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Molecular identification of the vaccine strain from the inactivated bovine viral diarrhea virus (BVDV) vaccines

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Abstract

Since the 1980's, several kinds of inactivated bovine viral diarrhea virus (BVDV) vaccines have been used to immunize domestic animals such as cattle and goat in Korea. Immunogenicity of the BVDV vaccines has been checked by the Korean Veterinary Authority using laboratory animals. In this study, we applied a molecular method to investigate the genetic characterization of the BVDV genes in six commercial inactivated BVDV vaccines, and determined the efficiency of two extraction reagents (i.e., sodium citrate or isopropyl myristate) to separate the vaccine antigens from the antigen/adjuvant complexes. Six partial non-coding regions (288 bp) were successfully amplified with specific primer sets, which demonstrated that sodium citrate is more efficient in extracting viral RNA from inactivated gel vaccines than isopropyl myristae. In addition, we identified the virus strains from the vaccines by analyzing the nucleotide sequences of the 5' non-coding region (NCR) of BVDV. The nucleotide similarity of the partial 5' NCR ranged from 95.1 to 100% among BVDV vaccine strains, respectively, indicating that a few manufacturers used different BVDV strains to produce their vaccines.

Key words : Inactivated BVDV vaccine, Isopropyl myristate, Molecular identification, Sodium citrate

INTRODUCTION

Bovine viral diarrhea virus (BVDV), a pestivirus, is the causative agent of bovine viral diarrhea-mucosal disease (BVD-MD) in cattle. BVDV belongs to the *Flaviviridae*, and has a genome size of 12.5 kb consisting of a 5' non-coding region (5' NCR) of 385 nucleotides, a single large open reading frame and 3' NCR of approximately 230 nucleotides. BVDV is one of the most common bovine pathogens with worldwide distribution and causes significant economic losses (Houe, 1995; Houe, 1999). BVDV infection causes various clinical symptoms such as nasal discharge, diarrhea, high fever and respiratory disease, and abortion or early embryonic death in pregnant cattle. On the basis of the 5' NCR gene analysis, BVDV is segregated into two genotypes, BVDV-1 and BVDV-2. Recently, based on the comparison of sequences derived from three genetic regions, i.e., 5' NCR, Npro, E2, BVDV-1 has been subdivided into at least 15 BVDV-1 genetic subgroups (Pellerin et al, 1994; Ridpath and Bolin, 1998; Vilcek et al, 1994; Vilcek et al, 2004). BVDV-2 has been identified in many countries including Korea (Choi and Song, 2011; Jones et al, 2001; Kim et al, 2006; Tajima et al, 2001; Yang et al, 2007) and is associated with more severe clinical signs, including hemorrhagic diarrhea and abortion (Choi and Song, 2011; Ridpath and Bolin, 1998). During the first trimester of gestation, viral infection of dams may produce persistently infected (PI) calves because of the effect of immune tolerance. PI animals are the main source of virus transmission because they continuously shed large amounts of virus into the environ-

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ment and are permanent carriers of BVDV furthermore, they can potentially increase mucosal disease outbreak. It is well known that mucosal disease only occurs when PI calves are exposed to different type of BVDV (Brock et al, 1991).

The Korean Veterinary Authority permitted the use of six inactivated BVDV vaccines for immunizing cattle and other species of animals such as goats in the 1980's. Laboratory animals have been used since then to monitor the immunogenicity of the vaccines; however, it is difficult to measure the quantity of vaccine antigen without conducting immunogenicity studies with animals as the vaccine strain is killed by inactivating agents such as formaldehyde and binary ethylenimine (BEI) during the manufacturing process. In addition, identification of antigen in the inactivated BVDV vaccine is difficult as the antigen is combined with adjuvant and has no special features like hemagglutination activity (Houe, 1999). To overcome these difficulties in identification of the BVDV antigen, we used a molecular method to amplify the target gene of the viral RNA extracted from six commercially available inactivated BVDV vaccines. This study also investigated the extraction condition of the antigen from commercially available inactivated gel-type BVDV vaccines and compared the genetic characterization of BVDV vaccine strains using generalized reverse transcription polymerase chain reaction (RT-PCR) and nucleotide sequence analysis.

MATERIALS AND METHODS

Vaccines and pretreatment

The five commercial inactivated BVDV vaccines produced by the Korean animal vaccine companies and one vaccine imported from foreign country were used in this study. Two methods were used to recover BVDV antigen from the gel vaccines (Choi et al, 2010). First, 200 μl of the inactivated vaccine was mixed thoroughly with 800 μl isopropyl myristate (Sigma, Steinbeim, Germany) for 5 min. The mixture was centrifuged at 3,000× g for 10 min, and the water phase was collected and used for the extraction of RNA. Second, 1 ml of inactivated vaccine was mixed with 0.1 g of sodium citrate (Sigma, USA) on a rotator at 37°C for 12 hrs. The mixtures were frozen and thawed three times and centrifuged at 7,000× g for 5 min, and then the supernatant was collected to use for the extraction of RNA. Korean BVDV isolate, KD26-1 was used for the positive control.

Extraction of viral RNA and RT-PCR

Viral RNA was extracted from the six inactivated vaccines using an RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted RNA was diluted in 50 μl of RNase- and DNase-free water. RT-PCR was performed using specific primer sets (panpestiF and panpestiR) that amplify 5' NCR gene of BVDV (Table 1) (Yang et al, 2007; Vilcek et al, 2004). The RT-PCR was performed in a reaction mixture containing 2 μl of denatured RNA, 1 μl of each primer (50 pmol), 10 μl of 5× buffer (12.5 mM $MgCl_2$), 2 μl of dNTP mix, 2 μl of enzyme mix (reverse transcriptase and Taq polymerase), and 32 μl of distilled water (Qiagen, Hilden, Germany). The cycling profile consisted of cDNA synthesis at 42°C for 30 min, followed by 35 cycles of 95°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR products were visualized using electrophoresis on 1.8% agarose gels containing ethidium bromide.

Table 1. List of the oligonucleotide primers used for RT-PCR of the BVDV vaccine

Primer	Nucleotide sequences (5'-3')	BVDV gene	Size of amplicon (bp)
PanpestiF	CAC TGC ACA CGC ATC AAG AC	5'NCR	288
PanpestR	ACC TGA GCT GGC CTA ATT GC		

Cloning and sequencing

All of the PCR products that were purified using the gel extraction kit (iNtRON Biotechnology, Seongnam, Korea) were ligated with pGEM-T easy vector (Promega, USA) according to the manufacturer's protocol. Plasmid DNA was isolated from amplified *Escherichia coli* (DH5α), and recombinant plasmids were identified using *EcoRI* enzyme digestion (Bioneer, Deajeon, Korea). The sequences of the purified plasmids were analyzed using an MJ Research PTC-225 Peltier Thermal Cycler and ABI PRISM BigDye™ Terminator Cycle Sequencing kits with AmpliTaq DNA polymerase (FS enzyme; Applied Biosystems, USA) according to the manufacturer's protocols. Single-pass sequencing was performed for each template using universal primers (e.g., SP6 and T7). The fluorescent-labeled fragments were purified from the unincorporated terminators using an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems, USA). Both DNA strands were sequenced to verify the sequences.

Phylogenetic analysis

The nucleotide sequences, accession numbers, and names of the strains used for the phylogenetic analysis in this study were obtained from the GenBank database. Each 5' NCR gene sequence (245 bp) of six commercial vaccines was compared with those of the other known BVDV strains using Clustal W (Larkin et al, 2007). Genetic distances were calculated using the Kimura-2 correction parameter and phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates in MEGA4 (Tamura et al, 2007).

RESULTS

Comparison between two pretreatment methods

Before extracting the viral RNA from the inactivated vaccines, two reagents (sodium citrate or isopropyl myristate) were used to separate BVDV antigens from the antigen/adjuvant complex in the vaccine. One specific positive reaction was detected when supernatant of vaccines without any treatment were used to recover BVDV antigens, and six weak positive reactions were detected when isopropyl myristate was used, while five specific strong positive reactions were detected when sodium citrate was used to recover BVDV antigens (Fig. 1). A 288 bp fragment of 5' NCR from all six inactivated BVDV vaccines treated by both sodium citrate and isopropyl myristate was successfully obtained using the 5' NCR primer set used in this study.

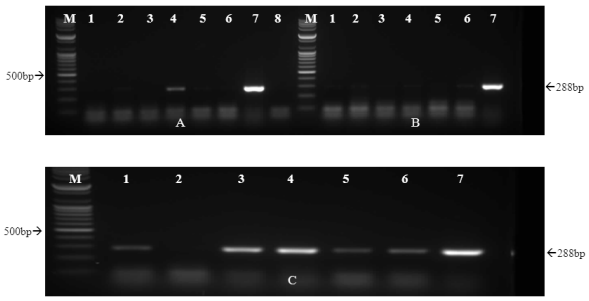

Fig. 1. Amplification of the 5' NCR from the BVDV using RNA extracted from non-treated antigen (A) isopropyl myristate-treated antigen (B), and sodium citrate-treated antigen (C) with specific primer sets for the 5' NCR of BVDV. The expected size was 288 bp. M: 100 bp DNA ladder; lane 1~6: company A, B, C, D, E, F, lane 7: positive, lane 8: negative, respectively.

Analysis of the nucleotide sequences in 5' NCR

The 245 nucleotide sequences obtained from six inactivated BVDV vaccines were identified and compared with 23 BVDVs retrieved from GenBank to analyze the relationship of BVDV vaccine strains. The phylogenetic tree based on the nucleotide sequence analysis of the 5' NCR revealed that BVDV vaccine strains were clustered into BVDV type I (Fig. 2). The nucleotide similarity of the partial 5' NCR ranged from 95.1 to 100% among the six BVDV vaccine strains and 80.5 to 99.6% between the Korean BVDV field isolates and the inactivated vaccine strains. Nucleotide sequences of the 5' NCR of BVDV vaccine strains were compared with that of representative Korean BVDV isolate, KD26-1 (Fig. 3). The one nucleotide deletion and 13 nucleotide substitutions were identified in the company D strain.

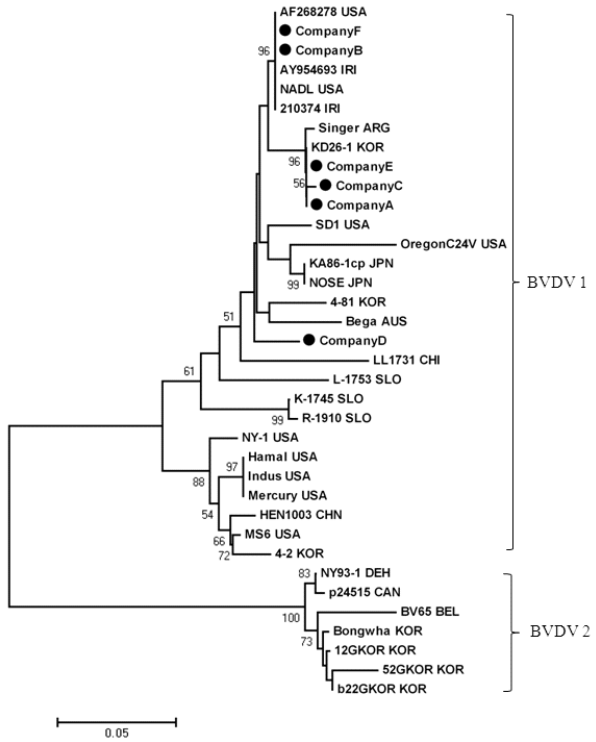

Fig. 2. Phylogenetic tree based on the partial 5' NCR nucleotide sequences of the vaccine strains and other sequences obtained from the GenBank database. Numbers at each key node indicate the degree of bootstrap support and only those with > 50% support are shown.

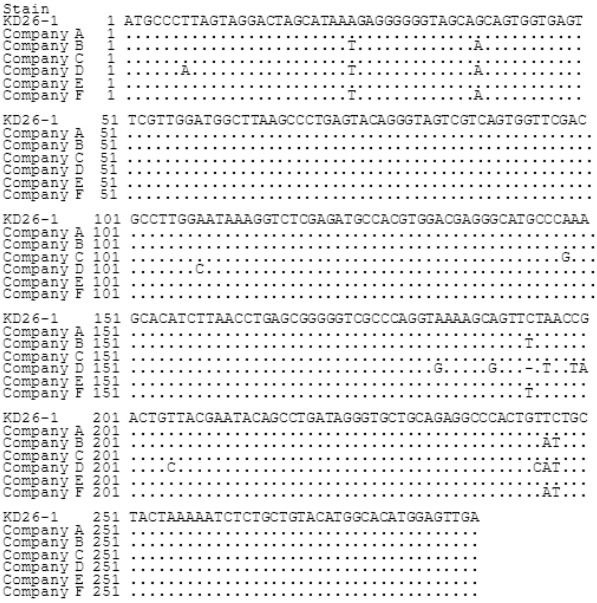

Fig. 3. Comparison of the nucleotide sequences of non-coding region between the vaccine strains and representative Korean BVDV isolate (KD26-1). Dots indicate nucleotides agreeing with the first line sequence.

However, there was no change of nucleotide sequence in company A and C strains.

DISCUSSION

Many countries throughout the world have produced live attenuated or inactivated BVDV vaccines for the purpose of immunization of ruminants (Greiser-Wilke et al, 2003). Most countries use cell culture with MDBK cells to produce BVDV antigens. Both live attenuated and inactivated vaccines have been used to immunization of cattle and its application was different depending on the eradication policy of each country. Live attenuated vaccines could induce some adverse effects including infection of foetus like field BVDV when they were administered to cattle in the early stages of pregnancy (Greiser-Wilke et al, 2003; Liess et al, 1984). In contrast, inactivated BVDV vaccines are safe. However, the efficacy and potency to prevent BVDV infection is limited, therefore, the tests are needed in cattle or small animals (van Oirschot et al, 1999). The Korean Veterinary Authority has performed immunogenic test using experimental animals such as goats and guinea pigs according to the Korean standard assay of veterinary biological products before officially approving the inactivated BVDV vaccine (European pharmacopoeia, 2007). However, it is time consuming, expensive to check immunity of animals inoculated with BVDV vaccine and it requires highly trained technicians. Thus, molecular methods were used to identify the BVDV vaccine antigen in this study. Aluminum hydroxide, the most common adjuvant, is used to manufacture the inactivated BVDV vaccine (Jordão et al, 2011). We used two kinds of reagents to separate vaccine antigens from the antigen/adjuvant complex in the vaccines. The 5' NCR is involved in the translation of the pestivirus gene and is important for the efficient expression of the first gene product (p20) of the BVDV (Poole et al, 1995). We focused on the 5' NCR to identify the vaccine strain by RT-PCR. As shown in Fig. 1, sodium citrate-treated antigen was more strongly detected by RT-PCR than isopropyl myristate-treated antigen, indicating that the vaccine antigen is rendered more accessible to RNA ex-

traction by pretreatment with sodium citrate rather than isopropyl myristate. Although previous study reported that extraction and amplification of target genes from all inactivated oil emulsion vaccines could be done using isopropyl myristate, several factors such as the vaccine virus, the method of virus inactivation, the valence of the vaccines, and the composition of the adjuvant were known to affect the easiness of antigen extraction from vaccines (Choi et al, 2010; Maas et al, 2002).

The 5' NCR nucleotide sequences from the extracted vaccine antigens were compared with the gene sequences of BVDV strains. The phylogenetic tree based on the nucleotide sequences of the 5' NCR revealed that the vaccine strains were classified into BVDV type 1 related groups, and that the nucleotide similarity of the partial 5' NCR (245 bp) ranged from 95.1 to 100% among the six vaccine strains, indicating that a few vaccine manufacturers used different BVDV strains. However, a wider range similarity in the nucleotide was identified between the Korean BVDV isolates and the inactivated vaccine strains.

Mutation of the nucleotide sequences in several positions can help to distinguish vaccine strains from other BVDV strains. It is well known that RNA viruses have a high mutation rate during replication due to both the lack of proofreading and post-replication error correction by RNA polymerase (Steinhauer and Holland, 1987). The BVDV seed strain used to produce vaccines in Korea is regulated by the Korean Veterinary Authority, and changes of seed strains are not allowed without permission. When nucleotide sequences were compared with the partial 5' NCR of BVDV vaccine strains, substitutions of nucleotides were identified in the vaccine strains. These changes in nucleotide sequence can be used as "genetic markers" of the vaccine strains so that changes in the vaccine seed strain could be monitored.

We successfully amplified the 5' NCR of viral antigens extracted from six gel vaccines containing aluminum hydroxide adjuvant using two different reagents. However, the gene of BVDV type 2 was not amplified in six extracted antigens using the two different reagents. Further studies are needed to resolve this problem. The real-time RT-PCR method using another specific primer sets may be an alternative way to quantify the viral antigens in the inactivated BVDV vaccines. Although the amplified region targeted in this study is comparatively short, this method may be an efficient tool for identifying the BVDV vaccine strains from the gel-type BVDV vaccines and possibly other types of inactivated vaccines as well.

In addition, the BVDV vaccine produced in Korea contained BVDV-1 strain only. For the prevention of cattle from BVDV-2 infection, it is necessary to develop new BVDV vaccine containing various BVDV genotypes.

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