Segregation of Bovine Viral Diarrhea Virus Isolated in Japan into Genotypes

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ABSTRACT. It was suggested that 3 strains of bovine viral diarrhea virus (BVDV) isolated from persistently infected calves in Tochigi prefecture in Japan belonged to BVDV type II. It was recognized lack of PstI site on the 5'-untranslated region of genome of them as well as BVDV type II reported previously. Inoculated with the 3 strains, the calves showed the mild decrease of platelet counts which was specific clinical sign of BVDV type II. We should report that the 3 strains were the first BVDV type II isolated in Japan. Neutralizing antibody titers of the antisera against the 3 strains using laboratory strains as neutralizing virus were lower than those of them using homologous strains. Therefore, it was indicated that the difference between BVDV type I and BVDV type II in the antigenicity. — KEY WORDS: antigenicity, BVDV, genotype, thrombocytopenia, vaccine.


Bovine viral diarrhea virus (BVDV) is a member of the genus Pestivirus within the family Flaviviridae [28]. BVDV infections in cattle have been reported throughout the world, and the wide spectrum of clinical syndromes associated with this positive-stranded and enveloped RNA virus make it one of the most important viral pathogens of cattle. The clinical signs produced by BVDV infections are characterized by transient fever and leukopenia, with or without diarrhea [4]. Particularly, cattle persistently infected with non-cytopathogenic BVDV suffer from mucosal disease if they are superinfected with a cytopathogenic BVDV [4]. The majority of reported infections are mild and often subclinical. However, some viral strains have been associated with much more severe disease including fatal hemorrhagic diarrhea and fatal thrombocytopenia. The pathogenesis of these clinical outcomes are still unclear.

Serologic subgroups of BVDV are not recognized, but several reports document significant genomic and antigenic heterogeneity among BVDV. It was reported that BVDV could be segregated into two genotypes by phylogenetic analysis based on comparison of sequences from the 5'-untranslated region (UTR) of genome [24]. Researching the sequences of 5'-UTR and antigenicity of new BVDV strains isolated from the veal calves in Quebec in 1993, Pellerin et al. [22] proposed to divide BVDV into two groups. The group I comprises the classical BVDV including commonly used laboratory and vaccine strains, and the group II comprises the newly described BVDV strains associated with thrombocytopenia and hemorrhaging [22]. They suggested that the different genotype including thrombocytopenic strains of BVDV were closely related to the group II. It is known that BVDV is distinguished BVDV type II from BVDV type I, on the 5'-UTR of genome [15]. It made it easy that BVDV was distinguished BVDV type II from BVDV type I.

BVDV infection associated with thrombocytopenia in Japan has not been reported in any literature until now. In the present study, we segregated BVDV strains isolated in Japan into BVDV type I or type II using genomic criteria, pathogenicity and antigenicity.

MATERIALS AND METHODS

Cell culture and viruses: Bovine testicle (BT) cells used for propagation of viruses, viral titration and serum neutralizing test were grown in Eagle’s minimum essential medium supplemented with 10% fetal calf serum (GM) [18]. All cell cultures and fetal calf serum were determined to be free of BVDV before use by RT-PCR [14]. BVDV used were listed in Table 1. These viruses were propagated in BT cells and stored at -80°C before use.

Template viral RNA for RT-PCR and RT-PCR: Viral RNA was extracted from the supernatant of BT cell culture infected with each virus by TRIzol LS Reagent (LIFE TECHNOLOGIES). After ethanol precipitation, viral RNA was suspended in 0.2% diethylpyrocarbonate-treated water to prevent degradation of RNA. RT-PCR was carried out as described by Harpin et al. [15].

Detection of PstI site by restriction enzyme digestion: The restriction enzyme digestion was performed by extraction of 20 μl of the PCR products with phenol and chloroform and followed by a precipitation step with ethanol according to standard procedures. The digestion was then carried out at 37°C for 24 hr using restriction enzyme PstI (Takara Co., Japan). The digested products were analyzed...
Table 1. The viruses used in the study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biotype</th>
<th>Origin</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADL</td>
<td>CP</td>
<td>U.S.A.</td>
<td>type I</td>
</tr>
<tr>
<td>Singer</td>
<td>CP</td>
<td>U.S.A.</td>
<td>type I</td>
</tr>
<tr>
<td>Osloss</td>
<td>CP</td>
<td>Germany</td>
<td>type I</td>
</tr>
<tr>
<td>890</td>
<td>NCP b)</td>
<td>U.S.A.</td>
<td>type II</td>
</tr>
<tr>
<td>KS86(+)+</td>
<td>CP</td>
<td>Japan</td>
<td></td>
</tr>
<tr>
<td>Tokachi</td>
<td>CP</td>
<td>Japan</td>
<td></td>
</tr>
<tr>
<td>K CP</td>
<td>CP</td>
<td>field isolate</td>
<td></td>
</tr>
<tr>
<td>Nose CP</td>
<td>Japan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. 12–43 NCp</td>
<td>Japan</td>
<td>vaccine strain</td>
<td></td>
</tr>
<tr>
<td>SW90–1</td>
<td>NCP</td>
<td>Japan d)</td>
<td>persistent infection</td>
</tr>
<tr>
<td>SW90–2</td>
<td>NCP</td>
<td>Japan d)</td>
<td>persistent infection</td>
</tr>
<tr>
<td>SW90–3</td>
<td>NCP</td>
<td>Japan d)</td>
<td>persistent infection</td>
</tr>
<tr>
<td>SW90–4</td>
<td>NCP</td>
<td>Japan d)</td>
<td>persistent infection</td>
</tr>
<tr>
<td>SW90–7</td>
<td>NCP</td>
<td>Japan d)</td>
<td>persistent infection</td>
</tr>
<tr>
<td>1</td>
<td>CP</td>
<td>Japan</td>
<td>mucosal disease</td>
</tr>
<tr>
<td>3</td>
<td>CP</td>
<td>Japan</td>
<td>mucosal disease</td>
</tr>
</tbody>
</table>


by 2% agarose gel electrophoresis and visualized with ethidium bromide under UV light.

Experimental inoculation with BVDV type II: Animals used in the experiment were 4 Holstein calves of 100 to 200 kg body weight. These calves were healthy and determined to be free of BVDV and neutralizing antibodies to BVDV. They were housed in individual isolation facilities for the duration of the experiments. A calf was infected intravenously with strain SW90–1, SW90–2, SW90–3 or SW90–7 (10⁴TCID₅₀/head) and collected blood everyday for 2 weeks. Each calf was inspected daily, and the rectal temperature and the presence of anorexia and diarrhea were recorded.

Virus recovery and titration: Whole blood samples added heparin were centrifuged for 10 min at 760 × g. Then, the buffy coats were stored at 80°C before use for virus recovery and titration. Virus titration procedure was modified method based on the interference method using the strain Nose as described previously [17, 19]. The titer of non-cytopathogenic strain was calculated by Behrens-Kärber’s method.

Hematological test: Daily blood samples were collected into sterile tubes from the cervical vein of all the calves for 2 weeks after inoculation. Immediately a portion of each blood sample was removed into special tube for the automatic cytometer machine. Total white blood cells (WBC) and total red blood cells (RBC) were counted on the machine, Celtac MEK-5155 (Nihon Kohden Co., Japan). And a portion of each sample was used for platelet count. They were counted manually by using a hemacytometer following Brecher-Crokite method.

Neutralizing antibody test: Sera collected from calves at 4 weeks postinoculation were examined for neutralizing antibody to the homologous strains, strain NADL, Osloss, Nose and No. 12–43. Serial 2-fold dilution of sera inactivated at 56°C for 30 min were prepared in GM. 0.05 ml of each dilution was delivered in each of 4 wells of the 96-well plastic plate and 0.05 ml of each virus (200 TCID₅₀/ml) was added in all wells. After 1 hr in a incubator (37°C), 0.1 ml of GM containing BT cells was added. Neutralizing antibody titers were determined by the method described previously [26].

RESULTS

Segregation of the viruses into genotypes: To investigate PstI site, first of all, a portion of 5’-UTR corresponding to the site was amplified by RT-PCR. The specific fragment of the expected size (256 bp) was observed in all the viruses (data not shown). Fragments digested by PstI should be about 175 bp and 88 bp according to NADL sequence [8].

After the digestion of PCR products with PstI, fragments of all the viruses except strain 890, SW90–1, SW90–2 and SW90–7 were about 175 bp on electrophoretic analysis (Fig. 1). Expected 88 bp-fragment was not clearly observed because of too short band.

Fragments of strain 890, SW90–1, SW90–2 and SW90–7 were still 250 bp after the digestion of PCR products with PstI (Fig. 1).

Clinical observation of experimental inoculation: Clinical signs of disease in the calves inoculated with strain SW90–1, SW90–2 and SW90–7 were not observed. The calf inoculated with strain SW90–3 became febrile at 6 days postinoculation and developed watery diarrhea from 3 to 7 days postinoculation.

Virus recovery: As shown in Fig. 2, viruses were recovered from all the calves. Their titers increased until 6 days postinoculation, and subsequently decreased gradually. Each maximal titer was 10⁴.25 to 10⁴.75 TCID₅₀/0.05 ml.

Hematological changes: There is no significant change of RBC-number in calves inoculated with strain SW90–1, SW90–2 and SW90–7 for the duration of the experiment. Only strain SW90–2 induced drop of RBC-number (1.042 to 886 × 10¹¹/mm³) from 4 days (1.042 × 10¹¹/mm³) to 11 days (886 × 10¹¹/mm³) postinoculation (data not shown).

In the present study, the calves inoculated with strain SW90–2, SW90–3 and SW90–7 developed leukocytopenia from 2 days postinoculation (Fig. 3). And it was observed only a few drop of WBC-number in the calf with strain SW90–1.

The notable hematological alteration was the decrease of platelet counts. The platelet counts in calves inoculated...
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with strain SW90–2 and SW90–7 mildly diminished from 2 or 3 days postinoculation. In the calf inoculated with strain SW90–1, slight decrease was shown at 6 and 7 days postinoculation. But thrombocytopenia was not observed in the calf with strain SW90–3 (Fig. 4).

Neutralizing antibody titers: At 4 weeks postinoculation, each serum neutralizing antibody titer against each homologous strain respectively was 64 to 512 as shown in Fig. 5. But anti-strain SW90–1, SW90–2 and SW90–7 serum titers against the laboratory strains, NADL, Osloss, Nose and No. 12–43, were significantly lower than those against each homologous strain (Fig. 5). The differences were more than 32-fold.

DISCUSSION

The genetic and antigenic diversity between different strains of BVDV are quite varied. However, it has become clear that two major groups of virus exist which differ significantly with respect to both their genetic and antigenic make-up. These two groups are referred to BVDV type I and type II. Classification of type I and type II are based on significant differences in the genetic sequence of 5'-UTR of the genome [24].

The fragments of strain SW90-1, SW90-2 and SW90-7 amplified by RT-PCR were not digested with PstI as similar to that of strain 890 which is a reference strain of BVDV type II. The other isolates as well as reference strains of BVDV type I were classified as BVDV type I. Therefore, it was suggested that strain SW90–1, SW90–2 and SW90–7 belonged to BVDV type II because of lack of PstI site on the 5'-UTR.

We certainly recognized the infection of each strain to each calf because of (1) virus recovery from their buffy coats, (2) antibody response in experimental inoculations, (3) clinical signs after virus inoculation. In the present...
study, we have shown that mild thrombocytopenia is consistently observed in calves after inoculation with the 3 strains. Though BVDV type II induces severe thrombocytopenia and hemorrhages [5, 9, 10, 22, 24], the thrombocytopenia-inducing ability of these strains was not strong. Generally, it was known that the consequences of infection of cattle with BVDV vary from an inapparent infection to a severe fatal disease [1, 6]. There are various virulence against cattle among classical strains belonging to BVDV type I. The outcome of an acute infection is probably related to several factors including strain of virus, age of host, immune and physiologic status of the host. Within each genotype, there are several different strains of BVDV that can differ significantly in their ability to cause disease. On the other hand, severe outbreaks of disease associated with acute post-natal BVDV infection have been reported sporadically for many years [11], but viruses isolated from such outbreaks may only induce mild illness in experimentally infected animals [20]. One explanation for this might be cell culture attenuation, via repeated in vitro passage [2]. Virulence attenuation by passage in cell culture during isolating procedure may be responsible for diminution of thrombocytopenia induction. It is possible that these strains were attenuated for passage in cell culture during isolating procedure.

The results of serological comparison suggested that BVDV type II, strains SW90–1, SW90–2, and SW90–7, were antigenically different from the laboratory strains, NADL, Osloss, Nose and No. 12–43. On the other hand, BVDV type I, strain SW90–3, was antigenically closer to the laboratory strains than them. The viral envelope-associated glycoproteins are named E0 (gp48), E1 (gp25), E2 (gp53) [7]. E2 is by far the immunodominant protein [12, 21]. Tijssen et al. [27] analyzed the E2 of BVDV type II with respect to their sequences and serological crossreactivities with E2 of NADL belonging to BVDV type I. They reported that the virus-neutralizing titers of the anti-BVDV type I sera were significantly lower for the BVDV type II as compared to those for the homologous BVDV, and BVDV type II can be serologically distinguished from NADL strain identified as a BVDV type I [27]. Our results also indicated the difference between BVDV type I (NADL) and type II (SW90–1, SW90–2, SW90–7) in the antigenicity.

We should report that strain SW90–1, SW90–2 and SW90–7 were the first BVDV type II isolated in Japan. Some viruses characterized as BVDV type II escaped neutralization by antibodies raised by vaccination with a virus identified as BVDV type I [3]. In the present study, strain SW90–1, SW90–2 and SW90–7 were antigenically farthest from strain No. 12–43 which is Japanese vaccine strain among the laboratory strains used in the study. This results seem to predict the inefficiency of BVDV vaccine. Further studies are now under way in our laboratory to examine cross-protection between BVDV type I and type II and evaluate the efficacy of BVDV type I vaccine against BVDV type II viruses.

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REFERENCES


