Evidence of a humoral immune response against the prokaryotic expressed N-terminal autoprotease (N^{pro}) protein of bovine viral diarrhoea virus

Niranjan Mishra[1*], Katherukamem Rajukumar[1], Shruti Shrikant Pitale[1], Anil Prakash[2], Ram Kumar Nema[1], Sthita Pragnya Behera[1] and Shiv Chandra Dubey[1]

[1]*High Security Animal Disease Laboratory, Indian Veterinary Research Institute, Anand Nagar, Bhopal 462 021, India*

[2]*Department of Biotechnology, Barkatullah University, Bhopal 462 026, India*

**Corresponding author (Fax, +91 755 2758842; Email, mishranir@rediffmail.com)*

Bovine viral diarrhoea virus (BVDV) is an economically important pathogen of cattle and sheep belonging to the genus *Pestivirus* of the family Flaviviridae. Although the BVDV non-structural N-terminal protease (N^{pro}) acts as an interferon antagonist and subverts the host innate immunity, little is known about its immunogenicity. Hence, we expressed a recombinant BVDV N^{pro}–His fusion protein (28 kDa) in *E. coli* and determined the humoral immune response generated by it in rabbits. The antigenicity of the N^{pro} protein was confirmed by western blot using anti-BVDV hyperimmune cattle, sheep and goat serum, and anti-N^{pro} rabbit serum. When rabbits were immunized with the N^{pro} protein, a humoral immune response was evident by 4 weeks and persisted till 10 weeks post immunization as detected by ELISA and western blot. Despite N^{pro}-specific antibodies remaining undetectable in 80 serum samples from BVDV-infected sheep and goats, BVDV hyperimmune sera along with some of the field cattle, sheep and goat sera with high BVDV neutralizing antibody titres were found positive for N^{pro} antibodies. Our results provide evidence that despite the low immunogenicity of the BVDV N^{pro} protein, a humoral immune response is induced in cattle, sheep and goats only with repeated BVDV exposure.

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1. Introduction

Bovine viral diarrhoea virus (BVDV) is an economically important pathogen of cattle and sheep, and causes significant respiratory and reproductive disease worldwide. Bovine viral diarrhoea virus type 1 (BVDV-1), BVDV-2 along with the border disease virus (BDV) and classical swine fever virus (CSFV) belong to the genus *Pestivirus* of the family Flaviviridae. The family Flaviviridae comprises two additional genera, *Flavivirus* and *Hepacivirus*. Two biotypes of BVDV exist depending on the presence or absence of cytopathic effect in cultured cells – cytopathic (cp) and non-cytopathic (ncp). Both cp and ncp BVDVs can cause acute infections, but only ncp BVDV gives rise to persistent infection (Baker 1987).

The BVDV genome consists of a single-stranded RNA about 12.3 kb in length. A long open reading frame (ORF) flanked by untranslated regions (UTRs) at both ends (5′ and 3′ UTR) is translated into a poly-protein of about 4000 amino acids and is cleaved into four structural (capsid [C] and three envelope [E^{rns}, E1 and E2] proteins) and 7–8 non-structural proteins (N^{pro}, p7, NS2-3 or NS2 and NS3, NS4A,

Keywords. Bovine viral diarrhoea virus; N^{pro} protein; expression; immunogenicity; humoral immune response

Abbreviations used: BDV, border disease virus; BVDV, bovine viral diarrhoea virus; cp, cytopathic; CSFV, classical swine fever virus; DTT, dithiothreitol; ELISA, enzyme linked immunosorbent assay; HRPO, horseradish peroxidase; IFN, interferon; IPTG, isopropyl-*β*-D-1-thiogalactoside; ncp, non-cytopathic; N^{pro}, N-terminal protease; OD, optical density; ORF, open reading frame; PBS, phosphate buffered saline; RT-PCR, reverse transcription polymerase chain reaction; TBS, Tris-buffered saline; UTR, untranslated region

NS4B, NS5A and NS5B) by viral and host cell proteases (Meyers and Thiel 1996). Despite the similarity in genome organization among all three genera of Flaviviridae, a unique feature of pestiviruses is the occurrence of an additional gene that encodes a non-structural 20 kDa protein, N-terminal protease (N^{pro}) at the 5′-end of the ORF (Rice 1996). N^{pro} is a papain-like cysteine protease and is responsible for generation of the mature N-terminus of the nucleocapsid protein (Rumenapf *et al.* 1998). Besides its protease activity, several studies have suggested that BVDV N^{pro} may act as an interferon (IFN) antagonist, which may account for the establishment of persistent infection and immunotolerance in cattle (Horscroft *et al.* 2005; Gil *et al.* 2006). However, disruption of the IFN response does not require the protease activity of N^{pro} (Gil *et al.* 2006; Hilton *et al.* 2006). It has also been shown that N^{pro} interferes with the primary cellular antiviral defence in CSFV-infected pigs (Ruggli *et al.* 2003). No report is available on the determination of humoral immune response against BVDV N^{pro} protein, although evidence of T-cell proliferation in cattle has been provided earlier (Collen and Morrison 2000).

In BVDV-infected animals, the humoral immune response allows detection of antibodies against the structural envelope proteins E2 and E^{rns}, as well as against the highly conserved non-structural protein NS3 (p80). BVDV N^{pro} protein does not appear to be immunogenic since sera from convalescent cattle do not contain antibodies to N^{pro} protein (Donis and Dubovi 1987). Although considerable knowledge has been gained regarding the modulation of IFN induction in host cells, little is known about the immunogenicity of the BVDV N^{pro} protein. The aim of this study was to determine the immune response against BVDV N^{pro}, which is a subject of considerable interest.

2. Materials and methods

2.1 *Construction of recombinant N^{pro} plasmid*

The N^{pro} coding region of a cattle BVDV-1 isolate Ind 1449 (Mishra *et al.* 2004) was amplified by reverse transcription polymerase chain reaction (RT-PCR) employing AMV reverse transcriptase and Tfl DNA polymerase enzymes (Access RT-PCR system, Promega), and primers corresponding to the N^{pro} sequence (forward primer: 5′-CATGGAGTTGATCACAAATGA-3′; reverse primer: 5′-GCAGCTTGAAACCCATAGGG-3′) at an annealing temperature of 55°C. The amplified N^{pro} DNA (504 bp) was purified from gel and cloned into the pGEMT-Easy vector (Promega) by the T/A cloning strategy to generate the recombinant plasmid N^{pro}-P. The recombinant plasmids were sequenced in both directions using a V.3.1 cycle sequencing kit (Applied Biosystems, USA) and an ABI 3130 automatic DNA sequencer (ABI, USA) employing M13 forward and reverse sequencing primers. After releasing the N^{pro} from the plasmid N^{pro}-P by *Eco*RI, the gel-purified DNA was subcloned into the *Eco*RI cloning site of the multisystem expression vector pTriEx-2-Neo (Novagen) bearing a histidine tag at the C-terminus to construct the expression plasmid pTriEx-2-Neo/N^{pro}.

2.2 *Transformation and selection of recombinant*

Transformation of *E. coli* Origami (DE3) p Lac1 competent cells (Novagen) with the expression plasmid pTriEx-2-Neo/N^{pro} was carried out according to the instructions of the manufacturer (Novagen). The cells were then plated onto LB agar containing 15 μg/ml kanamycin (Calbiochem), 12.5 μg/ml tetracycline (Calbiochem), 34 μg/ml chloramphenicol (Calbiochem) and 50 μg/ml carbenicillin (Amresco), and incubated at 37°C overnight. Individual colonies were then picked up and grown in LB broth containing antibiotics. The insertion of N^{pro} along with the presence of the ribosome-binding site, the start and stop codons provided by the vector were confirmed by sequencing the recombinant plasmids.

2.3 *Expression of N^{pro} as a fusion protein in* E. coli

A starter culture of the pTriex-2-Neo/N^{pro} recombinant in Origami cells was prepared by inoculating 100 μl of culture into 3 ml of LB broth containing 1% glucose and antibiotics, and grown overnight at 37°C with shaking at 250 rpm. Log-phase cultures (1.0 OD) were then induced with isopropyl-β-D-1-thiogalactoside (IPTG) at final concentrations of 0.5 mM, 0.75 mM and 1.0 mM. The cultures were grown at 37°C with shaking at 250 rpm for 4 h and 1 ml culture was collected at 0, 1, 2, 3 and 4 h following induction. The culture-bearing expression vector without any BVDV insert treated similarly served as a negative control. The total cell pellet (TCP) obtained after different hours of induction was analysed on 12.5% SDS-polyacrylamide gels run at 40 V and stained with Coomassie blue to detect the expression of N^{pro} as a fusion protein.

2.4 *Purification of N^{pro} fusion protein*

The culture showing expression of N^{pro} fusion protein was further fractionated into soluble lysate and inclusion body fractions using Bug buster reagent (Novagen), protease inhibitor cocktail set III (Calbiochem, Germany), recombinant lysozyme and benzonase (Novagen) according to the manufacturers' instructions. As the recombinant protein was in the form of inclusion bodies, it was solubilized in 1X His-binding buffer (Novagen) containing 6 M urea and purified by His-bind affinity chromatography under denaturing conditions as per the manufacturer's instructions (Novagen). The N^{pro}

protein was eluted from the resin by elution buffer (200 mM of imidazole in 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The flow-through, wash and elution fractions were electrophoresed on 12.5% SDS-polyacrylamide gels following standard procedures. The purified fractions of N^{pro} fusion protein were then pooled and subjected to dialysis using Tris-HCl buffer twice with dithiothreitol (DTT) and twice without DTT for 12 h each for refolding into the native state. The protein content was ascertained by spectrophotometry and aliquots were stored at –70°C.

2.5 *Immunizaion of rabbits against N^{pro} protein*

To determine whether BVDV N^{pro} protein is immunogenic, three male rabbits (Soviet Chinchilla) were immunized by intramuscular injection of 100 μg of purified BVDV N^{pro} fusion protein (1 ml volume) emulsified in 1 ml of complete Freund adjuvant (1:1 molar ratio) for the first immunization, and incomplete Freund adjuvant for subsequent immunizations at 2 and 4 weeks after the first immunizaion. Two more rabbits of the same age and breed were mock immunized (with adjuvants) as control. The sera from N^{pro}-immunized and control rabbits were collected at weekly intervals and stored at –20° C until use.

2.6 *Western blotting*

For western blotting, the proteins (40 ng per lane) were electrophoresed in 12.5% SDS-polyacrylamide gels at 40 V. Transfer of proteins onto a nitrocellulose membrane was carried out in a semi-dry electrophoretic transfer apparatus (Hoefer) at 40 V for 1 h using semi-dry transfer buffer (Tris–Glycine–Methanol). The membranes were then blocked in Tris-buffered saline (TBS) containing 1% non-fat dry milk (Sigma), washed with TBS-Tween 20 (0.05%) and incubated with 1/100 dilutions of BVDV-1 hyperimmune cattle, sheep and goat sera (raised during our previous work), N^{pro}-immunized rabbit serum or 1/1000 dilution of anti-histidine (RGS) monoclonal antibody conjugated to horseradish peroxidase (HRPO, Qiagen) in blocking buffer at 37°C for 1 h. After washing, the membranes were incubated with 1/5000 dilution of anti-mouse/rabbit, 1/2000 dilution of anti-bovine or 1/1500 dilution of anti-sheep/goat IgG conjugated to HRPO (Sigma) for 1 h at 37°C. The membranes were finally soaked in chromogen/substrate solution (DAB (3,3′-diaminobenzidine) and urea-H_2O_2, Sigma) for colour development.

2.7 *Humoral immune response against BVDV/N^{pro} protein*

The immune response against N^{pro} protein in rabbits was determined both by indirect ELISA and western blot. For ELISA, the purified and refolded N^{pro} fusion protein was coated at 150 ng/well (found optimum by titration) onto 96-well polystyrene microtitre plates (Greiner, Germany) and kept overnight at 4°C. After blocking with 3% lactalbumin hydrolysate in phosphate buffered saline (PBS) at 37°C for 1 h, the wells were washed. Rabbit sera at 8 weeks' post immunization were diluted two-fold starting at 1: 20 onwards and added to the wells in duplicate, and the plates were incubated at 37°C for 1 h. Anti-rabbit donkey IgG-HRPO at 1/10 000 dilution was used as conjugate and tetramethyl benzidine (TMB, Sigma) was used as substrate. Colour development was undertaken at room temperature and absorbance was determined at 450 nm. The cut-off level for a positive result at each dilution was established at three times the mean optical density (OD) of the mock-immunized rabbit serum. The antibody response against N^{pro} protein in rabbits was determined till 10 weeks post immunization by ELISA. Similarly, sera from rabbits collected at different weeks following immunization with N^{pro} protein were tested by western blot for correlating the immune response. Sera available from our earlier work from sheep and goats (80) acutely infected experimentally with BVDV, and field cattle, sheep and goat sera samples (30) submitted for detection of BVDV-neutralizing antibodies were also tested for the presence of anti-N^{pro} antibodies.

3. Results

3.1 *N^{pro} protein expression in* E. coli

One of the transformants able to grow in the presence of carbenicillin, kanamycin, tetracycline and chloramphenicol was selected for further characterization and expression. The insertion of the BVDV N^{pro} gene in this recombinant *E. coli* strain was detected by PCR, which generated a 504 bp DNA product. The expression level in the inclusion body and lysate was analysed at hourly intervals to determine the optimal conditions for harvesting. As revealed by SDS-PAGE analysis (figure 1), when induced with 1 mM IPTG, a fusion protein with the expected molecular weight of approximately 28 kDa (20 kDa for N^{pro} and 8 kDa for a part of the vector along with His-tag) was expressed in the inclusion body fraction of bacteria, which was subsequently purified. The highest level of expression was achieved 4 h post induction (data not shown). The expressed protein was not found in the cell supernatant (data not shown).

3.2 *Characterization of recombinant N^{pro} protein*

The antigenicity of the recombinant N^{pro} protein was characterized by western blot using cattle, sheep and

goat hyperimmune sera specific to BVDV as well as N^{pro}-immunized rabbit serum. The 28 kDa N^{pro} fusion protein could be recognized by BVDV hyperimmune cattle, sheep and goat sera, besides anti-N^{pro} rabbit sera, while there was no reactivity with the negative control sera (figure 2). The purity of the affinity-purified fusion protein was ascertained by SDS-PAGE (data not shown) followed by western blot with anti-His monoclonal antibodies, which revealed a single band of 28 kDa indicating the purity of the protein (figure 2).

3.3 *Specificity of anti-N^{pro} antibody*

At 8 weeks' post immunization, the rabbit sera were tested for the presence of anti-N^{pro} antibodies by indirect ELISA. The average OD values for both immunized and healthy rabbits starting from a dilution of 1:20 up to 1:2560 are shown in figure 3. The specificity of the anti-N^{pro} antibody was evident as demonstrated by a rise in the OD values in immunized rabbits as compared with controls. Considering the cut-off values as described in Materials and methods,

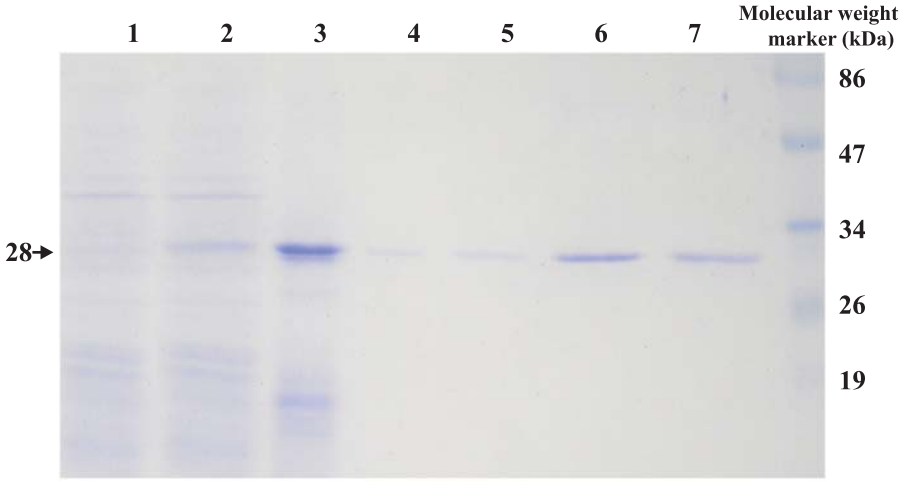

Figure 1. Expression of bovine viral diarrhoea virus (BVDV) N-terminal protease (N^{pro}) in *E. coli*. Bacteria containing the recombinant pTriEx-2-Neo were induced with 1 mM IPTG. The total cell pellet (lane 2), inclusion body (lane 3) and elution fractions (lanes 4–7) of the His-bind affinity chromatography were separated by 12.5% SDS-PAGE. As a negative control, *E. coli* strain transformed with wild-type vector was induced similarly and the total cell pellet (lane 1) was analysed. Molecular weight markers (in kDa) are shown on the right.

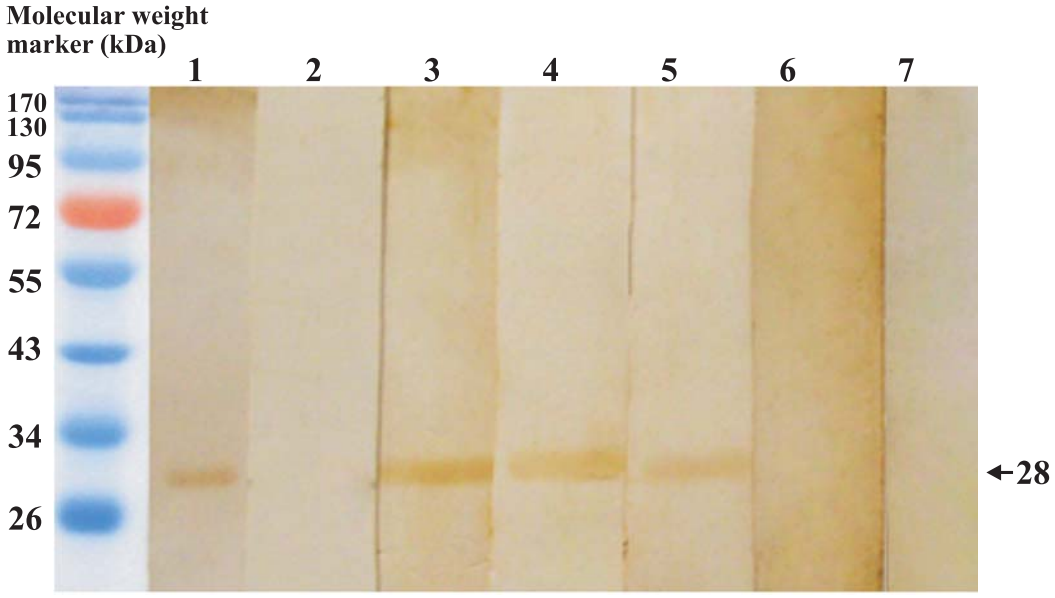

Figure 2. Characterization of recombinant N-terminal protease (N^{pro}) protein by western blot. The purified recombinant protein was separated by 12.5% SDS-PAGE followed by western blot with anti-bovine viral diarrhoea virus (BVDV) hyperimmune cattle (lane 3), sheep (lane 4) and goat (lane 5) serum, as well as anti-His monoclonal antibody (lane 1). BVDV antibody-free cattle (lane 2), sheep (lane 6) and goat (lane 7) sera were used as the negative control.

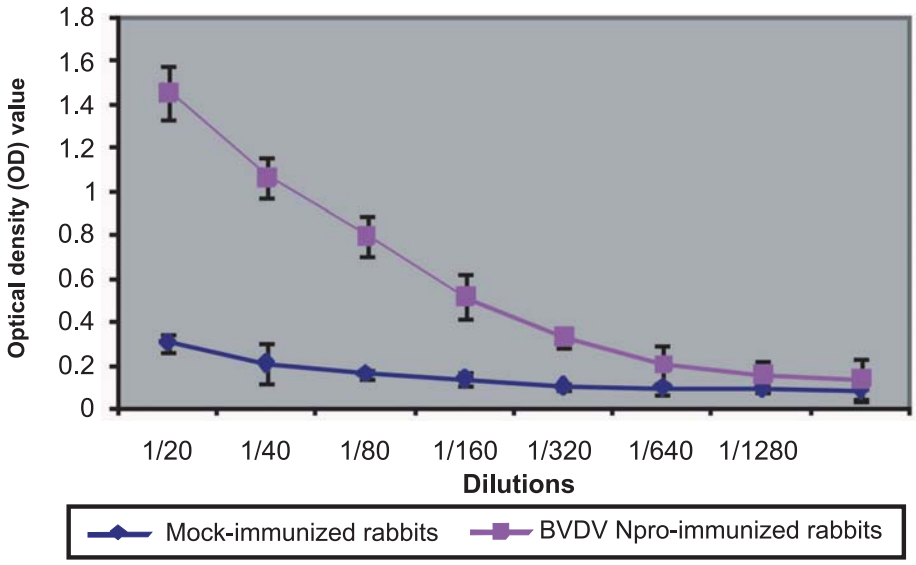

Figure 3. Reactivity of N-terminal protease (N^{pro})-immunized rabbit sera with recombinant N^{pro} protein on ELISA. At 8 weeks' post immunization, rabbit sera diluted 1:20 to 1:2560 were tested by indirect ELISA. The results are the means of the optical density (OD) values. Sera with OD values three times higher than those of mock-immunized rabbits were considered positive for the presence of N^{pro} antibodies.

the end-point dilution showing a positive result for all the three immunized rabbits was 1:320. Similarly, rabbit sera at 8 weeks' post immunization reacted with the N^{pro} fusion protein while there was no reactivity with mock-immunized rabbit sera on western blot (figure 4).

3.4 *Immune response against N^{pro} protein in rabbits*

The immune response against N^{pro} protein was ascertained both by western blot and ELISA using sera collected at weekly intervals from immunized and mock-immunized rabbits. As shown in figure 4A, on western blot, a 28 kDa band without background was observed in N^{pro}-immunized rabbit sera from 4 to 10 weeks of immunization, while no band of a similar size was detected in mock-immunized rabbit sera. This showed that a detectable anti-N^{pro} antibody response was evident by 4 weeks following immunization. The same sera samples were subsequently also tested by ELISA at a predetermined dilution of 1:40. As shown in figure 4B, the N^{pro} antibody response was again evident by 4 weeks and persisted till 10 weeks following immunization. The end-point dilutions showing a positive reactivity were 1:160 at 4 weeks' post immunization and 1:320 at 6, 8 and 10 weeks' post immunization.

3.5 *N^{pro} antibody response in ruminants*

To evaluate whether antibody against N^{pro} protein is detectable in BVDV-infected ruminants, the serum samples (80) collected at weekly intervals from experimentally infected sheep and goats were tested by indirect ELISA. The results showed that in acutely infected sheep and goats, N^{pro} antibodies were not detectable while antibodies against BVDV structural E2 protein and non-structural NS3 protein were present (data not shown). However, when sera from sheep and goats repeatedly exposed to BVDV for raising hyperimmune serum were tested, N^{pro} antibodies were detected. Testing of 30 BVDV neutralizing antibody-positive sera samples from cattle, sheep and goats showed that N^{pro} antibodies were detectable in some of the field sera (7) that showed a high BVDV neutralizing antibody titre (1/2048 to 1/8192 against BVDV-1, and 1/32 to 1/128 against BVDV-2). However, the N^{pro} antibody titre was low (1:160) in most cases. The field sera that were positive for BVDV N^{pro} antibody by indirect ELISA were also found reactive to N^{pro} protein by western blot (figure 5).

4. Discussion

The BVDV genome encodes a 4000 amino acid polyprotein which is cleaved further to generate four structural and 7–8 non-structural proteins (N^{pro}, p7, NS2/NS3, NS4A, NS4B, NS5A, NS5B). Among the non-structural proteins, the NS3/NS2-3 proteins are highly immunogenic and detectable following BVDV infection (Donis and Dubovi 1987; Corapi *et al.* 1990). Earlier studies did not find any detectable antibodies to N^{pro} protein after natural or experimental BVDV infection in cattle (Donis and Dubovi 1987). Although the autocatalytic nature and IFN antagonistic property of N^{pro} protein have been established, little is known about its immunogenicity. In this study, we have

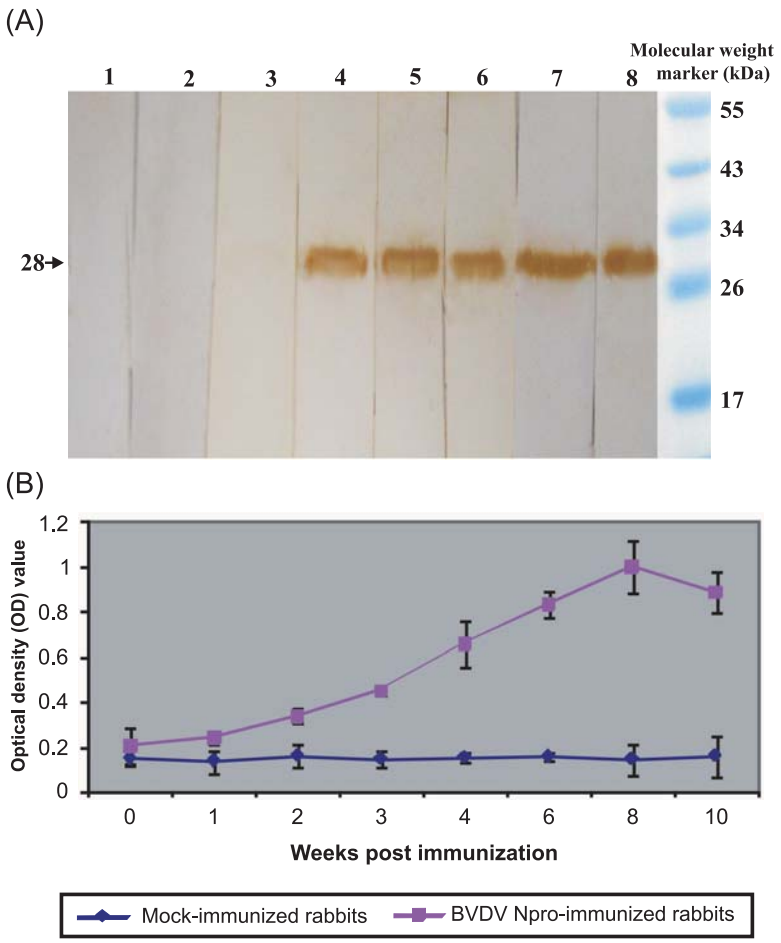

Figure 4. Humoral immune response to bovine viral diarrhoea virus (BVDV) N-terminal protease (N^{pro}) protein in rabbits. **(A)** The N^{pro} protein was resolved on 12.5% SDS-PAGE followed by western blot analysis with sera from rabbits collected at 2 (lane 2), 3 (lane 3), 4 (lane 4), 5 (lane 5), 6 (lane 6), 8 (lane 7) and 10 weeks (lane 8) following immunization. Mock-immunized rabbit serum (lane 1) served as the negative control. **(B)** Mock-immunized rabbit sera and N^{pro}-immunized rabbit sera collected at weekly intervals till 10 weeks post immunization were also tested by indirect ELISA for the presence of anti-N^{pro} antibodies.

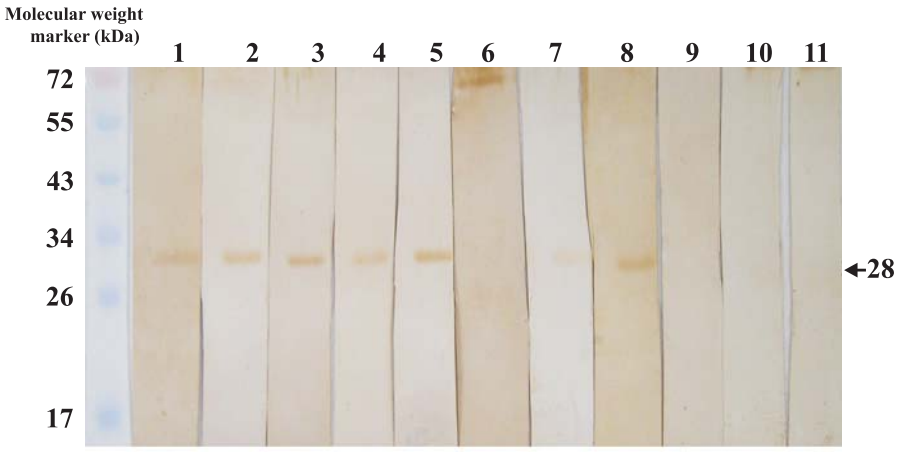

Figure 5. Evidence of anti-N-terminal protease (N^{pro}) antibodies in the sera of field cattle, sheep and goat. The recombinant N^{pro} protein was separated on 12.5% SDS-PAGE and the reactivity with bovine viral diarrhoea virus (BVDV) neutralizing antibody-positive field sera was ascertained by western blot. Lanes 1–4, cattle sera; lanes 5–7, sheep sera; and lanes 8–11, goat sera.

demonstrated that the BVDV N^{pro} protein is immunogenic at least in rabbits. We have also provided evidence that N^{pro} antibodies are detectable in cattle, sheep and goats only after repeated infection and a long exposure time, indicating the low immunogenicity of N^{pro}.

The coding region of the BVDV N^{pro} gene was successfully engineered into the multisystem expression vector pTriEx-2-Neo and expressed as the N^{pro}–His fusion protein in *E. coli*. We selected the vector and expression in *E. coli* for two reasons. First, other non-structural proteins of BVDV such as NS4B and NS5A as fusion proteins in *E. coli* have earlier been expressed for their further characterization (Van Olphen and Donis 1997). Second, we expressed the helicase domain of NS3 in *E. coli* using the same expression vector and successfully utilized the recombinant protein for the development of a monoclonal antibody-based competition ELISA for detection of BVDV antibodies in cattle and buffaloes (Bhatia *et al.* 2008). We used Freund complete and incomplete adjuvants in conjunction with recombinant protein in our study to increase the immunogenic potential of the N^{pro} protein, as earlier studies could not find any detectable N^{pro} antibodies following BVDV infection.

Testing of sera from immunized rabbits both by ELISA and western blot showed that N^{pro}-specific antibodies are elicited since antibodies against N^{pro} were absent in mock-immunized rabbits. The induction of a humoral antibody response was followed in a group of three rabbits following immunization with recombinant N^{pro} fusion protein. Anti-N^{pro} antibody response in rabbits was detected at 4 weeks and persisted till 10 weeks following immunization. Taken together, the results demonstrate that, although weak, the BVDV N^{pro} protein is immunogenic and induction of antibody response is successful in rabbits. Evidence of T-cell proliferation by N^{pro} has been provided by an earlier study, showing that recombinant BVDV N^{pro} and/or capsid are targets for CD4+ T-cell responses in acutely infected cattle (Collen and Morrison 2000). It has also been demonstrated that BVDV/C protein induces both humoral and cellular responses in mice, although antibodies against BVDV/C are not detectable in naturally or experimentally BVDV-infected cattle (Elahi *et al.* 1999). Our results provide evidence that BVDV N^{pro} induces a humoral immune response in rabbits. Although not determined in this study, it is likely that T-cell responses are also induced by N^{pro}. There is a possibility of a difference in immune response if the N^{pro} protein is presented in its native conformation instead of the native-like conformation used in our study. However, the likelihood of this appears low since N^{pro} protein is synthesized in limited quantity in BVDV-infected cells and is not exposed on the surface of the whole virus.

We assumed that the detection of antibodies against N^{pro} would identify animals undergoing active BVDV infection, since the amino-terminal coding sequence of the pestivirus N^{pro} is involved in translation initiation and its autoprotease activity is a prerequisite for synthesis of viable BVDV (Wiskerchen *et al.* 1991; Rumenapf *et al.* 1998). Contrary to our assumption, antibodies against N^{pro} protein could not be detected in BVDV-infected (experimentally) sheep and goats either by ELISA or western blot, despite a humoral immune response being elicited in immunized rabbits. Similar results were observed earlier for two other BVDV non-structural proteins, NS4B and NS5A (Van Olphen and Donis 1997). The NS4B–GST fusion protein and NS5A–GST fusion protein expressed in *E. coli* were found immunogenic and antibodies recognized the NS4B-5A precursor in BVDV-infected cells, while antibodies against these two proteins remained undetectable in BVDV-infected animals. Pestiviruses subvert the innate immunity by preventing IFN α/β induction, a property attributed to the N^{pro} protein (Ruggli *et al.* 2003; Gil *et al.* 2006). The low immunogenicity of N^{pro} that is evident from the present study might help pestivirus persistence by avoiding the host immune response.

Although N^{pro} antibodies remained undetectable in experimentally infected sheep and goat sera, anti-BVDV hyperimmune cattle, sheep and goat sera and some of the BVDV neutralizing antibody-positive field cattle, sheep, goat sera were found positive for N^{pro} antibodies as revealed by ELISA and western blot. When analysed, it was evident that N^{pro} antibodies were detectable only in sera having high neutralizing antibody titres against BVDV structural E2 protein. Our results provide evidence that N^{pro} antibodies are detectable in cattle, sheep and goat sera only after repeated BVDV exposure, indicating a low immunogenicity of the N^{pro} protein. The lack of a strong humoral immune response in rabbits following immunization with the N^{pro} protein lends support to our hypothesis. Further studies are required to delineate the epitopes responsible for humoral and cellular immune responses.

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