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Research paper

Epitope mapping of bovine viral diarrhea virus nonstructural protein 3

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ABSTRACT

Six consecutive overlapped coding regions (F1–F6) of whole NS3 molecule of bovine viral diarrhea virus (BVDV) were cloned into pMAL-c2X plasmid vector and expressed in *Escherichia coli* cells (BL21 strain). The recombinant proteins were then purified by amylose resin to determine the most immunogenic domain(s) of the NS3 molecule. Evaluation of the recombinant proteins was carried out by indirect ELISAs using several bovine sera (previously characterized by virus neutralization test, a commercial ELISA kit, and a newly developed NS3-ELISA) and 6 monoclonal antibodies. The experiments showed that the most immunogenic domain of the NS3 protein was the fourth designed fragment (F4), a 122 amino-acid (AA) region of about 13.5 kDa (nucleotide 1003–1368; residue 335–456). Purified recombinant F4 was also evaluated as single ELISA antigen (F4-ELISA) for the detection of anti-BVDV antibodies in sera of infected cattle. Although this small recombinant fragment of NS3 protein was almost completely soluble and expressed more efficient respect to whole NS3 molecule, it did not show enough sensitivity and specificity to be a proper substitute for NS3 as ELISA antigen to detect specific antibodies against BVDV. However, statistical analyses showed a medium correlation between the results of the developed F4-ELISA and virus neutralization test (kappa coefficient = 0.63, $P < 0.001$), with the relative sensitivity and specificity of 78.05% and 84.91%, respectively, suggesting the potential use of this fragment as an ELISA antigen along with other antigens or monoclonal antibody(s) in a competitive ELISA.

1. Introduction

Bovine viral diarrhea (BVD) is an economically important disease of cattle with a worldwide distribution. BVD is caused by bovine viral diarrhea virus (BVDV), which belongs to *Pestivirus* genus within the family *Flaviviridae*. Economic impact of BVDV infections has led a number of

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countries in Europe to start eradication or control programs (Greiser-Wilke et al., 2003; Presi and Heim, 2010). Diagnosis of BVD relies on laboratory-based detection of its viral causing agent (particularly for the identification of persistently infected animals) or virus specific antibodies and one of the most common laboratory methods for this purpose is ELISA (Sandvik, 1999). E^{rns}, E2, and nonstructural protein 3 (NS3) are the most immunogenic proteins of BVDV (Collett, 1992). NS3 is one of the BVDV proteins frequently used as ELISA antigen for the detection of specific antibodies against the virus (Lecomte et al., 1990; Reddy et al., 1997; Vanderheijden et al., 1993; Zoth and Taboga, 2006). NS3 is an 80 kDa (p80) protein which contains an N-terminal serine protease domain and a C-terminal RNA helicase (Collett et al., 1991; Gorbalenya et al., 1989; Wiskerchen and Collett, 1991). Production of NS3 is essential for the viral RNA replication and cytopathogenicity and the efficiency of this production is regulated by a cellular chaperone (Agapov et al., 2004). This nonstructural protein is also highly conserved among pestiviruses and induces a strong humoral immune response in cattle exposed to live BVDV either naturally or by vaccination (Bolin and Ridpath, 1989). Recombinant NS3 has been expressed and evaluated as ELISA antigen in several studies but a major problem of those expressions was that generally, this protein is insoluble and aggregates as inclusion bodies in recombinant bacterial cells. Thus, expressed recombinant NS3 must be treated with urea to turn into a soluble protein for convenient and effective purification of the protein (Lecomte et al., 1990; Reddy et al., 1997; Vanderheijden et al., 1993; Deregt et al., 2005). Recently, we developed a simple indirect ELISA for detection of antibodies against BVDV using prokaryotically (*Escherichia coli*, BL21 strain) expressed NS3 protein as ELISA detector antigen (Mahmoodi et al., 2014). This recombinant NS3 protein was expressed with maltose binding protein (MBP) as a fusion partner. MBP increased the solubility of the recombinant NS3 molecule. Results of this newly developed ELISA (NS3-ELISA) were comparable to results obtained from a commercial ELISA kit (IDEXX, USA) and virus neutralization test (VNT). Here, we tried to determine immunogenic domain(s) of BVDV NS3 protein and evaluate them by ELISA to see if we can find a smaller part of the protein with almost equal sensitivity and specificity respect to whole NS3 molecule.

2. Materials and methods

2.1. Construction of NS3 recombinant plasmid

Full length coding region of NS3 gene (2049 bp) of BVDV (NADL strain) was cloned into pMAL-c2X plasmid vector followed by confirmation of its sequence using procedures previously described (Mahmoodi et al., 2013). The constructed recombinant plasmid was used as template DNA for amplification of coding regions of designed fragments of NS3 molecule by PCR.

2.2. Designing of NS3 fragments

Six consecutive overlapped fragments covering whole coding region of NS3 molecule were designed. There were five 24 bp (8 AA) overlapped regions between all these fragments. Fig. 1 represents applied strategy for designing of the NS3 fragments and characteristics of these fragments are presented in Table 1. Six pairs of primers were designed for PCR amplification of coding regions of these fragments using Primer3 web-based software. Recognition sites of *Hind*III and *Xba*I restriction enzymes were then added to the designed primers for cloning and orienting of the amplified fragments into pMAL-c2X plasmid vector. Sequences of these primers are shown in Table 2.

2.3. PCR amplification of coding regions of the NS3 fragments

For the amplification of each of these coding regions, 5 μl of diluted purified recombinant NS3 plasmid containing full length coding region of the NS3 molecule was added to 45 μl of PCR mix containing 5 U of *Pfu*-DNA polymerase (Bioneer, Korea), 5 μl of 10× *Pfu* PCR Buffer (+$MgCl_2$), 2 μl of 10 mM dNTPs mix, 0.5 μl (50 pmol) of each of the forward and reverse primers, and 36 μl of H_2O. The reaction was run under the following thermal cycling program; predenaturing at 94 °C for 3 min; denaturing at 94 °C for 1 min, annealing at 50 °C for 1 min (annealing temperature for the sixth fragment was 44 °C), extension at 72 °C for 1:30 min (29 repeats); final extension at 72 °C for 10 min. PCR products were analyzed by electrophoresis on 1% agarose gel in TAE buffer containing ethidium bromide (0.5 μg/ml).

2.4. Cloning of sequences coding the NS3 fragments in E. coli

The amplified DNA fragments were purified by a commercial gel extraction kit (BioNeer, Korea) and together with purified pMAL-c2X plasmid vector (New England Biolabs, USA) were digested by *Hind*III restriction enzyme (Fermentas) at 37 °C for 2.5 h and extracted from agarose gel using a commercial kit (BioNeer, Korea). Digested DNA fragments and plasmid vector underwent ligation processes at 22 °C for 2 h followed by an overnight incubation at 4 °C using T4 DNA ligase (Fermentas, Lithuania). *E. coli* cells (TG1 strain) were then transformed by each of the products of the ligation reactions using previously

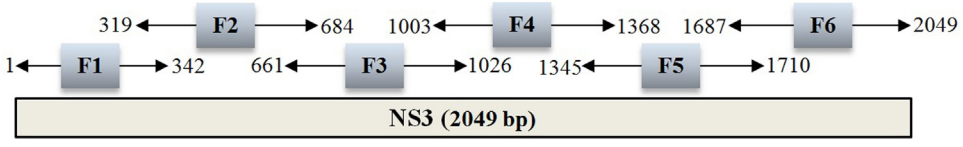

Fig. 1. The employed strategy for cloning of six consecutive overlapped fragments of full length coding region of NS3 molecule.