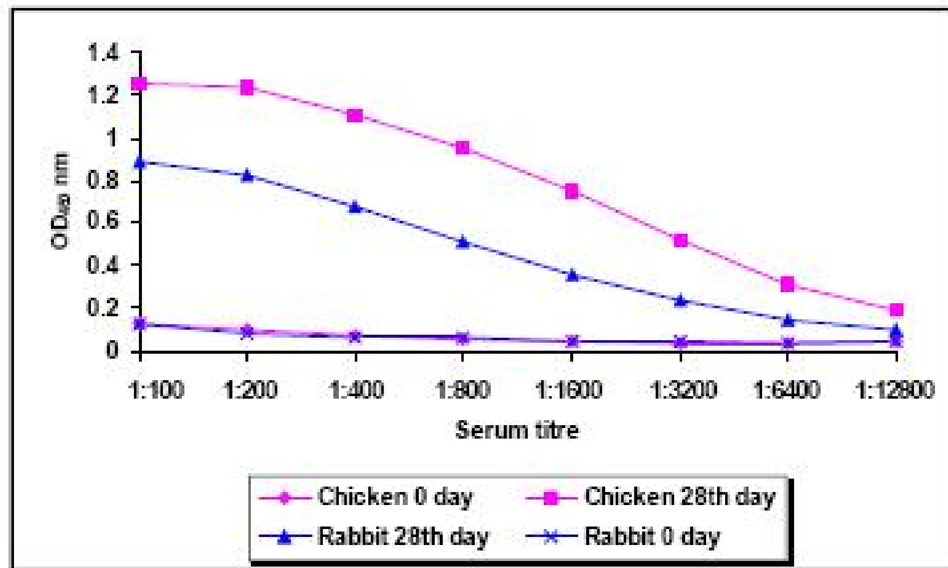


Fig. 16 : Antibody titre of polyclonal antisera raised against BuCGN in rabbit and chicken using direct ELISA

of buffalo conglutinin which were matching with the conglutinin 1 of nilgai and vice versa (Fig: 17 and 18). Blasting these putative peptides in NCBI database clearly indicates that these peptides sequences were conserved sequences of C-type lectin family, to which conglutinin also belongs. Thus, the identity of the rBuCGN and rNCGN was established.

4.2.5.2 Western blotting

The recombinant conglutinin of respective species were characterized by western blotting using anti-buffalo conglutinin antibody (1:400). The reactivity of 27kDa protein in western blot of p-RSET-BuCGN and p-RSET-NCGN transformed bacterial lysate, eluted protein fraction from affinity column and the re-natured protein in lane 1, 2 and 3, respectively confirms the presence of buffalo and nilgai conglutinin (Fig: 19 and 20).

4.3 Functional evaluation of rBuCGN and rNCGN

In order to confirm the functional activities of the rBuCGN and rNCGN, certain basic assays were performed which can determine the characteristic properties of conglutinin.

4.3.1 Sugar binding assay

Binding of the rBuCGN and rNCGN to the sugars like mannan in the presence of Ca^{+2} as analyzed using ELISA method is depicted in Fig; 21 and 22. In both the species, as the concentration of protein used were from 0.1 to 1000 ng/ml, the reactivity of the protein also increased and reached maximum value at 1000ng/ml.

4.3.2 Sugar binding inhibition assay

It is evident from Fig: 21 and 22 that sugar binding with rBuCGN was inhibited in the presence of 10mM EDTA. The maximum inhibition of 54.4% in case of rBuCGN and 59.11% in case of rNCGN was

(MATRIX SCIENCE) Mascot Search Results

```
User          :
Email         :
Search title  : Project: Proteomics, Spot Set: Proteomics\120419, Label: A21, Spot Id: 168862, Peak lis
MS data file  : C:\Program Files\applied biosystems\Proteomics\120419\ppw_A21_133490413518.txt
Database      : LudwigNR Q112_generic_forward (16818973 sequences; 5891363821 residues)
Taxonomy      : Other mammalia (881840 sequences)
Timestamp     : 24 Apr 2012 at 02:28:51 GMT
Protein hits  : E7DSS8 tr|E7DSS8|Conglutinin 1 (Fragment) Tax_Id=89462 [Bubalus bubalis]
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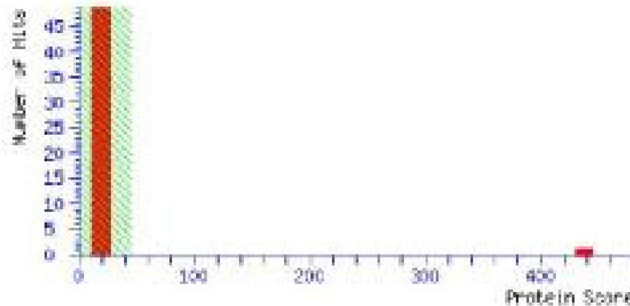
Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 44 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. E7DSS8 **Mass:** 17877 **Score:** 437 **Matches:** 9(8) **Sequences:** 7(6)
tr|E7DSS8|Conglutinin 1 (Fragment) Tax_Id=89462 [Bubalus bubalis]

☐ check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑	2	1023.4923	1022.4850	1022.5873	-0.1023	0	55	0.0048	1	U	R.VTILDGHLR.R
☑	9	1179.5323	1178.5250	1178.6884	-0.1634	1	46	0.039	1	U	R.VTILDGHLRR.F
☑	13	1260.4595	1259.4522	1259.6298	-0.1776	1	69	0.00016	1	U	R.FQNAFSQYRK.A
☑	16	1288.4801	1287.4728	1287.6360	-0.1632	1	58	0.0023	1	U	R.RFQNAFSQYR.K
☑	24	1463.4768	1462.4695	1462.6722	-0.2027	0	71	9.4e-05	1	U	R.SIAENEAVTQMVR.A
☑	26	1479.4305	1478.4232	1478.6671	-0.2439	0	(52)	0.0081	1	U	R.SIAENEAVTQMVR.A +
☑	31	1570.4763	1569.4690	1569.7093	-0.2403	0	102	9.4e-08	1	U	K.NAYLSNDDISTEGR.F
☑	33	1586.4385	1585.4312	1585.7042	-0.2730	0	(68)	0.00023	1	U	K.NAYLSNDDISTEGR.F
☑	41	1888.6073	1887.6000	1887.8407	-0.2407	0	35	0.36	1	U	R.FTYPTGEILVYSNWAK.

Proteins matching the same set of peptides:
E7DSS9 **Mass:** 17877 **Score:** 437 **Matches:** 9(8) **Sequences:** 7(6)
tr|E7DSS9|Conglutinin 1 (Fragment) Tax_Id=9917 [Boselaphus tragocamelus]

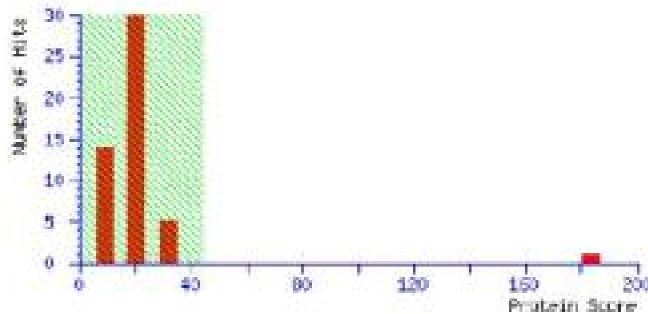
Fig. 17 : Mass spectrometric analysis of rBuCGN

Mascot Search Results

User :
Email :
Search title : Project: Proteomics, Spot Set: Proteomics\120419, Label: A22, Spot Id: 864863, Peak Lis
MS data file : C:\Program Files\Applied Biosystems\Proteomics\120419\pp4_A22_1334904613819.txt
Database : LudwigNR Q112_generic_forward (16618977 sequences; 5691348021 residues)
Taxonomy : Other mammalia (681640 sequences)
Timestamp : 24 Apr 2012 at 02:25:25 GMT
Protein hits : E7D888 tr E7D888|Conglutinin 1 (Fragment) Tax_Id=89462 [Bubalus bubalis]

Mascot Score Histogram

Ions score is $-10*\mathrm{Log}(P)$, where P is the probability that the observed match is a random event.
Individual ions scores > 44 indicate identity or extensive homology ($p<0.05$).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. E7D888 Mass: 17377 Score: 183 Matches: 4(3) Sequences: 4(3)
tr|E7D889|Conglutinin 1 (Fragment) Tax_Id=89462 [Bubalus bubalis]

Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
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4	1179.5688	1178.5615	1178.6884	-0.1269	1	48	0.023	1	U	R.VTILDGHLRR.F
6	1288.5056	1287.4983	1287.6360	-0.1377	1	65	0.00043	1	U	R.RFQNAFSQYK.E
14	1479.4591	1478.4518	1478.6671	-0.2153	0	17	27	4	U	R.SEAEMEAVTQMVR.A +

Proteins matching the same set of peptides:
E7D889 Mass: 17377 Score: 183 Matches: 4(3) Sequences: 4(3)

tr|E7D889|Conglutinin 1 (Fragment) Tax_Id=9917 [Boselaphus tragocamelus]

Fig. 18 : Mass spectrometric analysis of rNCGN

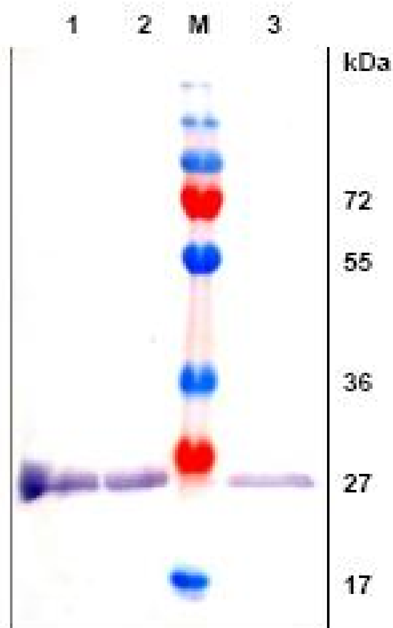

Fig. 19 : Western-blot analysis of buffalo recombinant conglutinin (rBuCGN) using anti-rBuCGN chicken poly clonal serum

Lane M : Pre-stained protein molecular weight marker
Lane 1 : pRSET-BuCGN transformed IPTG induced *E.coli* BL21 cell lysate
Lane 2 : Elute from Ni-NTA affinity column chromatography
Lane 3 : Dialysate

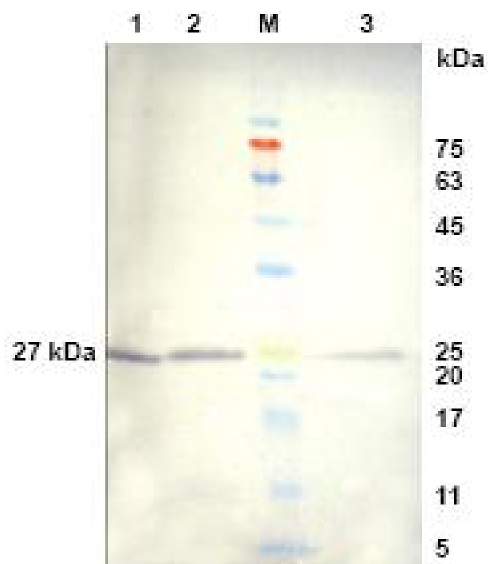

Fig. 20 : Western-blot analysis of nilgai recombinant conglutinin (rNCGN) using anti-rBuCGN chicken poly clonal serum

Lane M : Pre-stained protein molecular weight marker
Lane 1 : pRSET-NCGN transformed IPTG induced *E.coli* BL21 cell lysate
Lane 2 : Elute from Ni-NTA affinity column chromatography
Lane 3 : Dialysate

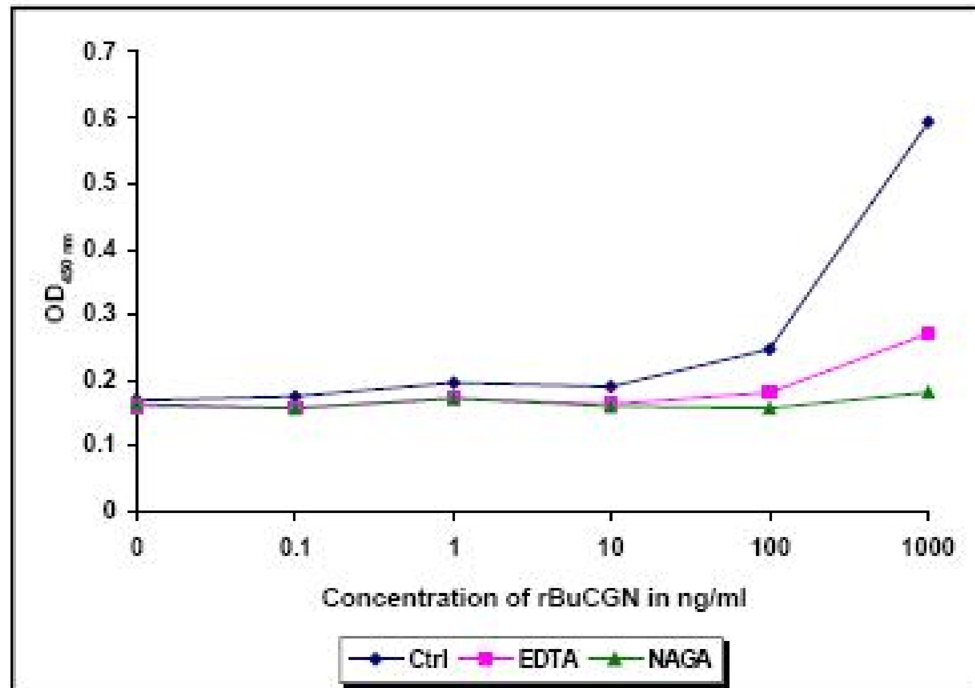

Fig. 21 : Inhibition of binding of rBuCGN to coated mannan by 20 mM N-acetyl-D-glucosamine and 10 mM EDTA

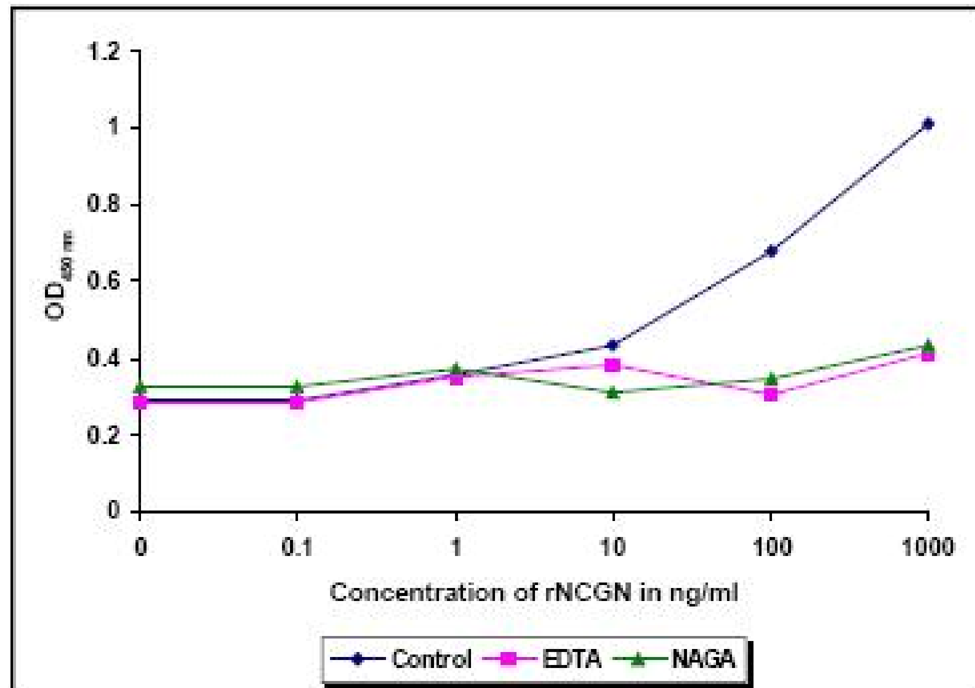

Fig. 22 : Inhibition of binding of rNCGN to coated mannan by 20 mM N-acetyl-D-glucosamine and 10 mM EDTA

observed at 1000 ng/ml. Similarly, inhibition in mannan binding with rBuCGN and rNCGN were observed in presence of 20mM NAGA with maximum inhibition of 69.61% and 57.24%, respectively at 1000 ng/ml (Fig: 23).

4.3.3 LPS binding assay

Binding of the rBuCGN and rNCGN to bacterial LPS were analyzed using ELISA method and the results are depicted in Fig: 24 and 25. In both the species, as the concentration of protein used were from 0.1 to 1000 ng/ml, the binding affinity of recombinant conglutinin towards the bacterial LPS were found to increase only in absence of the calcium. However, when calcium was included in the buffer (Tris buffered saline), no reactivity was observed at any concentration.

4.3.4 Haemagglutination inhibition assay

Agglutination of chicken erythrocytes by 4 HAU of New Castle Disease viruses was observed in the presence or absence of calcium ions Fig: 26. However, no inhibition of viral agglutination reaction was observed in the presence of either rBuCGN or rNCGN in the concentration range of 2.5 -20 μg/ml.

4.3.5 Virus neutralization assay

rBuCGN and rNCGN used in neutralization of BHV-1 propagated in MDBK cells were found to reduce the relative BHV-1 titre , compared to virus control (Fig: 27). The reductions in the titre upon treatement with both the recombinant proteins were clearly depicted in the table 2.

4.4 Standardization and validation of sandwitch ELISA

In order to detect conglutinin in buffalo serum, sandwitch ELISA was standardized using anti-buCGN rabbit hyperimmune serum as

Table 2 : Relative titer values of the BHV-1 after incubation of the virus with the different concentrations of rBuCGN and rNCGN (24hrs) and reduction in the relative titre percentage

	rBuCGN 4.5 µg/ml	rBuCGN 2.25 µg/ml	Virus control	rNCGN 4.5 µg/ml	rNCGN 2.25 µg/ml
BHV-1 titre in $TCID_{50}$	3.16×10^4	1×10^4	4.64×10^4	1×10^4	2.15×10^3
Relative titre percentage	31.89	78.44	100	78.44	95.0
Reduction in the relative titre percentage	68.11	21.5	0.00	21.5	5

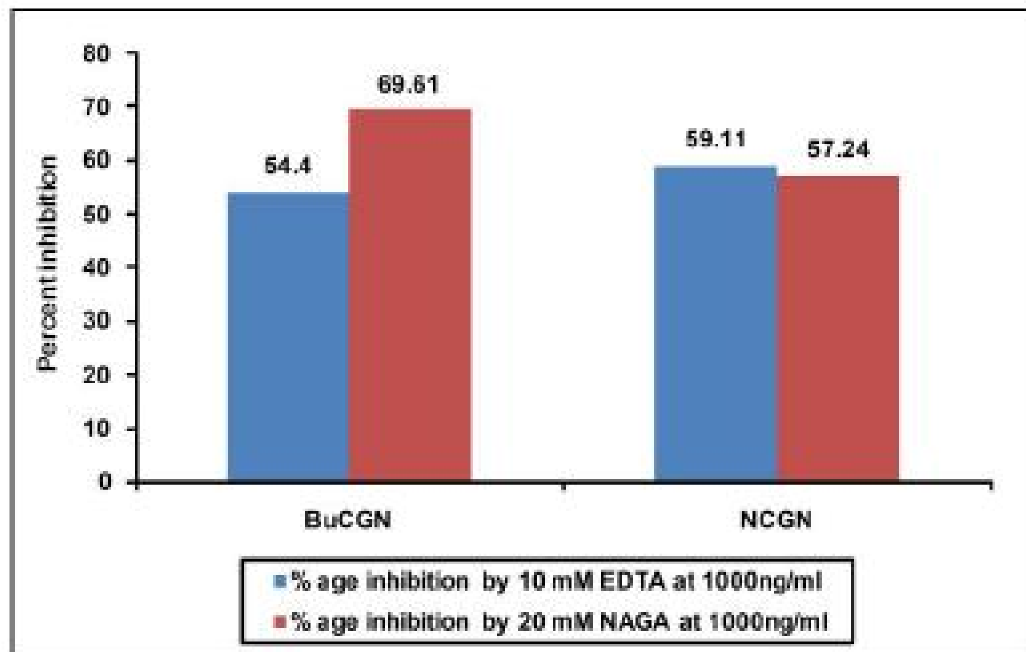

Fig. 23 : Percentage inhibition of binding of BuCGN and NCGN to coated mannan by EDTA and NAGA at 1000 ng/ml

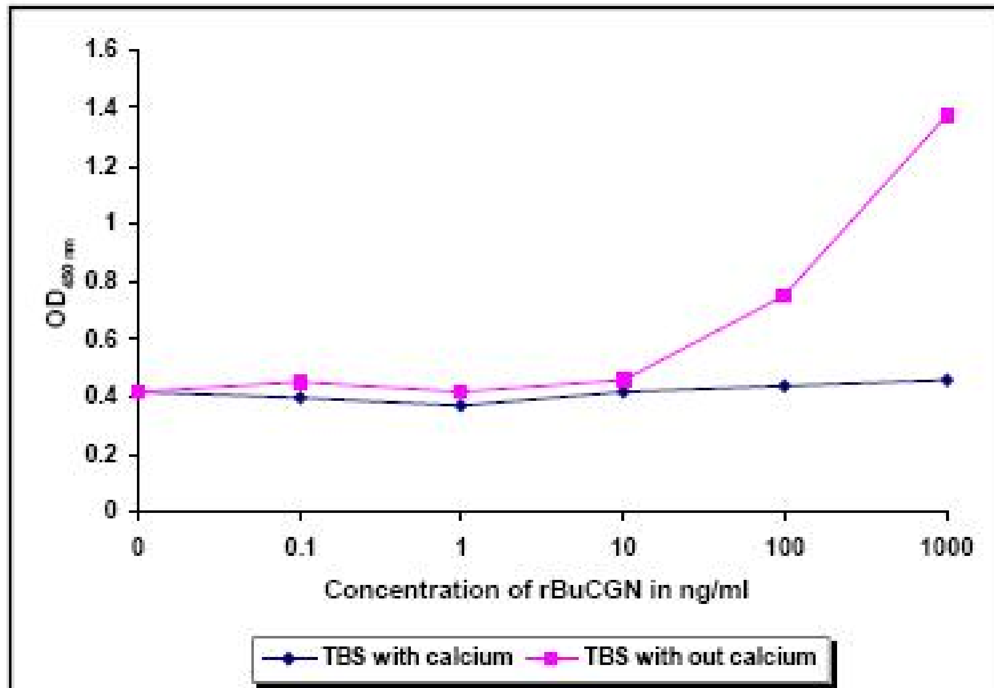

Fig. 24 : LPS binding assay of rBuCGN with and without 10 mM calcium chloride

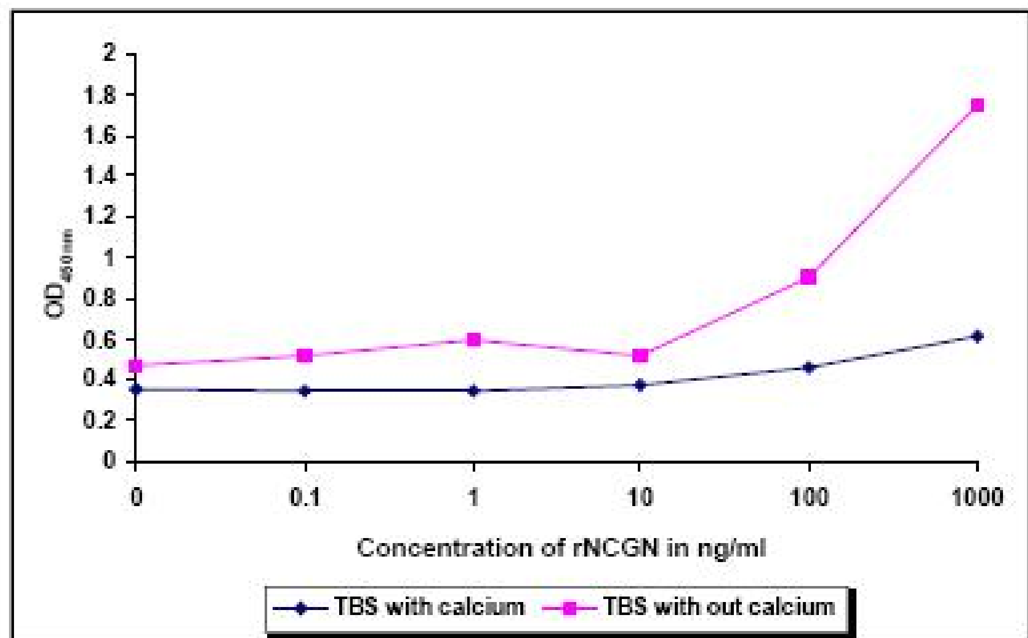

Fig. 25 : LPS binding assay of rNCGN with and without 10 mM calcium chloride

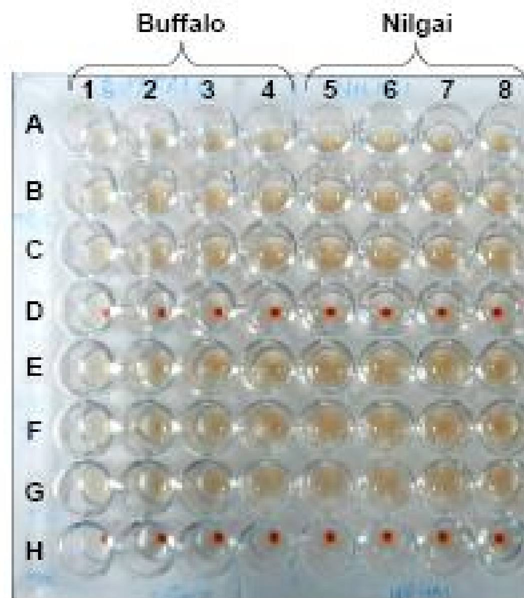

Fig. 26 : Haemagglutination inhibition of rBuCGN and rNCGN using 4HAU of NDV virus and 1% chicken RBC in TBS

A to D : TBS with 10 mM $CaCl_2$
E to H : TBS without 10 mM $CaCl_2$
C & G : 4HAU NDV virus + 1% chicken RBC
D & H : 1% chicken RBC in TBS
A & B : 1 : 2.5 µg/ml; 2 : 5 µg/ml; 3 : 10 µg/ml; 4 : 20 µg/ml of rBuCGN
5 : 2.5 µg/ml; 6 : 5 µg/ml; 7 : 10 µg/ml; 8 : 20 µg/ml of rNCGN
E & F : 1 : 2.5 µg/ml; 2 : 5 µg/ml; 3 : 10 µg/ml; 4 : 20 µg/ml of rBuCGN
5 : 2.5 µg/ml; 6 : 5 µg/ml; 7 : 10 µg/ml; 8 : 20 µg/ml of rNCGN

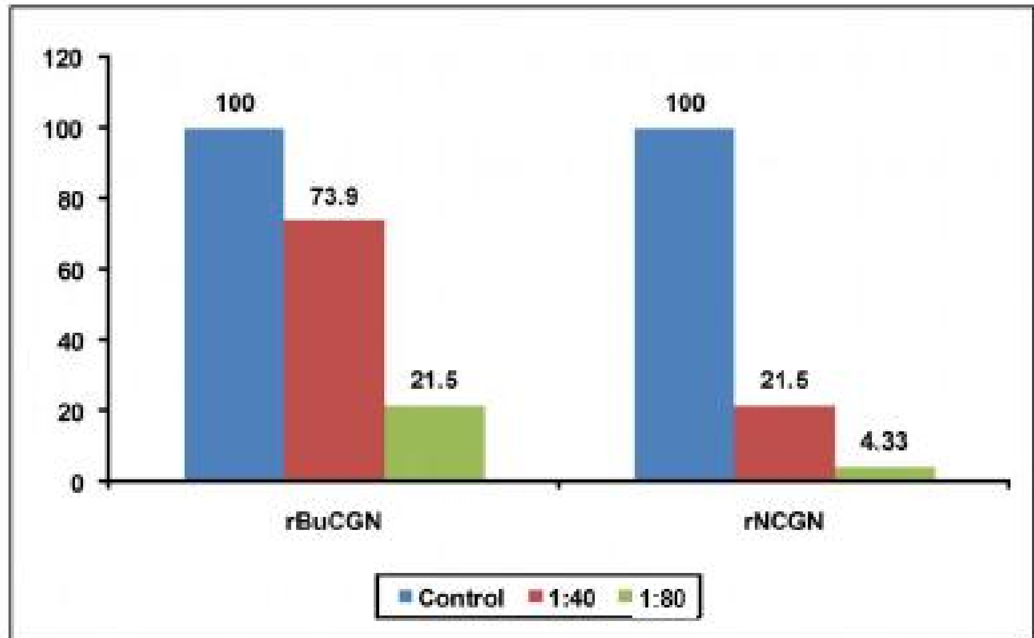

Fig. 27 : Relative plaque % of the BHV-1 (4 x 10^5 $TCID_{50}$) on MDBK cells upon incubation with rBuCGN or NCGN

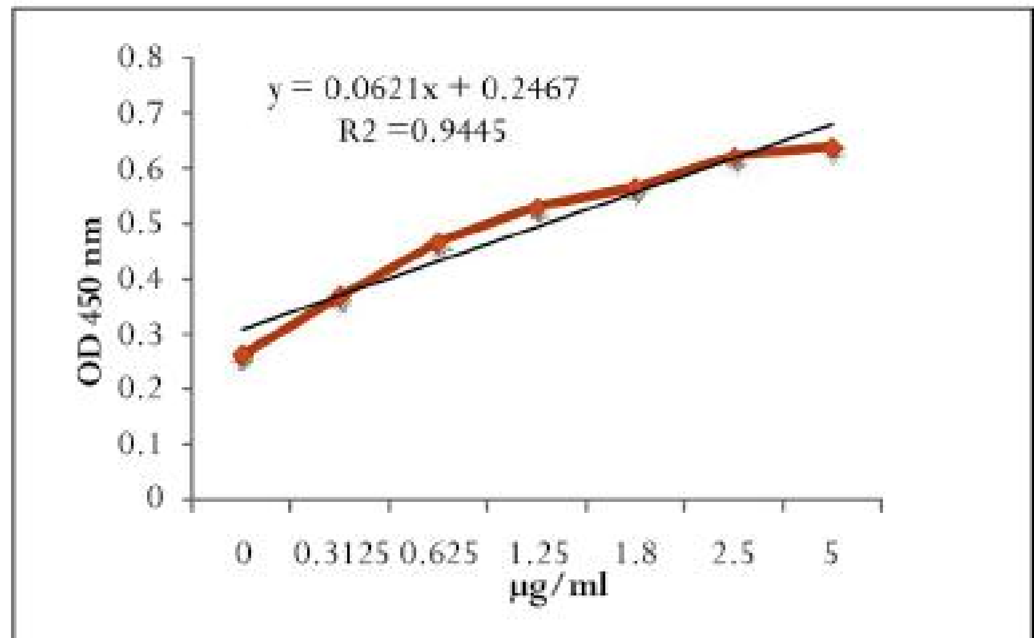

Fig. 28 : Quantitative sandwitch ELISA to quantitate native serum conglutinin

capture antibody, buffalo recombinant conglutinin (rBuCGN) as standard antigen, anti-buCGN poultry hyperimmune serum as detecting antibody. Linear regression (Y=0.0621+0.2467), R^2 =0.9445) was obtained when concentration (in X- axis 0.3125 to 5 μg/ml) Vs. OD450 (in Y-axis) was plotted in the standard curve (Fig: 28). Out of nine buffalo sera, no reactivity was observed in three samples. Conglutinin concentration in six positive samples, as calculated using the straight line equation, was found to be in the range of 1.9 to 53.9 μg/ml).

Discussion

5 Discussion

Conglutinin, a high molecular-weight C-type lectin which was originally detected in bovine serum, belongs to the family of collectins that bind sugar residues in a Ca^{2+}-dependent manner and act as effector molecules in innate immunity. Collectins are secretory pattern recognition receptors that can act as opsonins and cause the destruction of microorganisms by stimulating phagocytic cells. Studies on conglutinin revealed that it plays an important role in host immune defense mechanisms, showing antiviral and antibacterial activities both *in vivo* and *in vitro*. The defensive mechanisms were mainly mediated through binding to microbial surface carbohydrates, thereby inducing aggregation and preventing the spread of pathogens, and also by binding to complement, the degradation product iC3b, deposited on microbes (Lachmann *et al.*, 1967). The antimicrobial activity of the conglutinin mediated through the generation of oxygen metabolites by effector cells in the immune system has been noticed recently (Dec *et al.*, 2012). Though, the biological role of conglutinin is still not fully understood, yet several reports have indicated that this protein supports the immune system.

5.1 Characterization of cDNA encoding buffalo and nilgai conglutinin

The present study reports the presence of conglutinin mRNA in both buffalo liver and nilgai foetal liver for the first time. Moreover amplification of cDNA encoding the NCRD of conglutinin using primers

established for cattle (Wakamiya, 2005) suggested that at least primer binding region is conserved among buffalo, nilgai and cattle. The amplified product (497bp) contained 488bp conglutinin NCRD partial cds encoding the putative 162 amino acids and the additional flanking nucleotides covering restriction enzyme sites for directional cloning for expression studies. Nucleotide sequence alignment of the coding region revealed that the BuCGN is 96.5 % similar to bovine conglutinin, 93.9 % to sheep conglutinin, 99.9% to nilgai conglutinin on the other hand NCGN is 96.5% similar to bovine conglutinin and 93.4% similar to sheep conglutinin respectively. Likewise at amino acid level, BuCGN is 96.3 % similar to bovine conglutinin, 88.2 % to sheep conglutinin, and 100% to nilgai conglutinin on the other hand NCGN is 96.3% similar to bovine conglutinin and 88.8% similar to sheep conglutinin. Though the nucleotide sequence of buffalo and nilgai were found to vary at only one nucleotide but their amino acid sequences were found 100% similar. Five â-sheets in the predicted secondary structure of rBuCGN and rNCGN were observed for the first time. Recent report on conglutinin cross-reactivity to anti-bovine conglutinin antibody revealed that this versatile immunomodulatory lectin is not only confined to the members of the bovidae family but also exists in serum of the other monogastric animals like pig, horse, rabbit, sheep, goat etc (Kania *et al.*, 2010, Dec *et al.*, 2011). Though the presence of the conglutinin in other ruminants of bovidae family was reported recently using western blotting (Dec *et al.*, 2011) but present study is the first to report the characterization of this novel gene in buffalo and nilgai.

5.2 Characterization of recombinant buffalo and nilgai conglutinin

In the present study recombinant partial conglutinin of buffalo and nilgai origin comprising neck and carbohydrate binding domain

has been expressed in prokaryotic system for the first time. The expressed recombinant protein both rBuCGN and rNCGN were found to be 27kDa which is similar in molecular weight to that of recombinant bovine conglutinin (Wakamiya *et al.*, 2005). Expression as fusion protein in this system accounted for higher observed molecular mass (27kDa) than expected (17kDa) on the basis of coding region sequence. The full length of the conglutinin protein consists of 351 amino acids of which in the present study C-terminal (191-351) 162 amino acids have been expressed in prokaryotic system. Of these 162 amino acids, the presence of four cystein at 65, 137, 151, and 159 positions in forming the active dodecameric structure has been shown to be of functional importance for bovine conglutinin in physiological system. These positions for cysteine residues were also found conserved in predicted structure of both buffalo and nilgai conglutinin. The recombinant conglutinin of both the species were found to contain two Gly-X-Y repeats initially like G-E-S and G-L-A at 1^{st} and 4^{th} positions respectively, which is also present at the similar positions in the recombinant bovine conglutinin expressed by Wakamiya *et al.*, (2005). Potent N-glycosylation site Asn-Asn-Ser (N-N-S) which was present in bovine conglutinin (Lee *et al.,* 1991) was noticed in the recombinant conglutinin of both the species at 127-129 residues.

Further, mass spectrometric analysis of the recombinant protein identified the recombinant protein of both the species as conglutinin as number of hits which the MALDI-TOF analysis revealed for both the species were known to be conserved for C-type lectin family kinds of proteins including conglutinin. The conserved peptides sequences among bovine, buffalo and nilgai conglutinin thus depicted the evolutionarily and functionally conserved nature of conglutinin in the ruminant immune system. Cross reactivity of the rNCGN towards anti-buffalo conglutinin antibody was established by western blotting and ELISA which also suggests the evolutionarily and functionally

conservedness of the protein among wild and domestic ruminants. Similar observations showing the presence of the conglutinin in various bovid and non-bovid species using western blotting with polyclonal serum against bovine conglutinin have been recoded recently (Kania, *et al.*, 2010, Dec *et al.*, 2011).

5.3 Functional evaluation of recombinant buffalo and nilgai conglutinin

The recombinant buffalo and nilgai conglutinin was found to be functionally active. Present study clearly indicates that the binding of the rBuCGN and rNCGN to mannan is calcium dependent as the binding activity is inhibited in presence of the calcium chelating agent EDTA and is inhibited by N-acetyl glucosamine for which bovine conglutinin is known to possess much higher affinity than mannan. These observations are in corroboration with the findings of the Wakamiya for bovine conglutinin (2005).

However LPS binding of rBuCGN and rNCGN is not agreeable with that of the results of Lim *et al.,* (1994) because these proteins were found to be actively binding to the coated LPS in the absence of calcium. This difference could be attributed to the improper orientation of the calcium recognition domain in these species as compared to bovine conglutinin because of the presence of the glutamate (E) just before the calcium recognition domain instead of aspartate (D) and histidine (H) instead of phenyl alanine (F) in the vicinity of the calcium binding domain as compared to cattle.

Haemagglutination inhibition assay of the recombinant conglutinin rBuCGN and rNCGN revealed no HI activity against NDV in the present study. That could be attributed to the difference in the pattern of the orientation and variable compositions of hemagglutinin between influenza (orthomyxoviridae) and NDV virus

(paramyxoviridae). Although both the viruses can agglutinate chicken RBC but the HI is mainly noticed in influenza virus using bovine conglutinin by Wakamiya *et al.* (2005).

BHV-1 neutralization by rBuCGN and rNCGN in current study is the first report invitro antiviral activity of these proteins. Similar kind of influenza virus neutralizing effect of bovine conglutinin has been observed by Wakamiya *et al.*, (2005). This reduction in the titer values of neutralized virus may be attributed to the sugar binding ability of the rBuCGN and rNCGN towards the surface glycoprotein's of the viral particles.

5.4 Standardization and validation of sandwitch ELISA

Information on the assessment of the serum level of bovine conglutinin is very scanty. Studies carried out by Akiyama et al. (1992) reported the assessment of the serum concentration of the bovine conglutinin in sandwich ELISA using native conglutinin as standard. The present study indicated that sandwich ELISA using rBuCGN as standard antigen and the antiserum raised against rBuCGN in rabbit and chicken as capturing and detecting antibody, respectively, can be employed to show the existence of native conglutinin in serum and also to quantitate the normal serum level of this novel protein in. As such, there is no basal data regarding the mean serum concentration of the buffalo conglutinin till date. The present study observed 19 to 53.9 μg/ml conglutinin in sera samples in sand witch ELISA. This range appears to be wider than the range reported for bovine sera (8-38 μg/ml) by Holmskov *et al.* (1998). However, the assay developed in the current study has to be employed in the wide range of the serum samples with respect to age, sex, physiological state and pathological conditions of buffaloes to establish the normal values.

Summary and Conclusions

Summary and Conclusions

6.1 Summary

Conglutinin was known as soluble PRRs that belong to the family of innate immunity markers called collectins consisting C-type lectin domain, formerly thought to be confined to bovidae. The current study was undertaken to amplify, clone and characterize buffalo and nilgai conglutinin gene encoding Neck and Carbohydrate Recognition Domain, to express this region in prokaryotic system and then to characterize the recombinant protein (rBuCGN and rNCGN), followed by functional evaluation of these recombinant proteins using *in vitro* assays, development of sandwitch ELISA to detect the normal serum concentration of conglutinin in buffalo serum and elucidate the mean serum concentration of this novel protein. The outcome of the research has been summarised below.

6.1.1 Cloning and characterization

Total RNA was isolated from liver tissues of buffalo and nilgai with TRIzol, and cDNA was synthesized using oligoDT primers. The conglutinin genes of respective species were amplified with primers established for bovine conglutinin using proof reading enzyme. The amplicon (497bp) was cloned into cloning vector (pJET), the recombinant plasmids (pJET-BuCGN and pJET-NCGN) were characterized with *Pst* I restriction analysis released three fragments of 256bp, 566bp and 2.8 kd from both the recombinant plasmids.

6.1.2 Sequencing and sequence analysis

The recombinant plasmids (pJET-BuCGN and pJET-NCGN) were sequenced, the sequence obtained revealed 488bp nucleotide region encoding neck and CRD portion of conglutinin. This sequence when aligned with other available sequences using Megalign (DNA star) demonstrated >93% similarity with cattle and sheep conglutinin sequences. The secondary structure obtained for predicted amino acid sequence with online software Swiss prot and BuCGN and NCGN were found to contain 162 amino acids and molecular mass of 17kDa and possessed 5β sheets in the secondary structure. The presence of cysteine at 65, 137, 151, and 159 positions in forming the active dodecameric structure that has been shown to be of functional importance for conglutinin in physiological system was found to be conserved in the predicted structure of buffalo as well as nilgai conglutinin.

6.1.3 Sub cloning and expression

The insert (497bp) was released from pJET-BuCGN and rNCGN plasmids using *EcoR*I and *Xho*I double digestion and so obtained insert was re-ligated to prokaryotic expression vector pRSET-A and transformed into BL(21)-pLys competent cells. Positive colonies were grown in LB broth with 1mM IPTG as inducer for 4 h. Cells were pelleted every one hour up to 4h and expression profile was studied in 12% SDS-PAGE analysis.

6.1.4 Purification and protein analysis

The bacterial cells expressing recombinant protein (rBuCGN and rNCGN) were lysed and the expressed proteins were purified using Ni-NTA agarose affinity chromatography and eluted with urea buffer at pH 4.5. Eluted protein was renatured by slow dialysis and purification

was confirmed with SDS-PAGE analysis. Thus obtained proteins of both the species were subjected to mass spectrometric analysis and western blot analysis and confirmed it to be conglutinin of respective species.

6.1.5 Developing hyperimmune serum aginst rBuCGN in rabbit and chicken

Polyclonal antisera was raised in rabbit and chicken using rBuCGN and effective titer values of these sera used for the antibody related assays were elucidated using the direct ELISA. The titer of the chicken antiserum was calculated to be 1:6400 and that of rabbit was 1:3200.

6.1.6 Functional characterization of recombinant conglutinin

The sugar binding activity of conglutinin was checked by employing ELISA system. Conglutinin was allowed to bind yeast mannan coated to the microtiter plate in the presence of calcium chloride. Binding activity inhibited in presence of 20mM NAGA or 10mM EDTA suggested high affinity of rBuCGN and rNCGN to NAGA and requirement of Ca^{2+} for sugar binding activity of this protein respectively. LPS binding assay revealed that both rBuCGN and rNCGN bind to the coated LPS in calcium independent manner. None of the recombinant proteins of either species demonstrated HI activity against the 4HAU ND virus in presence of 1% chicken RBC. Virus neutralization assay clearly depicted that both rBuCGN and rNCGN possessed neutralizing effect on the multiplication of BHV-1 in MDBK cells.

6.1.7 Standardization and validation of sandwitch ELISA

Sandwitch ELISA was standardized using anti-rBuCGN rabbit hyperimmune serum as capture antibody, buffalo recombinant

conglutinin (rBuCGN) as standard antigen, anti-rBuCGN poultry hyperimmune serum as detecting antibody. Good correlation (R^2 0.94) was observed between conglutinin concentration upto 5µg/ml and absorbance. Six out of nine buffalo serum samples reacted in ELISA and conglutinin concentration in these positive samples, as calculated using the straight line equation, was found to be in the range of 1.9 to 53.9 µg/ml).

6.2 Conclusions

It can be concluded from the present piece of investigation that soluble innate immunity marker conglutinin is present in buffalo and nilgai and this is the first report of its molecular characterization in these wild and domestic ruminants. Neck and Carbohydrate Recognition Domain of this protein is encoded by 488 bp nucleotide sequences in both the species. This region expressed in prokaryotic system as 27kDa recombinant fusion protein possessed antigenic specificity of native conglutinin in buffalo. The present work was conceived with the hypothesis that nilgai conglutinin might stronger functional activities as compared to buffalo as observed in case of toll-like receptors (TLR2, TLR3, TLR4) in previous studies in our laboratory (Das et al., 2008; Dhara et al, 2007). However, similar primary and secondary structure and functional activities of rBuCGN and rNCGN observed in the present investigation are suggestive of similar immunity in buffalo and nilgai with respect to this soluble marker of innate immunity. However, the mean serum level of the protein may vary in these species and that has to be studied to further test the hypothesis of stronger innate immunity in nilgai versus buffalo as model for wild versus domestic ruminants. The recombinant protein of both species has been produced and quantitative sandwitch ELISA developed using anti-rBuCGN polyclonal sera in the current study

that has paved a way to establish the mean serum level of the native conglutinin in these ruminant species in future studies.

6.3 Suggestions for future work

1. Purification of native protein from serum samples and comparing the functional activities of recombinant versus native protein
2. Characterization of antiviral activity of the recombinant protein in other enveloped viruses
3. Establishing the normal values of mean serum concentration of conglutinin in buffalo and nilgai by testing large number of samples
4. Testing the presence of conglutinin in other wild and domestic species using anti-rBuCGN polyclonal serum.

Mini Abstract

Mini Abstract

Conglutinin, a high molecular-weight C-type lectin which was originally detected in bovine serum, belongs to the family of collectins that bind sugar residues in a Ca^{2+}-dependent manner and act as effector molecules in innate immunity. Current study revealed the cDNA sequence for NCR domains of BuCGN **HQ330990** and NCGN **HQ330991** and expressed these proteins in the prokaryotic system characterized them for the first time. Conglutinin NCR domain cDNA (497 bp) were amplified by proof reading DNA polymerase using primers established for *Bos taurus*. The amplicons so obtained were ligated into cloning vector, that was transformed into *E coli* DH5á competent cells and the recombinant plasmids (pJET-BuCGN and pJET-NCGN) were characterized by *Pst I* restriction analysis and sequencing. More than 90 % similarity was observed at nucleotide sequence and predicted amino acid level among the ruminant conglutinin. BuCGN was found to be 99.6% similar to NCGN at nucleotde level and 100% at predicted amino acid level. The predicted proteins of both the species (162 amino acids) were expected to possess five â-sheets in the secondary structure. For expression studies, 497 bp insert was released from the recombinant plasmids (pJET-BuCGN and pJET-NCGN) upon *EcoRI* and *XhoI* double digestion and subcloned into prokaryotic expression vector pRSET-A. Recombinant plasmids pRSET-BuCGN and pRSET-NCGN were transformed into *E-coli* BL21 competent cells. The recombinant colonies cultured in LB broth containing 1mM IPTG as inducer and collected at 0, 1, 2, 3 and 4 hrs after induction, demonstrated the expression of conglutinin as 27 kDa fusion protein in SDS-PAGE. The identity of recombinant proteins could be established by western blotting using anti-buffalo conglutinin poultry antiserum and mass spectrometry analysis. Both rBuCGN and rNCGN revealed high affinity for NAGA in functional assay as per characteristic feature. Inhibition of the sugar binding to mannan by EDTA suggested that this protein requires calcium ions for its functional activity. The binding of the rBuCGN and rNCGN towards LPS is calcium independent. Neither of the recombinant proteins revealed HA inhibition towards 4HAU NDV virus; however both rBuCGN and rNCGN were reduced the BHV-1 multiplication in MDBK cells. Sandwitch ELISA was standardized to detect and quantitate conglutinin level in buffalo serum using rBuCGN as standard. Reaction of nine buffalo sera in sandwitch ELISA suggested concentration in the range of 1.9-53.9 μg/ml.

लघु सारांश

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कलैक्टिन परिवार से संबधित एक प्रोटीन कॉनग्लूटिनिन, मूल रुप से गोजातीय सीरम में पहचाना गया जो कि कैल्शियम –निर्भर रीति से शर्करा अवशेषों से बन्धन करके सहज प्रतिरक्षा तंत्र में प्रभावी अणुओं का कार्य करता है । प्रस्तुत शोध में प्रथम बार भैंस व नीलगाय के कॉनग्लूटिनिन के ग्रीवा व शर्करा–पहचान क्षेत्रों को कोड़ित करने वाले सी.डी.एन.ऐ क्रमों को दर्शाया गया तथा इन प्रोटीनो को प्रोकैरिओटिक तंत्र में अभिव्यक्त करके उनका अभिलक्षण किया गया। नीलगाय व भैंस के जिगर से पृथकीकृत समस्त आर.एन.ए. से ओलिगो–डी.टी प्राईमरस् का प्रयोग कर सी.डी.एन.ए. का निर्माण किया गया। कॉनग्लूटिनिन के ग्रीवा व शर्करा–पहचान क्षेत्रों के 497 बेसयुग्म सी.डी.एन.ए. का गाय के लिये स्थापित प्राईमरस् का उपयोग कर प्रूफ रीड़िग डी.एन.ए.–पोलिमेरेस् द्वारा संवर्धन किया गया। इस प्रकार प्राप्त संवर्धित उत्पादों को क्लोनिंग वेक्टर में जोड़कर उसे ई.कोलाई डी.एच.5अल्फा समर्थ कोशिकाओं में रुपान्तिरित किया तथा रिकॉम्बिनैंट प्लॅास्मिड पी–जेट–बीयू.सी.जी.एन. व पी–जेट–एन.सी. जी.एन. को पी.एस.टी.1 ऐंजाईम विश्लेषण द्वारा अभिलक्षित किया गया। रूमन्थियों के कॉनग्लूटिनिन मे न्यूक्लिोटाईड़ कम व संभावी अमीनो अम्लो के कम के स्तर पर 90% से अधिक समानता पाई गई। नीलगाय व भैंस के कॉनग्लूटिनिन न्यूक्लिोटाईड स्तर पर 99.6% व संभावी अमीनो अम्लो के स्तर पर पूरे 100 प्रतिशत समान थे। दोनो प्रजातियों के 162 अमीनो अम्लो के संभावित प्रोटीन की सैकेन्डरी संरचना मे पॉँच बीटा–शीट की संभावना पाई गई। अभिव्यक्ति विश्लेषण के लिये, 497 बेस युग्म के जुडाव को रिकॉम्बिनैंट प्लॅास्मिड पी–जेट–बीयू.सी.जी.एन. व पी–जेट–एन.यू.सी.जी.एन. से ईको.आर.–1 व एक्स.एच.ओ.–1 दोहरे पाचन द्वारा पृथक किया गया तथा उसे प्रोकैरिओटिक अभिव्यक्ति वेक्टर पी.आर.सेट–ऐ. मे एकपुंजक किया गया। रिकॉम्बिनैंट प्लॅास्मिड पी.आर.सेट–बीयू.सी.जी.एन. व पी.आर.सेट–एन.यू.सी.जी.एन. को ई.कोलाई बी.एल.21 समर्थ कोशिकाओं में रुपान्तिरित किया । रिकॉम्बिनैंट बैक्टीरिया का 37^0 सैल्सियस पर एक मिली मोलर आई.पी.टी.जी.–युक्त एल.बी.माध्यम में संवर्धन किया गया। उत्पन्न करने के 0,1,2,3,4 घंटे के पश्चात एकत्रित बैक्टीरिया कोशिकाओ ने एस.डी.एस.पेज में 27 किलो डाल्टन के फ्यूज़न प्रोटीन की अभिव्यक्ति दर्शायी। इन प्रोटीनों की पहचान भैंस कॉनग्लूटिनिन के प्रतिरक्षी युक्त मुर्गी के सीरम द्वारा वेस्टर्न ब्लॉटिंग में तथा मास–सपेक्ट्रोमीटरी द्वारा स्थापित की गई। नीलगाय व भैंस के रिकॉम्बिनैंट कॉनग्लूटिनिन ने एन.ए.जी.ए. शर्करा के प्रति अधिक आकर्षण दर्शाया जो कि कॉनग्लूटिनिन विशेष गुण है। ई.डी.टी.ऐ द्वारा इस आकर्षण की रूकावट ने कैल्शियम की आवश्यकता को सुझाया। एल.पी.एस. के प्रति आर.–बीयू सी.जी.एन व आर.–एन.सी.जी.एन. का बन्धन कैल्शियम–निर्भरता से मुक्त पाया गया। दोंनो में से किसी भी प्रोटीन ने 4 एच.ए.यूनिट रानीखेत विषाणु के प्रति हिम आश्लेषण नहीं दर्शाया । परन्तु दोंनो ने गोजातीय हर्पीस विषाणु–1 के एम.डी.बी.के. कोशिकाओ में सवंर्धन को कम किया। इस प्रकार बैक्टीरिया–तंत्र में अभिव्यक्त रिकॉम्विनैंट प्रोटीन क्रियात्मक रूप से समरुप पाऐ गये। सीरम में कॉनग्लूटिनिन स्तर की पहचान व आंकलन के लिये आर.बीयू.सी.जी.एन. को मानक के रूप मे प्रयोग कर सैंडविच ऐलाईजा का मानकीकरण किया गया। इस जॉच मे नौ भैंसो के सीरम में क्रियाशीलता ने इस प्रोटीन का स्तर 1.9.53.9 माईक्रोग्राम प्रति मिलीलीटर सुझाया। भविष्य में भैंस के अधिक नमूनो में कॉनग्लूटिनिन स्तर को स्थापित करके यह जॉच सहज प्रतिरक्षा पहचान में सहायक सिद्ध होगी।

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Appendix

Appendix

Tris Acetate-EDTA (TAE) Buffer (50X)

Tris base	24.2 g
Glacial acetic acid	5.71 ml
0.5M EDTA (pH 8.0)	10 ml

Volume made upto 100ml with double distilled water, sterilized by autoclaving for 20minutes at 15psi on liquid cycle and stored at room temperature.

Ethidium Bromide Solution (10mg/ml)

10 mg of ethidium bromide is dissolved in 1 ml of autoclaved distilled water and wrapped with alluminium foil and atored at 4^0c.

Gel Loading Dye (6X)

Tris-HCl (pH 7.6)	10 mM
Bromophenol blue	0.03%
Glycerol	60%
EDTA	60 mM

Tris EDTA (TE) buffer

Tris-Cl (pH 8.0)	10mM
EDTA (pH 8.0)	1mM

Sterilized by autoclaving for 20min at 15psi on liquid cycle and stored at 4^0C

Stop mix for LMP agarose elution

10mM Ammonium acetate	2.5ml
0.5M EDTA (pH 8.0)	0.2ml

Volume made upto 5ml and sterilized by autoclaving for 20min at 15psi on liquid cycle and stored at 40C

Media for bacterial culture

LB (Luria-Bertani) Broth

Bact. Trypto	10.0 g
NaCl	10.0 g
Yeast Extract	5.0 g

Dissolved in 950 ml distilled water, pH was adjusted to 7.0 using 5N NaOH;volume made upto one liter and sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle

Luria Bertani (LB) Agar

1.5 % Bacto Agar was added in LB broth and sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle.

2M Mg^{++} Solution

2 M $MgCl_2$ and 2 M $MgSO_4$ were prepared separate;y and mixedin equal volume and filter sterilized through 0.22µm filters.

SOB medium

Tryptoone	2g
Yeast Extract	5g
NaCl	0.05g
250mM KCl	1ml
Deioniszed	90ml
Adust pH tp	7.0 with 5N NaOH

Volume made upto 100ml and sterilized by autoclaving for 20min at 15psi on liquid cycle and stored at 4^0C

SOC

Sterile SOB	800µl
Filtered- 2M Mg^{++}	4 µl
Filtered -1M Glucose	16 µl

TSS buffer

2X LB medium	20ml
DMSO	3 ml
30% w/v PEG	20ml
2M Mg^{++} solution	0.6ml

IPTG (100mM)

IPTG	23.83mg
Distilled water	1.0 ml

Sterilized by filtration through 0.22 mm filter and stored at -20^{0}C.

Solutions for plasmid isolation

Alkaline lysis solution (PI)

Glucose	50mM
Tris-Cl (pH8.0)	25mM
EDTA (pH 8.0)	10mM

Solution prepared from standard stocks and sterilized by autoclaving for 15min at 15 psi on liquid cycle.

Alkaline lysis solution (PII)

NaOH	0.2N
SDS	1.0%

Freshly prepared and stored at room temperature.

Alkaline lysis solution (PIII)

5M Potassium acetate	60.0ml
Glacial acetic acid	11.5 ml
DDW	28.5ml

The resulting solution is 3M with respect to potassium and 5M with respect to acetate and stored at 4^{o}C.

Solution for SDS-PAGE

Acrylamide-Bisacrylamide solution (30%)

Acrylamide	30 gms

Bisacrylamide	0.8 gms

Volume made to 100ml with distilled water.

Separating gel buffer (pH 8.4 – 8.6)

Tris	73.2 gms
TEMED	0.46 ml
SDS	1.6 gms

Adjust the pH to- 8.4-8.6 using 1N HCl and make the volume to 200ml.

Stacking gel buffer (pH6.8)

Tris	12.1 gms
TEMED	232 µl
SDS	3.2 gms

Adjust the pH to 6.8 using 1N HCl and then make up the volume to 100ml with distilled water.

Sample buffer (PH 6.8) (5X)

Tris	1.89gms
SDS	5gms
Glycerol	25 ml

Bromophenol blue few crystals

Adjust the pH and make up the volume to 50ml with distilled water.

Electrode Buffer

Tris	1.5 gms
Glycine	7.2 gms
SDS	0.5 gms

Volume made to 500ml with distilled water.

Staining solution

Acetic acid	10%
Methanol	45%

Coomasie Brilliant –Blue R-250	0.15%

Destaining solution

Acetic acid	10%
Methanol	30%

Add distilled water to make final volume to 100 ml

Ammonium persulphate

20% solution in distilled water.

Reagents for Western Blot

Transfer Buffer

Tris Base	14.4g
Glycine	3.0g
Methanol	20%

Add distilled water to make volume upto 1000ml

Tris Buffered Saline

Sodium Chloride	8g
Potassium Chloride	0.2g
Tris Base	3g

Add 800ml distilled water and adjust the pH to 8.0 with 1N HCl and make the volume to 1000ml, sterilized by autoclaving for 20minutes at 15psi on liquid cycle and stored at room temperature.

TBS-Tween

TBS	1000ml
Tween-20	500 µl

Blocking Buffer

Skimmed milk powder	3g
TBS	100ml

Phosphate buffered saline

Sodium chloride	8.06g
Potassium chloride	0.20g

Na_2HPO_4	1.44g
NaH_2PO_4	0.20g

Add distilled water upto 800ml and adjust the pH to 7.4 using 1N HCl and make the volume to 100ml, sterilized by autoclaving for 20minutes at 15psi on liquid cycle and stored at room temperature.

Substrate buffer for development

TMB	6mg
Hydrogen peroxide	10µl

Add PBS to make volume to 10ml citrate buffer.

Vitae

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