Studies on antigenic and pathogenic characterizations and detection of *Bovine viral diarrhea virus 2*

（牛ウイルス性下痢ウイルス2の抗原性および病原性並びに検出法に関する研究）

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Preface

A new transmissible disease in cattle was detected in New York State, U.S.A. in 1946. The disease was characterized by leucopenia, high fever, depression, diarrhea and dehydration, anorexia, salivation, nasal discharge, gastrointestinal erosions, and hemorrhages in various tissues. This was the first reported outbreak of bovine viral diarrhea (BVD) (Olafson, McCallum and Fox 1946). After that outbreak in the U.S.A., a similar but more severe BVD outbreak in cattle was reported in Canada. This report was considered the first description of mucosal disease (MD) in cattle. MD is the most dramatic clinical evidence of BVD. MD was characterized by fever, anorexia, depression, profuse salivation, nasal discharge, gastrointestinal hemorrhages, erosions, ulcers and severe diarrhea with watery feces that were sometimes mixed with blood. Furthermore, MD usually affected only a few animals in a herd but had a very high case fatality rate. Although MD was thought to be a disease distinct from BVD, it was determined that the viral agents isolated from BVD and MD were the same and that BVD and MD were actually different disease manifestations caused by the same agent (Gillespie et al. 1961, Kniazeff et al. 1961, Thomson and Savan 1963). Thus,

several years later, the disease became officially known as bovine viral diarrhea-mucosal disease (Kennedy et al. 1968). In Japan, a viral agent was unexpectedly isolated from a cell culture originating from a bovine fetus and it caused fever and leucopenia in the 1950s. Cases characterized by diarrhea, MD, congenital anomalies and abortion were reported in the 1970s. At present, BVD is an economically important disease of cattle having been reported throughout the world and is caused by infection with bovine viral diarrhea virus (BVDV) (Baker 1987, Brinton 1980).

BVDV is a small positive-sense single-stranded RNA virus classified in the genus *Pestivirus* within the family *Flaviviridae* (Thiel et al. 2005). There are currently four recognized species within the pestivirus genus: BVDV 1, BVDV 2, border disease virus, and classical swine fever virus (CSFV), previously known as hog cholera virus. Pestivirus virions are enveloped, spherical particles of 40 to 60 nm in diameter. The virions are made up of a central capsid, composed of the virally encoded C protein and the genome RNA, surrounded by a lipid bilayer. The Pestivirus genome is approximately 12.3 Kb in length (Collett et al. 1988a, b, Moormann et al. 1990, Deng and Brock, 1992, Ridpath and Bolin 1995, 1997). The long open

reading frame (approximately 4000 codons) is bracketed by relatively large 5' (360-390 bases) and 3' (200-240 bases) untranslated regions (UTRs). The highest nucleic acid sequence identity among pestiviruses is found in the 5' UTR (Ridpath and Bolin 1997).

It was discovered in 1957 that the viral agent isolated in acute BVD did not cause cytopathology in vitro, meaning that infected cells in culture appeared normal (Lee and Gillespie, 1957). Gillespie et al. later reported the first isolation of a cytopathogenic strain of BVDV, designated Oregon C24V (Gillespie et al. 1960). The discovery of the cytopathogenic strain allowed the development of serum neutralization and plaque neutralization assays.

As stated above, there are two biotypes of BVDV: one is non-cytopathogenic (ncp) BVDV, which did not induce cytopathogenic effects (CPE) in any cell culture, and the other is cytopathogenic (cp) virus which shows clear CPE in bovine cell cultures. Ncp BVDV, which is a major biotype in the field, can cause persistent infection in fetuses. McClurkin et al. inoculated cows or their fetuses directly with ncp BVDV strain or cp BVDV strain. (McClukin et al. 1984). Of interest, but not fully appreciated at the time, was the failure to produce any persistently infected (PI) calves with the

cp BVDV strain. Later, Brownlie et al. (1989) infected pregnant cattle with a cp BVDV strain but could not produce persistent infection. Thus, it became generally accepted that only ncp BVDV strain could produce persistent infection. By the end of the 1960s, it had become evident that cattle with MD had persistent viremia and often failed to produce neutralizing antibodies to the virus (Malmquist 1968). Particularly, born cattle persistently infected with ncp BVDV should be infection source in bovine population. PI cattle with ncp BVDV suffer from MD if they are superinfected with a cp BVDV (Bolin et al. 1985).

The majority of reported infections of BVDV are mild and often subclinical. However, some viral strains have been associated with much more severe diseases including fatal hemorrhagic diarrhea and fatal thrombocytopenia. Thrombocytopenia with hemorrhage associated with BVDV infection was first reported in 1987 within a summary of case reports for dairy herds in the northeastern United States (Perdrizet et al. 1987). Hemorrhages associated with BVDV infection in young veal calves were also observed with increasing frequency in the late 1980s in the northeastern United States (Corapi et al. 1990). Concurrent to this research, a highly

virulent form of BVD, termed hemorrhagic syndrome, was reported in Canada and the United States (Corapi et al. 1989, Corapi et al. 1990, Carman et al. 1998). Phylogenetic analysis of BVDV strains, isolated from animals suffering from hemorrhagic syndrome, grouped them separately from the BVDV strains commonly used, at that time, in vaccine production, diagnostic tests, and research (Pellerin et al. 1994, Ridpath et al. 1994). The newly recognized group of BVDV was designated BVDV genotype II, and the group containing the strains used in vaccines, detection, and research was termed BVDV genotype I. The names of these two genotypes were later modified to BVDV 1 and BVDV 2 in keeping with taxonomic conventions in use with other viruses (Heinz et al. 2000). Prevalences of BVDV 2 in North America, Europe and South America have been reported. Field strains of BVDV may belong to one of two different genotypes, BVDV 1 and BVDV 2. Genotyping is grouping based on comparison of genomic sequences in the 5' UTR. Ideally, genotypes would be associated with practical observations such as geographic distribution, antigenic variation, or variations in virulence. The differentiation between the BVDV 1 and BVDV 2 genotypes meets these practical considerations. Therefore, it is worthwhile to segregate BVDV

strains isolated in Japan into BVDV 1 or BVDV 2.

Although immunization with some vaccines containing BVDV 1 alone was protective against acute BVD caused by BVDV 2 (Cortese et al. 1998, Carman et al. 1998), apparent vaccine breaks did occur (Ridpath et al. 1994). With the emergence of BVDV 2, the concern for incorporation of BVDV 2 in vaccines escalated with a report that vaccines containing BVDV 1 appeared not to be protective against infection with BVDV 2 (van Campen et al. 2000). The efficacy of the Japanese vaccine containing BVDV 1 against BVDV 2 should also be evaluated.

The observation that fetal bovine sera frequently contained BVDV and neutralizing antibodies against BVDV (Kniazeff,Rimer and Gaeta 1967, Malmquist, 1968) was an increasing concern in the 1970s for cell culture work because contaminated cultures could have undesirable consequences for research and vaccine production. It is difficult to detect an ncp BVDV strain contaminating a vaccine. Tamoglia found that 8% of licensed live IBR vaccines were contaminated with BVDV, raising concerns that such vaccines might give rise to fetal abortions (Tamoglia 1968). In order to ensure that BVDV-free vaccines are produced, it is therefore essential to use valid tests

and to develop effective quality assurance programmes, stage by stage, throughout the manufacturing process (Falcone, Tollis and Conti 2000).

As mentioned above, BVDV, especially new BVDV 2, is a very important agent of livestock hygiene and quality control of vaccines. Regrettably, it is not cleared the presence of BVDV 2 in Japan, efficacy of commercial vaccines used in Japan against BVDV 2 and potentiality of detection of extraneous active BVDV in bovine vaccine by RT-PCR. The purposes of this study were to clarify these uncertainties.

The present thesis consists of three chapters. In the first chapter, BVDV strains isolated in Japan are segregated into genotypes. In the second chapter, the efficacy of the BVD vaccine used in Japan against BVDV 2 strain 890 is evaluated. In the last chapter, it is shown that the active BVDV contaminating bovine live viral vaccines could be detected by means of the detection of negative-sense RNA of BVDV with RT-PCR method.

Chapter 1

Segregation of Bovine viral diarrhea virus isolated in Japan into genotypes

INTRODUCTION

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 phylogenic analysis based on comparison of sequences from the 5'-UTR of genome (Ridpath, Bolin and Dubovi 1994). Researching the sequences of 5'-UTR and antigenicity of new BVDV strains isolated from the veal calves in Quebec in 1993, Pellerin et al. proposed to divide BVDV into two groups (Pellerin et al. 1994). The group I comprises the newly described BVDV strains associated with thrombocytopenia and hemorrhaging (Pellerin et al. 1994). They suggested that the different genotype including thrombocytopenia strains of BVDV were closely related to the group II. It is known that BVDV is distinguished as the two genotypes, termed BVDV 1 and BVDV 2, by phylogenic analysis of sequences of the 5'-UTR of genome. Moreover, new BVDV strains isolated in North America since 1980s and associated with thrombocytopenia and very high mortalities belong to the BVDV 2 (Bolin and Ridpath 1992, Corapi, French and Dubovi 1989, Pellerin et al. 1994). Subsequently, it was demonstrated that BVDV 2 did not have *Pst* I site, which is present in all known BVDV 1, on the 5'-UTR of genome

(Bolin and Ridpath 1992). It made it easy that BVDV was distinguished BVDV 2 from BVDV 1. BVDV infection associated with thrombocytopenia in Japan has not been reported in any literature until now. In the chapter, the author segregated BVDV strains isolated in Japan into BVDV 1 or BVDV 2 using genomic criteria, and demonstrated those pathogenecity 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 (FCS) (Nakamura et al. 1997). All cell cultures and FCS were determined to be free of BVDV before use by RT-PCR (Harasawa and Tomiyama 1994). BVDV used were listed in Table 1. Strains NADL and Singer are cp viruses isolated in U. S. A. and used as laboratory strains (Gutekunst and Malmquist 1963, Pellerin et al. 1994, Yu et al. 1994). Strain Osloss is a cp virus isolated in Germany (Renard, Dina and Martial 1987). Strain 890 is a ncp virus isolated from the cattle developing severe thrombocytopenia in U. S. A. (Bolin and Ridpath 1992, Pellerin et al. 1994). Strains KS86(+), Tokachi, K and Nose are cp viruses isolated in Japan (Hashiguchi et al 1978, Kodama et al 1974, Shimizu, Murakami and Satou 1989). They are used as laboratory strains. Strain No.12-43 is a vaccine strain contained in BVD live vaccine used in Japan. Shiribeshi 2, Shiribeshi 4, Shiribeshi 129, 419, 420, 437, 443, 763 and 799 are ncp virus isolated from PI calves in Hokkaido province of Japan. Strain 20 is cp virus isolated from a cattle developing MD in Hokkaido

province of Japan. Strains SW90-1, SW90-2, SW90-3, SW90-4 and SW90-7 are ncp viruses isolated from PI calves in Tochigi prefecture from 1986 to 1991. Strains 1 and 3 are cp virus isolated from cattles developing MD in Tochigi prefecture. All of these viruses were propagated in BT cells and stored at −80℃ 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. (Harpin et al. 1995).

Detection of *Pst* I site by reaction enzyme digestion

The restriction enzyme digestion was performed by extraction of 20 μL of the PCR products with phenol and chloroform and followed by precipitation step with ethanol according to standard procedures. The digestion was then carried out at 37℃ for 24hr using restriction enzyme *Pst*

I (Takara Co., Japan). The digested products were analyzed by 2% agarose gel electrophoresis and visualized with ethidium bromide under UV light.

Experimental inoculation with BVDV 2

Animals used in the experiment were 4 Holsteins 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 BVDV strain SW90-1, SW90-2, SW90-3 or SW90-7 ($10^4$$TCID_{50}$/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℃ before use for virus recovery and titration. Virus titration procedure was modified method based on the interference method using BVDV strain Nose as described previously

(Kodama et al. 1979, Nakamura et al. 1995). The titer of ncp strain was calculated by Behrens-Karber'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, Celltac MEK-5155 (Nihon Kohden Co., Japan). And a portion of each sample was used for platelet (PLT) 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, NADL, Osloss, Nose and No. 12-43 of BVDV. Serial 2-fold dilution of sera inactivated at 56℃ for 30 min were prepared in Eagle's minimum essential medium supplemented

with 10% FCS. 50 μ L of each dilution was delivered in each of 4 wells of the 96-well plastic plate and 50 μ L of each virus (200 $TCID_{50}$/0.1mL) was added in all wells. After 1 hr in a incubator (37℃), 0.1 mL of Eagle's minimum essential medium supplemented with 10% FCS containing BT cells was added. Neutralizing antibody titers were determined by the method described previously (Shimizu and Satou 1987).

RESULTS

Segregation of the viruses into genotypes

To investigate *Pst* I 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 bps) was observed in all the viruses (data not shown). Fragments digested by *Pst* I should be175 bps and 88 bps according to NADL sequence (Collett et al. 1988c).

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

Fragments of strain 890, SW90-1, SW90-2 and SW90-7 were still 250 bps after the digestion of PCR products with *Pst* I (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 $10^{2.25}$ to $10^{2.75}$ $TCID_{50}/50\,\mu L$.

Hematorogical changes

There is no significant change of RBC- number in calves inoculated with strain SW90-1, SW90-3 and SW90-7 for the duration of the experiment. Only strain SW90-2 induced drop of RBC-number (1,042 to 886 $\times 10^4/mm^3$) from 4 days (1,042 $\times 10^4/mm^3$) to 11 days (886 $\times 10^4/mm^3$) 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 PLT counts. The PLT counts in calves inoculated 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 week 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 homorogous 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 1 and BVDV 2. The classification of BVDV 1 and BVDV 2 is based on significant differences in the genetic sequence of 5'-UTR of the genome (Ridpath, Bolin and Dubovi 1994).

The fragments of strain SW90-1, SW90-2 and SW90-7 amplified by RT-PCR were not digested with Pst I as similar to that of strain 890 which is a reference strain of BVDV 2. The other isolates as well as reference strain of BVDV 1 were classified as BVDV 1. Therefore, it was suggested that strain SW90-1, SW90-2 and SW90-7 belonged to BVDV 2 because of lack of Pst I 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 2

induces severe thrombocytopenia and hemorrhages (Bolin and Ridpath 1992, Corapi et al. 1990, Corapi, French and Dubovi 1989, Pellerin et al. 1994, Ridpath, Bolin and Dubovi 1994), 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 (Baker 1987, Brinton 1980). There is various virulence against cattle among classical strains belonging to BVDV 1. The outcome of an acute infection is probably related to several factors including strain 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 (David et al. 1994), but viruses isolated from such outbreaks may only induce mild illness in experimentally infected animals (Paton 1995). One explanation for this might be cell culture attenuation, via repeated in vitro passage (Bezek, Gröhn and Dubovi 1994). 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 2, 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 1, 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) (Collett et al. 1988b). E2 is by far the immunodominant protein (Donis, Corapi and Dubovi 1988, Paton, Lowings and Barrett 1992). Tijssen *et al.* analyzed the E2 of BVDV 1 (Tijssen et al. 1996). They reported that the virus-neutralizing titers of the anti-BVDV 1 sera were significantly lower for the BVDV 1 as compared to those for the homologous BVDV, and BVDV 2 can be serologically distinguished from strain NADL identified as a BVDV 1 (Tijssen et al. 1996). It was also indicated the difference between BVDV 1 (NADL) and BVDV 2 (SW90-1, SW90-2, SW90-7) in the antigenicity. 【J. Vet. Sci. 60: 579-583. 1998】

It should be reported that strain SW90-1, SW90-2 and SW90-7 were the first BVDV 2 isolated in Japan. Some viruses characterized as BVDV 2 escaped neutralization by antibodies raised by vaccination with a virus

identified as BVDV 1 (Bolin, Littledike and Ridpath 1991). 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. These results seem to predict the inefficiency of BVDV vaccine.

ABSTRUCT

It was suggested that 3 strains of BVDV isolated from persistently infected calves in Tochigi prefecture in Japan belonged to BVDV 2. It was recognized lack of *Pst* I site on the 5'-UTR of genome of them as well as BVDV 2 reported previously. Inoculated with the 3 strains, the calves showed the mild decrease of platelet counts which was specific clinical sign of BVDV 2. The author should report that the 3 strains were the first BVDV 2 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 1 and BVDV 2 in the antigenicity.

Table 1. The viruses used in the study

Strains	Biotype	Origin	Genotype
NADL	cp[a)]	U.S.A.	BVDV 1a
Singer	cp	U.S.A.	BVDV 1
Osloss	cp	Germany	BVDV 1b
890	ncp[b)]	U.S.A.	BVDV 2
KS86(+)	cp	Japan	BVDV1
Tokachi	cp	Japan	BVDV1
K	cp	Japan	BVDV1
Nose	cp	Japan	BVDV1a
No.12-43	ncp	Japan	BVDV1a
Shiribeshi2	ncp	Japan[c)]	BVDV1b
Shiribeshi4	ncp	Japan[c)]	BVDV1b
Shiribeshi129	ncp	Japan[c)]	BVDV1a
419	ncp	Japan[c)]	BVDV1b
420	ncp	Japan[c)]	BVDV1
437	ncp	Japan[c)]	BVDV1
443	ncp	Japan[c)]	BVDV1
763	ncp	Japan[c)]	BVDV1
799	ncp	Japan[c)]	BVDV1
20	cp	Japan[c)]	BVDV1
SW90-1	ncp	Japan[d)]	BVDV2
SW90-2	ncp	Japan[d)]	BVDV2
SW90-3	ncp	Japan[d)]	BVDV1
SW90-4	ncp	Japan[d)]	BVDV1
SW90-7	ncp	Japan[d)]	BVDV2
1	cp	Japan[d)]	BVDV1
3	cp	Japan[d)]	BVDV1

a) Cytopathogenic strain. b) Non-cytopathogenic strain. c) Isolated in Hokkaido Province. d) Isolated in Tochigi Prefecture from 1986 to 1991.

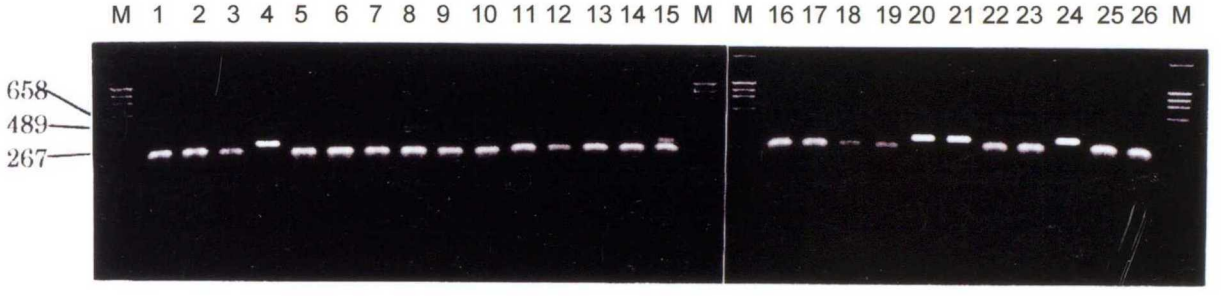

Fig. 1. Electrophoretic analysis of RT-PCR amplification products digested by the restriction enzyme *Pst* I. M: pHY marker; Lane 1: NADL; Lane 2: Singer; Lane 3: Osloss; Lane 4: 890; Lane 5: KS86(+); Lane 6: Tokachi; Lane 7:K; Lane 8: Nose; Lane 9: No. 12-43; Lane 10: Shiribeshi 2; Lane 11: Shiribeshi 4; Lane 12: Shiribeshi 129; Lane 13: 419; Lane 14: 420; Lane 15: 437; Lane 16:443; Lane 17:763; Lane 18: 799; Lane 19: 20; Lane 20: SW90-1; Lane 21:SW90-2; Lane 22: SW90-3; Lane 23: SW90-4; Lane 24: SW90-7; Lane 25: 1; Lane 26: 3.

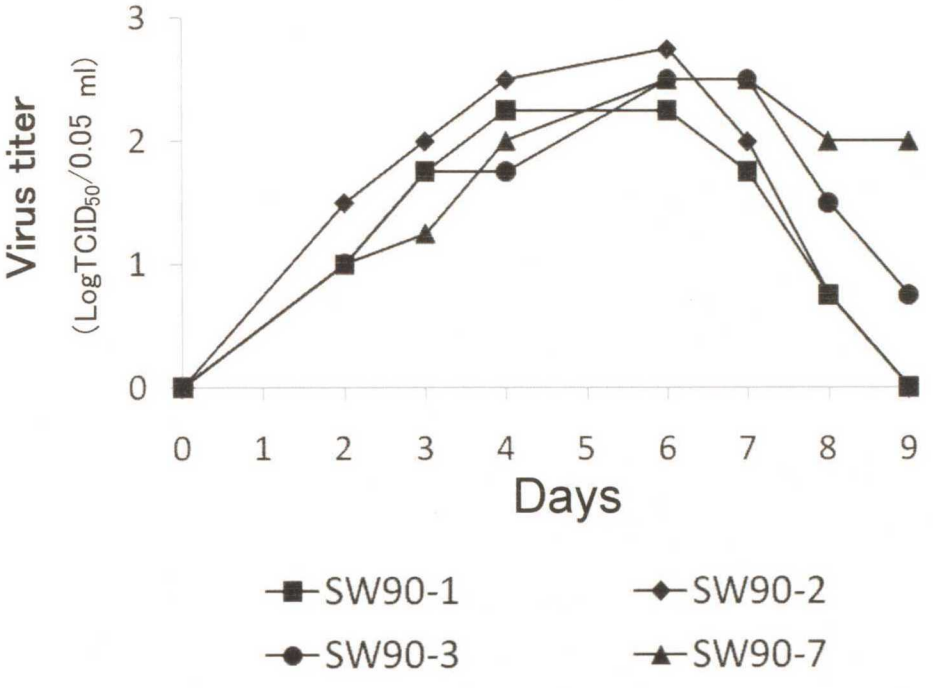

Fig. 2. Virus titers in calves inoculated with the 4 strains.

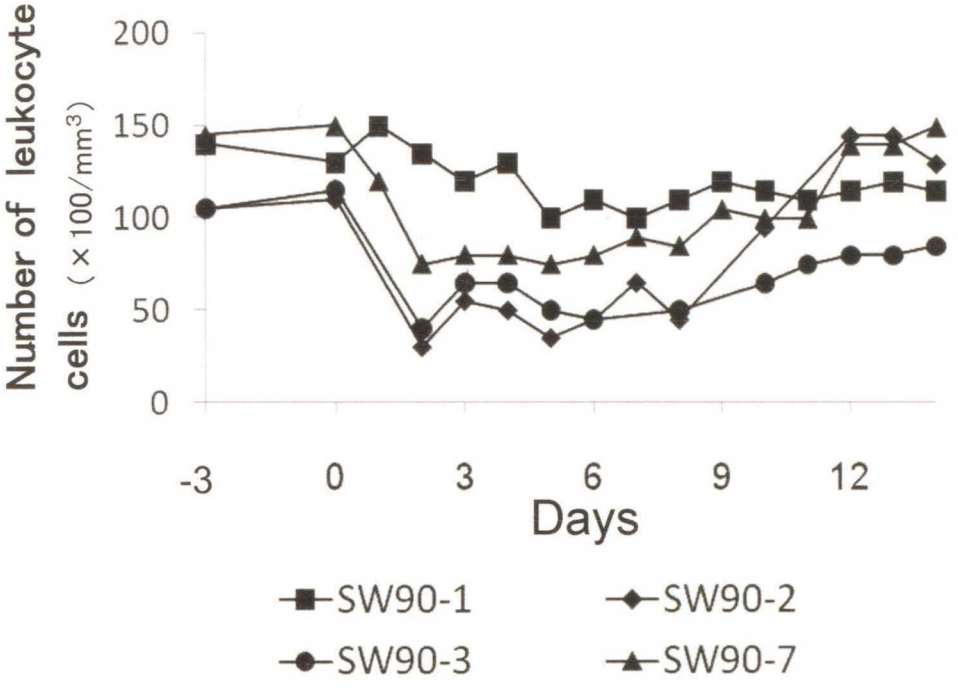

Fig. 3. Development of leukocytopenia in calves after inoculation with the 4 strains.

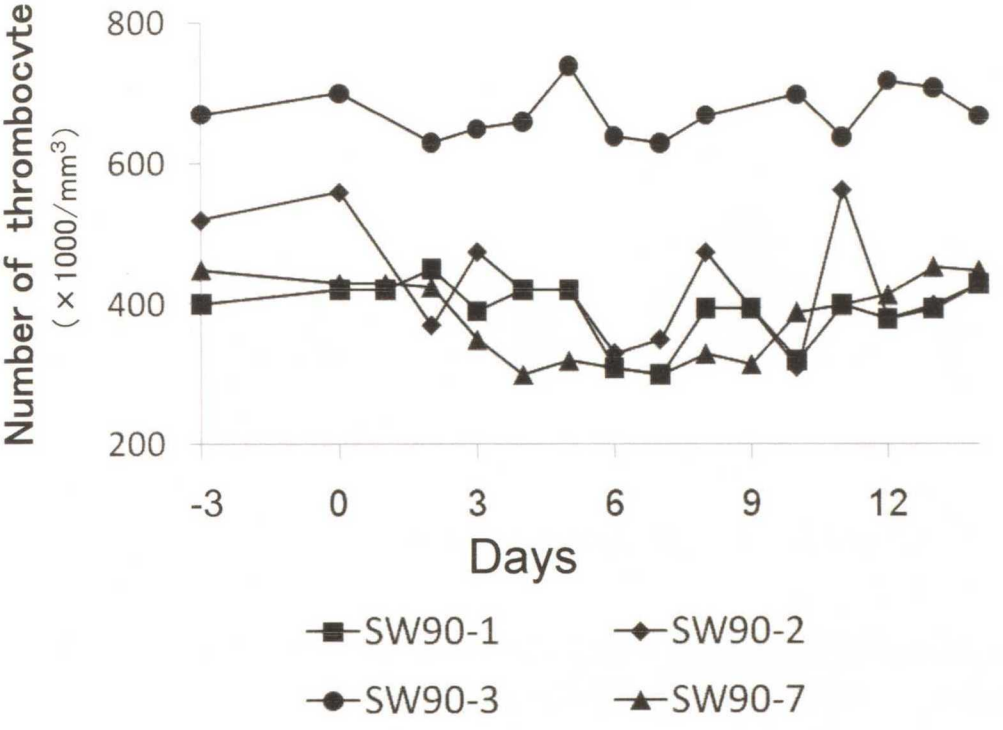

Fig. 4. Development of thrombocytopenia in calves after inoculation with the 4 strains.

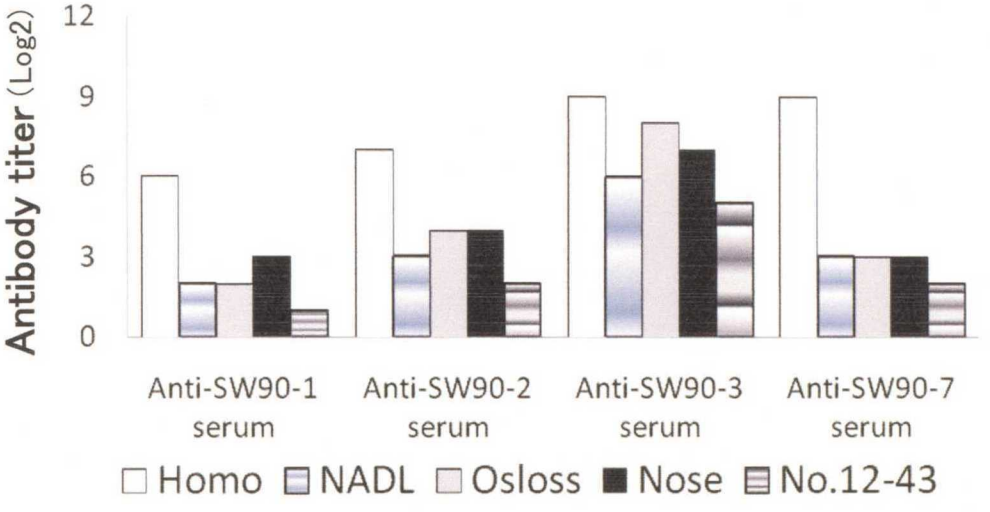

Fig. 5. Neutralizing antibody titers using the homologous strains (SW90-1, SW90-2, SW90-3 and SW90-7), laboratory strains (NADL, Osloss and Nose) and vaccine strain (No.12-43).

Chapter 2

Efficacy of bovine viral diarrhea vaccine used in Japan against *Bovine viral diarrhea virus 2* strain 890

INTRODUCTION

BVDV has been segregated into two genotype, BVDV 1 and BVDV 2, by phylogegenetic analysis based on comparison of sequence from the 5'-untranslated region of the genome (Ridpath, Bolin and Dubovi 1994). Originally, the new genotype, BVDV 2, was identified in severe outbreaks of acute hemorrhagic disease in U.S.A. and Canada (Carman et al. 1998, Corapi, French and Dubovi, 1989) and subsequently identified in Europe and South America (Canal et al. 1998, Nagai et al. 1998, Wolfmeyer et al. 1997).It was identified in Japan as a result of previous chapter. In addition to the classical clinical signs, some virulent strains of BVDV 2 cause severe thrombocytopenia with hemorrhage (Corapi, French and Dubovi 1989, Pellerin et al. 1994) Regretably, the pathogenesis of the clinical outcome is still unclear.

Antigenic heterogeneity in BVDV has been demonstrated by some investigators (Bolin and Ridpath 1992, Bolin, Littledike and Ridpath 1991, David et al. 1994, Donis, Corapi and Dubovi 1988, Paton, Lowings and Barett 1992, Ridpath, Bolin and Dubovi 1994, Shimizu, Murakami and Satou 1989), but their serotypes have not been established yet. Especially, BVDV 2 is antigenically distinct from BVDV 1 (Pellerin et al. 1994, Ridpath,

Bolin and Dubovi 1994). In previous chapter, the author reported that three strains of BVDV 2 isolated in Japan antigenically differed from BVDV 1 strain No. 12-43, which is the vaccine strain, used in Japan. Therefore, it was feared that BVDV 2, escaping neutralization by vaccine-induced antibody, might contribute to vaccine failure (Bolin, Littledike and Ridpath 1991). Although there are some reports of heterotypic protection by BVDV 1 vaccine against BVDV 2 (Cortese et al. 1998, Dean and Leyh 1999, Makoschey et al. 2001), the protective efficacy of the vaccine has to be determined on a case-by-case basis, as antigenicity of BVDV varies widely.

The purpose of the study reported in this chapter was to determine the efficacy of a Japanese modified-live BVDV vaccine containing ncp biotype of BVDV 1 in protecting calves from diseases caused by a virulent strain of BVDV 2 strain 890 which is a representative strain of BVDV 2.

MATERIALS AND METHODS

Animals

Five healthy calves of 3 to 5 months, bodyweight 100 to 200 kg, determined to be free of BVDV and neutralizing antibodies to BVDV, were randomly divided into two groups.

A group consisting of three calves (Calves A, B and C) was administrated intramuscular with a single dose of the combined live vaccine containing modified live infectious bovine rhinotracheitis (IBR) virus, bovine parainfluenza 3 virus and BVDV 1 strain No. 12-43 which is used to prevent these infectious diseases in Japan. The other group (Calves D and E) served as the unvaccinated control group. Four weeks after the administration of the vaccine, both groups were infected intravenously with BVDV 2 strain 890 ($10^4$$TCID_{50}$/head). General clinical observation was performed daily during the whole experiment. Special attention was paid to depression, anorexia and diarrhea. The clinical parameters were recorded daily as described previously (Makoschey et al. 2001). The total daily clinical scores were calculated. The rectal temperature of each calf was recorded every morning from 4 days before the challenge to 2 weeks after the challenge.

Virus isolation

For isolation of the challenge virus, buffy coats were collected from all calves after the challenge. Whole blood samples added heparin were centrifuged for 10 min at 760×g. Then, the buffy coats were stored at −80℃ before use for virus isolation. The buffy coats were delivered into a well of 6 wells plates per a buffy coat and 3mL of Eagle's minimum essential medium supplemented with 10% FCS containing BT cells was added to each well. The cultures were incubated at 37℃ for 5 days in a CO_2 incubator. The culture fluid was stored at −80℃ after the incubation and replaced with Eagle's minimum essential medium supplemented with 2% FCS containing 104 TCID50/mL of strain Nose. The cultures were further incubated for 3 days and observed for any CPE. The cultures absent from CPE were considered as infected with ncp BVDV strain 890 is ncp biotype. Additionally, the existence of BVDV strain 890 in the stocked culture fluid was checked by RT-PCR and detection of the *Pst* I site on the products was performed as described previous chapter.

Hematological test

Blood sample were collected from all the calves 2 and 4 days prior to the challenge, on the day of the challenge, and on every day for 2 weeks after the challenge. The number of total red blood cells (RBC), total white blood cells (WBC) and total platelet (PLT) were counted immediately with an automatic cytometor machine, PCE-170 (ERMA Inc.).

Neutralizing antibody test

Sera collected from all the calves every week after the vaccination were examined for neutralizing antibody to BVDV 2 strain 890 and BVDV 1 strain No. 12-43 by the method described previous chapter.

RESULTS

A pronounced increase in rectal temperature was measured in both unvaccinated calves. Seven days after the challenge, the average rectal temperature of the unvaccinated calves had risen to 41.15℃ (Calf D: 40.9℃, Calf E: 41.4℃) (Fig. 6). On the following days, however, they had rectal temperatures within the reference range. On the other hand, the average rectal temperature of the vaccinated calves continued within the reference range throughout the duration of the experiment (Fig. 6).

The clinical signs were validated using an established scoring system. The clinical scores were recorded from 4 to 8 days after the challenge in the unvaccinated calves (Fig. 7). They showed clear peaks at 6 and 7 days after the challenge. In contrast, all the total daily scores were "0" in the vaccinated calves.

BVDV was not isolated from buffy coats collected from the vaccinated calves at 1 to 3 and 7 to 14 days after the challenge. But, BVDV was isolated from buffy coats collected from the unvaccinated calves at 4, 5 and 6 days after the challenge (Fig. 7). Furthermore, specific fragments of expected size (256 bps) were observed in electrophoresis as a result of RT-PCR. The fragments were still 256 bps after digestion of the restriction enzyme *Pst* I.

Thus it was confirmed the isolation of BVDV 2. In contrast, BVDV was not isolated from any vaccinated calves throughout the duration of the experiment, and no specific fragment was amplified, either.

There was no remarkable change of RBC count in all the calves after the challenge (data not shown). The unvaccinated calves had markedly decreased WBC count starting 2 days after the challenge (Fig. 8). The average WBC count was lower at 2 days after the challenge, then recovered gradually and reached the baseline value by the end of the experiment. On the other hand, the average WBC count in the vaccinated calves did not decrease as drastically as in the unvaccinated calves (Fig. 8). The PLT count of one unvaccinated calf (Calf D) decreased temporarily from 2 days to 4 days after the challenge, but there was no marked change of average PLT count (data not shown).

As expected, neutralizing antibody titers against BVDV 1 strain No. 12-43 (component of the vaccine) in sera of the vaccinated calves began to increase from 2 weeks after the vaccination, and reached their maximum titers 5 weeks or 6 weeks after the vaccination (Fig. 9A). On the other hand, neutralizing antibodies were only detected in low titers in the sera collected

from the unvaccinated calves 3 weeks after the challenge (Fig. 7A). As shown in Fig. 9B, all calves had negative results for neutralizing antibody against BVDV 2 strain 890 until 5 weeks after the vaccination, but they had high neutralizing antibody titers from 2 weeks after the challenge (6 weeks after the vaccination). The first detectable titers of the vaccinated calves (Fig. 9B) were just a little higher than those of the unvaccinated calves (Fig. 9B).

DISCUSSION

It was reported that BVDV 2 strain 890 infection *in vivo* induced marked thrombocytopenia (Bolin and Ridpath 1992). In the present experiment, however, marked thrombocytopenia was not observed. In another study, thrombocytopenia was not observed in calves following BVDV 2 strain 890 (Dean and Leyh 1999). Concerning the cause of it, two predisposing factors may be considered. One is the age of the calves used in the experiment. Though most calves used in the study by Bolin and Ridpath were 3- to 4-weeks-old, we used 3-month-old calves. Generally, the older an animal is, the more resistant it is to infectious diseases. The other factor is virulence attenuation of the BVDV 2 strain 890 by passage in cell culture (Bezek, Gröhn and Dubovi 1994).

The above results indicate that vaccination with Japanese modified live vaccine containing BVDV 1 strain No. 12-43 can protect young calves from infection with BVDV 2 strain 890. Such a heterotypic protection by BVDV 1 vaccine has been already reported by some investigator (Cortese et al. 1998, Dean and Leyh 1999, Makoschey et al. 2001). It seems that these vaccine strains are antigenically closely related to the challenge viruses in spite of the difference of genotype. In the present study, however, antibody

against BVDV 2 strain 890 (the challenge virus) was not detected in any sera from the vaccinated calves at the time of the challenge, though their antibody titers against BVDV 1 strain No. 12-43 were not less than 64. This result suggests that BVDV 1 strain No. 12-43 (the vaccine strain) is antigenically distinct from BVDV 2 strain 890 (the challenge virus); nevertheless protection was observed in all the vaccinated calves.

Though a difference between BVDV 1 and BVDV 2 in antigenicity has been already indicated, they have cross-reacted in neutralizing antibody tests to greater or lesser degrees as described in previous chaptert and report (Fulton and Burge 2001). Therefore, it could be conjectured that the challenge virus does not multiply adequately for development of the clinical signs because of the antibodies induced by the vaccination.

The antibodies against the challenge virus were detected in the sera of all calves from 2 weeks after the challenge (6 weeks after the vaccination) (Fig. 9B). It is generally acknowledged that increase of antibody titer is induces by the immune response against the increase of pathogen. Although calves that received the vaccine did not develop detectable viremia and were free of the clinical signs, as observed in the unvaccinated calves, the antibody

against BVDV 2 strain 890 was detected in the sera of them after the challenge. The author conjectures that the antibody response might be induced by a "secondary immune response" based on the vaccination. The vaccinated calves would be able to respond well to the challenge virus, BVDV 2 strain 890, because of an "immunological memory" condition induced by the vaccination. Secondary immune response is stronger than primary immune response. This theory is supported by the fact that the antibody titers against BVDV 2 strain 890 in the vaccinated calves were higher than those in the unvaccinated calves from two to three weeks after the challenge in this experiment (Fig. 9B).

In the present experiment, calves administrated the vaccine containing BVDV 1 were not affected by the challenge of BVDV 2. However, it is known that BVDV has antigenically and pathogenically much variation. BVDV 2 isolated in Japan may differ from BVDV 2 strain 890 antigenically. Therefore, it is suggested that it's necessary to confirm the effectiveness of the vaccine on several strains of BVDV 2 isolated in Japan. 【J. Vet. Sci. 65: 263-266. 2003】

ABSTRUCT

BVDV has been segregated into two genotype. To determine the efficacy of the commercially available BVDV 1 vaccine used in Japan against BVDV 2, calves were infected with BVDV 2 strain 890 after 4 weeks of administration of the vaccine. The vaccinated calves did not develop any clinical signs and hematological changes such as observed in unvaccinated calves after the challenge. Furthermore, the challenge virus was not recovered from the vaccinated calves throughout the duration of the experiment, whereas it was recovered from all unvaccinated calves. The BVDV 1 vaccine used in Japan is efficacious against infection with BVDV 2 strain 890.

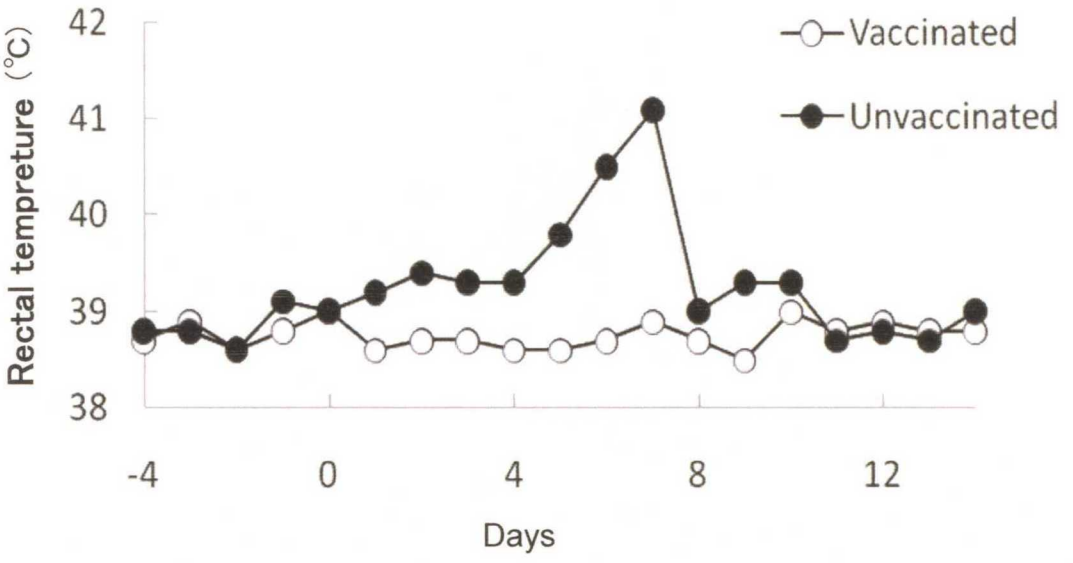

Fig. 6. Average rectal temperatures of calves after challenge with bovine viral diarrhea virus type 2 strain 890.

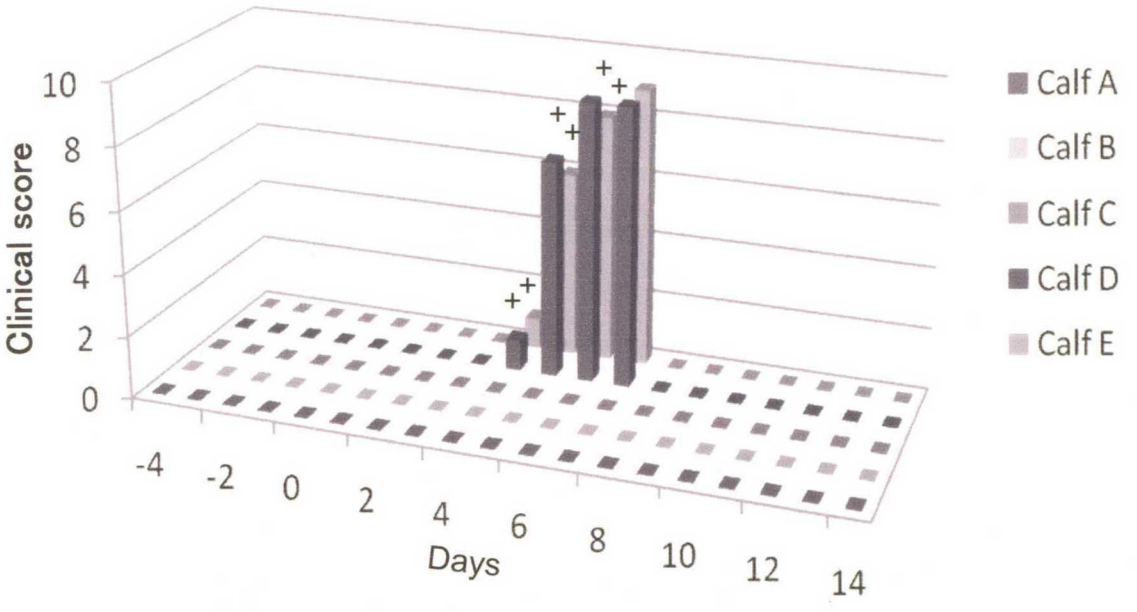

Fig. 7. Total clinical scores (Makoschey et al. 2001) of calves after challenge with bovine viral diarrhea virus type 2 strain 890. Cross symbol : Recovery and RT-PCR amplification of BVDV.

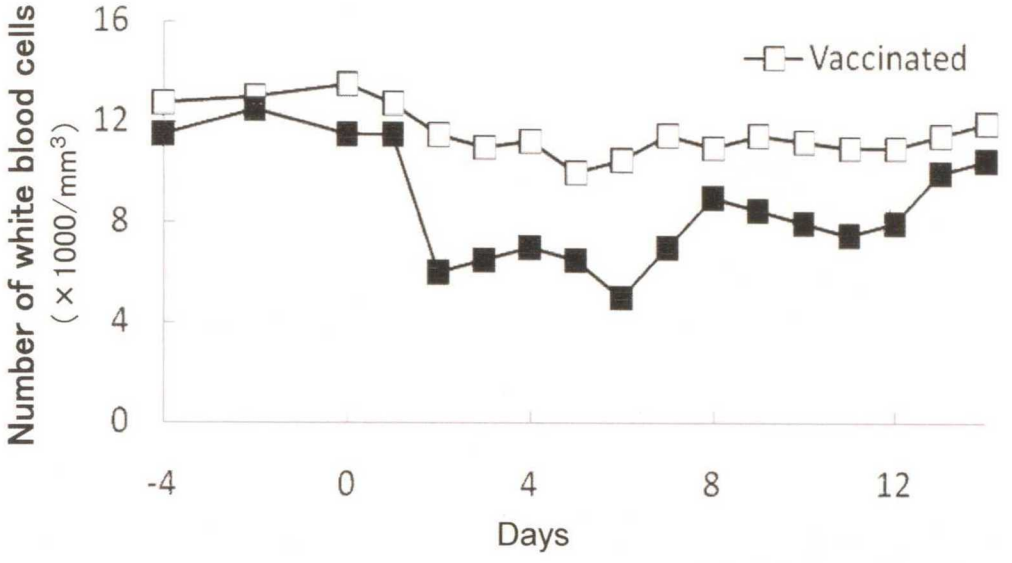

Fig. 8. Average white blood cells of calves after challenge with bovine viral diarrhea virus type 2 strain 890.

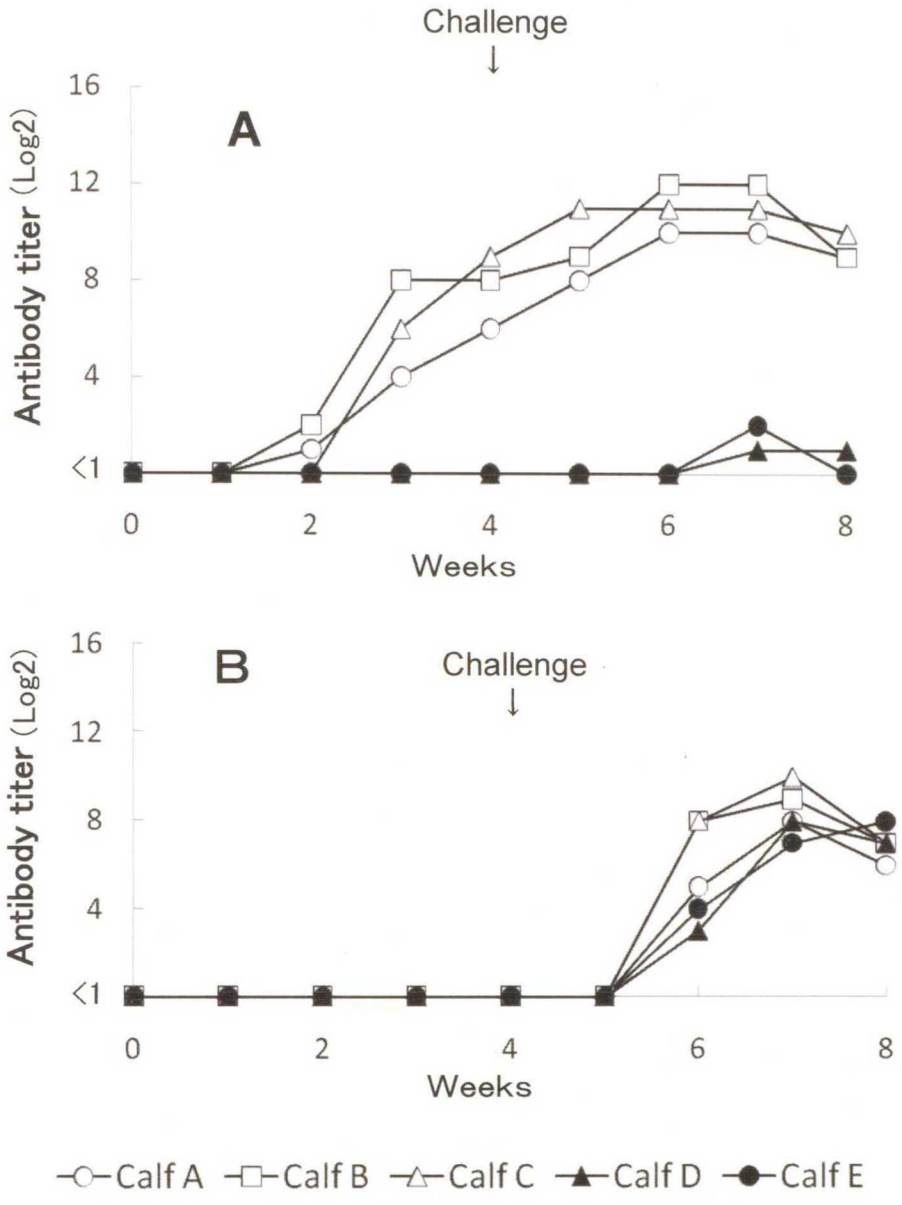

Fig. 9. Neutralizing antibody titers against bovine viral diarrhea virus type 1 strain No. 12-43 (A) and type 2 strain 890 (B) after the vaccination.

Chapter 3

Verification of the active bovine viral diarrhea virus (BVDV) contaminated in bovine live viral vaccines by means of the detection of negative-sense RNA of BVDV with RT-PCR

INTRODUCTION

Most of viral vaccine is made from vaccine strain cultured with biological material such as cells and fetal bovine serum. However, it means that the vaccines have some risks of contamination with extraneous infectious agent derived from biological materials. Bovine viral diarrhea virus (BVDV) is well known as contaminant within bovine serum and cells. If extraneous active BVDV is present in a live viral vaccine made with biological materials contaminated with active BVDV, it would cause serious problems for animal hygiene. Actually, it has been reported that Italy encountered an epizootic problem resulted as a result of the use of a live infectious bovine rhinotracheitis (IBR) vaccine contaminated with BVDV in cattle (Falcone et al 2003, Falcone, Tollis and Conti 2000).

Various assays by RT-PCR have been developed for the general detection of pestiviruses or the specific recognition between classical swine fever virus (CSFV) and BVDV (Ridpath and Bolin 1998, Sullivan and Akkina 1995, Vilček et al 1994, Wirt et al 1993). Since the RT-PCR has been recognized to be a very sensitive for the detection of BVDV in serum, cell cultures, seed virus and vaccines (Harasawa 1995, Sasaki et al 1996), those RT-PCR methods are recognized to be powerful tool for detection of viral

RNA. However, it is unfortunately impossible to determine whether the PCR products are amplified from RNA in active or inactive virus because of the use of a random primer or a specific antisense primer. Therefore, a RT-PCR method was developed for the detection of an active BVDV by amplification of negative strand viral RNA replicated in cells infected with BVDV (Aoki et al 2002). Previous report showed that the RT-PCR method was sensitive as same as biological method such as both of the exaltation of Newcastle disease method and the interference method, and also that it was applicable for detection of extraneous active CSFV in porcine live viral vaccines. The purpose of this study was to verify that extraneous active BVDV in a bovine live viral vaccine could be detected by the RT-PCR method

MATERIALS AND METHODS

Viruses and cells

Various ncp BVDV strains listed in Table 1 and cp BVDV Nose strain were used in this experiment. These viruses were propagated in primary bovine testicle cell cultures (BT cells) and stored at −80℃ before use. Virus titration for the ncp strains was done by the modified method based on the interference method with cp BVDV Nose strain as described previously (Nakamura et al 1995). The titer of each virus was calculated by Behrens-Kärber's method.

BT cells were prepared from the testicles of BVDV-free cattle and grown in Eagle's minimum essential medium supplemented with 10% FCS (Nakamura et al 1997). They were used for propagation of virus, the interference method and detection of negative-strand viral RNA.

Antisera

Antisera were produced and collected from SPF rabbits inoculated with vaccine strain of IBR live vaccine, Akabane live vaccine, Ibaraki live vaccine or bovine respiratory syncytial (RS) live vaccine.

RNA extraction and RT-PCR amplification (The RT-PCR method)

RNA extraction and RT-PCR amplification were carried out by the RT-PCR method described by Aoki et al. (Aoki et al 2002). Briefly, total RNA was extracted with TRIzol reagent according to the manufacture's protocol from BT cells inoculated with each BVDV or viral inoculums. All RT-PCR procedures were carried out using an RNA PCR Kit (AMV) Ver. 2.1 (Takara Shuzo Co., Ltd, Shiga, Japan). Complementary DNA was synthesized from negative-sense viral RNA using *Pst* 324α primer designed on the basis of the 324 primer (Vilček et al 1994). The sequence of the *Pst* 324α primer and position in the genome of BVDV NADL strain (Collett et al 1988c) are as follows: 5'-GCTAGCCATGCCCWYAGTAGGATAGCA-3' (sense: 100-127). Amplification of cDNA by PCR was carried out using primers 324 and 326 (Collett et al 1988c). After the amplification, electrophoretic analysis of the RT-PCR product was carried out on 2% agarose gel in Tris-borate-EDTA buffer.

RESULTS

Detection of various ncp BVDV strains by the RT-PCR method

This experiment has been tried to confirm whether detection of viral negative-sense RNA of BVDV based on the viral replication in the BVDV infected cells was possible in the case of any BVDV strains. Anti-sense viral RNA extracted from the 26 strain of ncp BVDV listed in table 1 was detected by the RT-PCR method. Specific fragments of the expected size (about 290 base pairs) were amplified from all of the viruses tested (Fig.10).

Comparison of sensitivity between the RT-PCR method and the interference method

In order to confirm a parallel of sensitivity for detection of virus between the RT-PCR method and the interference method, each of serial 10-fold diluted BVDV No.12-43 strain was inoculated onto BT cells grown as monolayer in 2 wells. At 3 days after inoculation, BT cells in a well were applied to the detection of ncp BVDV by the RT-PCR method. At 5 days after inoculations, infected cells in the other well were superinfected with cp BVDV Nose strain for the virus titration by the interference method. On the RT-PCR method, the expected bands (about 290 base pairs) were amplified

until 10^{-7} dilution by electrophoretic analysis (Fig.11). On the other hand, CPE developed by BVDV Nose strain was not observed until 10^{-7} dilution. These results indicate that there is no difference in virus titer of ncp BVDV No.12-43 strain between both methods. The sensitivity of the RT-PCR method was exactly the same as that of the interference method.

Detection of extraneous BVDV spiked in commercial bovine viral vaccines by the RT-PCR method

It has been examined whether it is possible to detect the negative-sense BVDV RNA from the commercial bovine viral vaccines which have been artificially spiked with ncp BVDV. Each of serial 10-fold dilutions of ncp BVDV No.12-43 strain was added to one dose of the BVDV free live vaccines for bovine (IBR live vaccine, Akabane live vaccine, Ibaraki live vaccine and Bovine RS live vaccine) which had been inspected beforehand by the interference method. After neutralization of vaccine strains with each antiserum, detection of spiked BVDV from each vaccine was carried out by both the RT-PCR method and the interference method. Spiked BVDV No.12-43 strain was detected until 10^{7} -dilution in all the examined vaccines

by both the RT-PCR method and the interference method, respectively (Table3).

Detection of extraneous active BVDV in the commercial bovine live viral vaccines

Six batches of IBR live vaccine, 4 batches of Akabane live vaccine, a batch of Ibaraki live vaccine and a batch of bovine RS live vaccine were examined by the RT-PCR method and the interference method for detection of extraneous ncp BVDV. It has confirmed that these vaccines except Batch No.2 of IBR live vaccine have not contained extraneous BVDV by the interference method. On the other hand, ncp BVDV was detected in Batch No.2 of IBR live vaccine by the same method. By the RT-PCR method, the specific fragments of the expected size (about 290 base pairs) were observed in Batch No.2 of IBR live vaccine. (Fig.12)

DISCUSSION

All of the ncp BVDV strains examined in this study were detected by the RT-PCR method. On the basis of the nucleotide sequence of the 5'-untranslated region (5'-UTR), BVDV strains are phylogenetically categorized into genotype 1 (BVDV-1) and genotype 2 (BVDV-2) (Collett et al. 1988c, Thiel et al. 2005). The RT-PCR method was not influenced by the genotype because the method could detect BVDV-2 as well as BVDV-1.

BVDV-1 strains can be further divided into at least 11 genetic subgroups (Vilček et al. 2001). Although we did not reveal genetic subgroups of all BVDVs examined in this study, the RT-PCR method is expected to be available whatever genetic subgroup the BVDV comes under because the 5'-UTRs of various strains of BVDV were detected by RT-PCR with the same primers, 324 and 326 (Matsuno et al. 2007, Sakoda et al. 1999).

All of the bovine live viral vaccines used in Japan were examined for the presence of extraneous active ncp BVDV by the interference method which is one of most sensitive methods. In this study, the RT-PCR method was no less sensitive than the interference method. Therefore, the RT-PCR method may have potential as an alternative method.

Spiked BVDV No. 12-43 strain could be detected by both the RT-PCR

method and the interference method until 10^7-dilution in bovine live viral vaccines. The results suggested that ingredients in those vaccines did not interfere with the RT-PCR.

A total of 12 batches of bovine live viral vaccines were examined to determine the alternative method could be applied for commercial vaccines for cattle. As expected, the results agreed with previous results obtained using the interference method.

Although recent advances in biology and technology have significantly improved the ability to produce veterinary biologicals of high purity, efficacy and safety, the presence of pestiviruses in raw materials of animal origin is a problem widely recognized by vaccine manufacturers (Falcone, Tollis and Conti 2000). The use of BVDV-contaminated fetal bovine serum (FBS) can result in infection of cell culture (Nuttall, Luther and Stott 1977). BVDV contamination can sometimes be difficult to detect and can lead to generation of cell and virus stocks and to final batch of products that also contain the contaminating virus (Falcone, Tollis and Conti 2000). Therefore, it is very important to examine for the presence of extraneous BVDV in an animal vaccine, especially in a bovine live viral vaccine.

In this study, it was verified that extraneous active BVDV in a bovine live viral vaccine could be detected by the RT-PCR method. The RT-PCR method is a rapid, easy and highly sensitive method that can be used for specific screening and for detection of extraneous active pestiviruses in animal viral vaccines (Aoki et al. 2002). The interference method requires 17days of incubation and superinfection with BVDV Nose strain. Compered with it, the RT-PCR method could be carried for 3 days after inoculation of sample on BT cells and does not require BVDV Nose strain. Therefore, the RT-PCR method is more rapid and easier than the interference method. Aoki et al. reported that the sensitivity of the RT-PCR method was higher than that of the interference method at a lower dose (less than 10 $TCID_{50}$). This study did not bring about the results that RT-PCR method was a highly sensitive method because it was not carried out comparison of the sensitivities of the RT-PCR and the interference methods at a lower dose.

The RT-PCR method can not be applied for bovine viral diarrhea vaccine because it has necessarily contained negative-strand viral RNA of BVDV derived from the vaccine strain. We conclude that the RT-PCR method is very available as a method for quality control of bovine live viral vaccines

except for bovine viral diarrhea live vaccine. 【Jap. J. Anim. Hyg. 35: 41-46. 2009】

ABSTRUCT

The RT-PCR developed for the detection of active pestiviruses by amplification of negative strand viral RNA was verified to detect extraneous active BVDV in bovine live viral vaccines. The RT-PCR method was confirmed to be no less sensitive than the interference method. Moreover, it was also shown that all of the non-cytopathogenic BVDV strains examined in this study were detected by this method. Furthermore, ingredients in bovine live viral vaccines did not interfere with the RT-PCR method. Twelve batches of bovine live viral vaccines were examined by both methods if the alternative method could be applied for commercial vaccines for cattle. As expected, the results agreed with previous results obtained using the interference method. The author concludes that the novel RT-PCR method is very useful for quality control of bovine live viral vaccines except for BVD live vaccine.

Table 2. The ncp BVDV used in the study

Strains	Origin	Genotype
NADL	U. S. A.	BVDV1
New York-1	U. S. A.	BVDV1
Indiana 46	U. S. A.	
Osloss	Germany	BVDV1
Kanto-Tozan	Japan	
KS86-1	Japan	
TK87-2	Japan	BVDV1
NM87-1	Japan	BVDV1
Nose	Japan	BVDV1
No.12-43	Japan	BVDV1
890	U. S. A	BVDV2
Shiribeshi 2	Japan	BVDV1
Shiribeshi 4	Japan	BVDV1
Shiribeshi 129	Japan	BVDV1
419	Japan	BVDV1
SW90-1	Japan	BVDV2
SW90-2	Japan	BVDV2
SW90-3	Japan	BVDV1
SW90-4	Japan	BVDV1
SW90-7	Japan	BVDV2
Ishikawa 1995-1	Japan	
Ishikawa 1995-2	Japan	
Ishikawa 1995-4	Japan	
Ishikawa 1995-5	Japan	
GBK E-	Japan	
K	Japan	

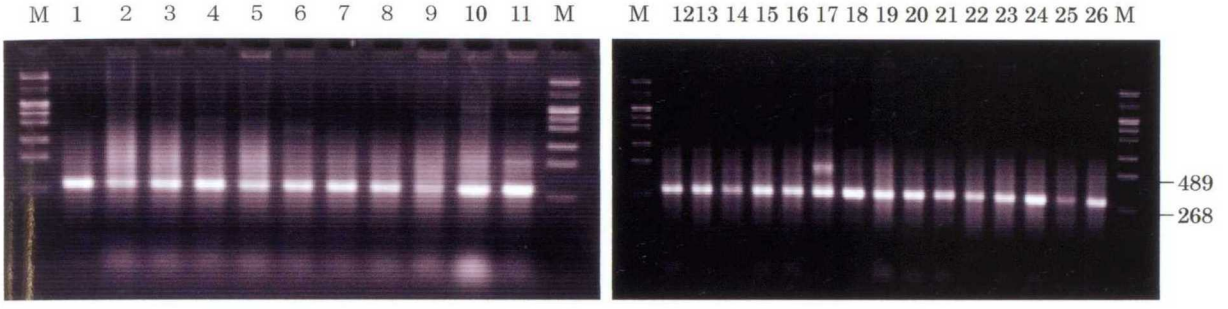

Fig. 10. Electrophoretic analysis of the RT-PCR products of various ncp BVDVs. Lane M: pHY marker, Lane 1: NADL, Lane 2: New York-1, Lane 3: Indiana-46, Lane 4: Osloss, Lane 5: Kanto-Tozan, Lane 6: KS86-1, Lane 7: TK87-2, Lane 8: NM87-1, Lane 9: Nose, Lane 10: No.12-43, Lane 11: 890, Lane 12: Shiribeshi 2, Lane 13: Shiribeshi 4, Lane 14: Shiribeshi 129, Lane 15: 419, Lane 16: SW90-1, Lane 17: SW90-2, Lane 18: SW90-3, Lane 19: SW90-4, Lane 20: SW90-7, Lane 21: Ishikawa1995-1, Lane 22: Ishikawa1995-2, Lane 23: Ishikawa1995-4, Lane 24: Ishikawa1995-5, Lane 26: GBK E-, Lane 26: K

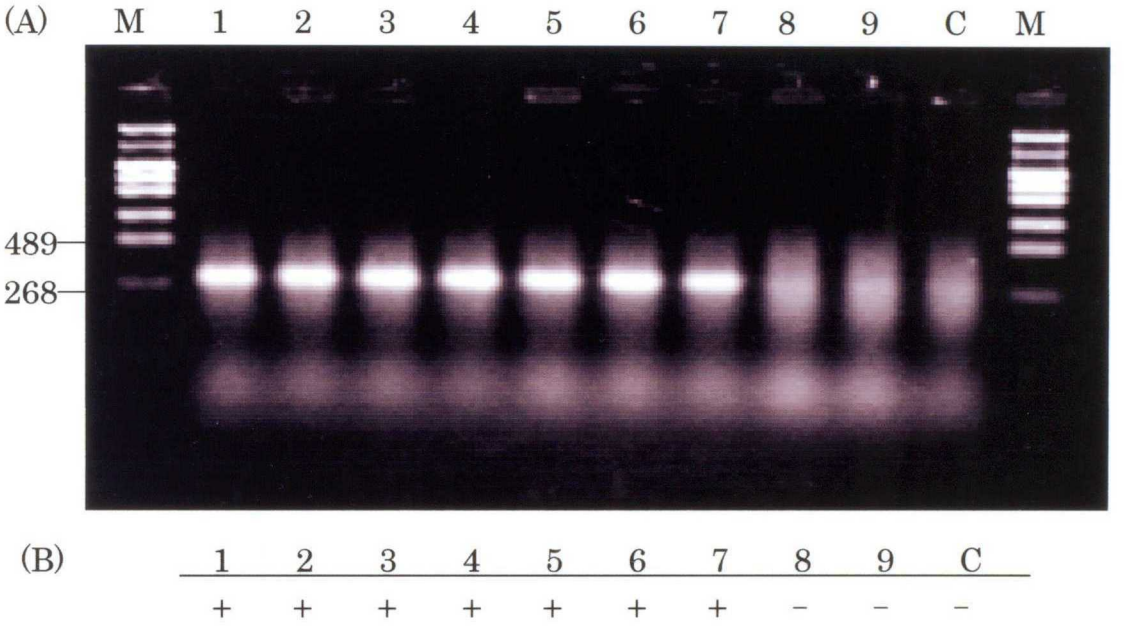

Fig.11. Comparison of the sensitivity of the RT-PCR method (A) and the interference method (B). Lanes 1-9: Serial 10-fold dilution of BVDV No.12-43, C: mock-infected BT cells, M: pHY marker, +: CPE was not observed.

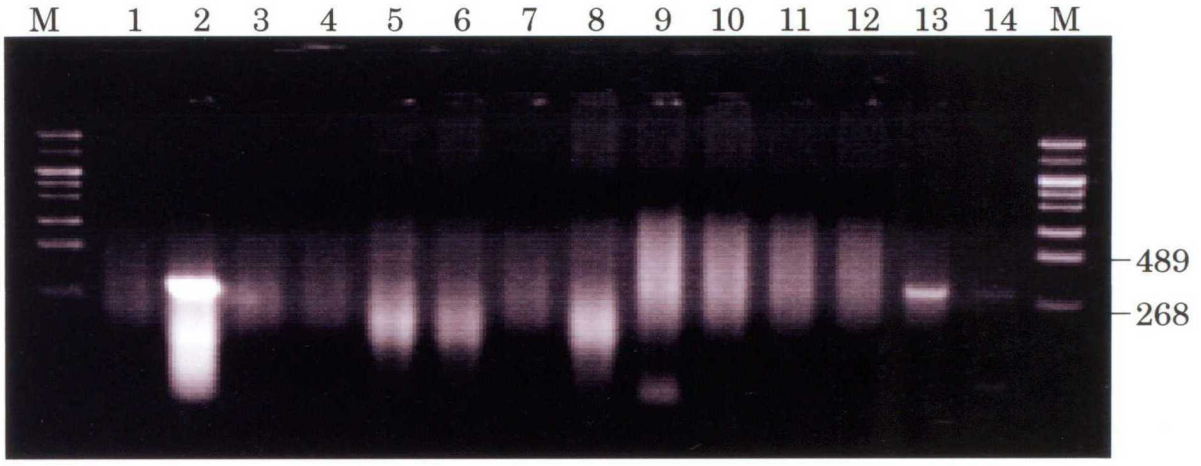

Fig.12. Detection of extraneous BVDV from bovine viral live vaccine. M: pHY marker, Lanes 1-6: Batch No.1-6 of IBR live vaccines in turn, Lane 7-10: Batch No.1-4 of Akabane live vaccines in turn, Lane 11: Ibaraki live vaccine, Lane 12: Bovine RS live vaccine, Lane 13: BVDV No.12-43 strain (positive control), Lane 14: mock-infected BT cells.

Table 3. Detection of spiked ncp BVDV from bovine viral live vaccine

Vaccine	Method	Dilution of spiked ncp BVDV [log]					
		5	6	7	8	9	C*
IBR	Interference	+	+	+	−	−	−
	RT-PCR	+	+	+	−	−	−
Akabane	Interference	+	+	+	−	−	−
	RT-PCR	+	+	+	−	−	−
Ibaraki	Interference	+	+	+	−	−	−
	RT-PCR	+	+	+	−	−	−
Bovine RS	Interference	+	+	+	−	−	−
	RT-PCR	+	+	+	−	−	−

* mock-infected BT cells

CONCLUSION

In the 1980s, BVD with symptoms of severe thrombocytopenia and hemorrhage occurred in Canada and the United States. The isolated causing hemorrhagic syndrome and acute severe BVD formed a new genetic group (genotype) of BVDV distinct from early strains. The new group was designated BVDV 2 and the group comprising the early strains was designated BVDV 1. The prevalences of BVDV 2 in Europe and South America were later reported.

Heterogeneity is the defining characteristic of BVDV. Variation between BVDV 1 and BVDV 2 has practical significance. In particular, variation in antigenicity has an impact on detection and management programs. BVDV 1 and BVDV 2 are antigenically distinct. Understanding the variation among BVDV strains is essential for successful control of BVDV. Therefore, the author segregated BVDV strains isolated in Japan into BVDV 1 and BVDV 2 using genomic criteria and demonstrated their pathogenecity and antigenicity.

Three BVDV strains isolated from PI calves in Tochigi Prefecture in Japan belonged to BVDV 2. While the pathogenicity of these strains was

weak, antigenicity of the strains differs from that of early strains. Therefore, the author demonstrated protection by a Japanese BVDV vaccine containing ncp BVDV 1 strain No.12-43 in calves against BVDV 2 strain 890. Four weeks after vaccination, the vaccinated and unvaccinated calves were challenged intravenously with BVDV 2 strain 890. The vaccinated calves were seronegative to BVDV 2 strain 890 at the time of challenge; however, the neutralizing titers to ncp BVDV 1 were >1:64. The vaccinated calves did not develop clinical signs or fever and did not show hematological change (decreased WBC) after challenge. The vaccinated animals were protected against blood leukocyte infection. The BVDV 1 vaccine used in Japan is efficacious against infection with BVDV 2 strain 890.

Although BVDV may be spread by animals that are either persistently or acutely infected, the main emphasis until now has been on the detection of PI animals. This is because the removal of PI animals is considered to be integral to an effective control strategy. Infection of the fetus with an ncp BVDV may result in the birth of a PI animal. Persistent infection has been established with both BVDV 1 and BVDV 2. Generation of PI animals peaks when infection occurs from about 30 until 90 days of

gestation and becomes less frequent as the fetus approaches 125 days of gestation (Roeder, Jeffrey and Cranwell 1986, Radostits and Littlejohns 1988). Unfortunately, a PI animal may be born from a cow that had been injected with a vaccine contaminated with a ncp BVDV at about 30 to 90 days of gestation. Accordingly, it is important to examine for the presence of extraneous BVDV in a bovine viral vaccine. The author verified that extraneous active BVDV in a bovine live viral vaccine could be detected by al RT-PCR method. As expected, the RT-PCR method is very useful for quality control of bovine live viral vaccines except for BVD live vaccines.

The author concludes that the present works are possible to contribute to develop a successful control of BVDV 2 in Japan and the quality control of a BVD live vaccine, which is very important tool for prevention of bovine infectious diseases.

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SUMMARY IN ENGLISH

Preface

The first report on bovine viral diarrhea (BVD) was outbreak in cattle observed in the U.S.A. in 1946. After the outbreak in the U.S.A., a similar but more severe BVD in cattle with mucosal disease (MD) was reported in Canada. Thus, several years later, the disease became officially known as bovine viral diarrhea-mucosal disease. At present, BVD is an economically important disease of cattle having been reported throughout the world and is caused by infection with bovine viral diarrhea virus (BVDV).

BVDV is a small positive-sense single-stranded RNA virus classified in the genus *Pestivirus* within the family *Flaviviridae*. There are currently four recognized species within the pestivirus genus: BVDV 1; BVDV 2; border disease virus and classical swine fever virus, previously known as hog cholera virus. The Pestivirus genome is approximately 12.3 Kb in length. The highest nucleic acid sequence identity among pestiviruses is found in the 5' UTR .

There are two biotypes of BVDV: one is non-cytopathogenic (ncp) BVDV which did not induce cytopathogenic effects (CPE) in any cell culture,

and the other is cytopathogenic (cp) virus, which shows clear CPE in bovine cell cultures. It became generally accepted that only ncp BVDV strain could produce persistently infected (PI) cattle. PI cattle with ncp BVDV suffer from MD if they are superinfected with a cp BVDV .

Hemorrhages associated with BVDV infection in young veal calves were also observed with increasing frequency in the late 1980s in the northeastern United States. Phylogenetic analysis of the BVDV strains grouped them separately from the BVDV strains commonly used, at that time, in vaccine production, diagnostic tests, and research. The newly recognized group of BVDV 2 and the group containing the early strains was termed BVDV 1. Prevalence of BVDV 2 in North America, Europe and South Africa have been reported. Ideally, genotypes would be associated with practical observations such as geographic distribution, antigenic variation, or variations in virulence. The differentiation between the BVDV 1 and BVDV 2 genotypes meets these practical considerations. Therefore, it is worthwhile to segregate BVDV strains isolated in Japan into BVDV 1 or BVDV 2.

With the emergence of BVDV 2, the concern for incorporation of

BVDV 2 in vaccines escalated with a report that vaccines containing BVDV 1 appeared not to be protective against infection with BVDV 2. The efficacy of Japanese vaccine containing BVDV 1 against BVDV 2 should also be evaluated.

The observation that fetal bovine sera frequently contained BVDV was an increasing concern in the 1970s. In order to ensure that BVDV-free vaccines are produced, it is therefore essential to use valid tests and to develop effective quality assurance programs, stage by stage, throughout the manufacturing process.

As mentioned above, BVDV, especially new BVDV 2, is a very important agent of livestock hygiene and quality control of vaccines. Regrettably, it is not cleared the presence of BVDV 2 in Japan, efficacy of commercial vaccine used in Japan against BVDV 2 and potentiality of detection of extraneous active BVDV in bovine vaccine by RT-PCR. The purposes of this study were to clarify these uncertainties.

The present thesis consists of three chapters. In the first chapter, BVDV strains isolated in Japan are segregated into genotypes. In the second chapter, the efficacy of BVD vaccine used in Japan against BVDV 2 strain

890 is evaluated. In the last chapter, it is shown that the active BVDV contaminating bovine live viral vaccines could be detected by means of the detection of negative-sense RNA of BVDV with RT-PCR.

Segregation of bovine viral diarrhea virus isolated in Japan into genotypes

It was demonstrated that BVDV 2 did not have *Pst* I site, which is present in all known BVDV 1, on the 5'-UTR of genome. It made it easy that BVDV was distinguished BVDV 2 from BVDV 1. BVDV infection associated with thrombocytopenia in Japan has not been reported in any literature until now. In the chapter, BVDV strains isolated in Japan are segregated into BVDV 1 or BVDV 2 using genomic criteria, and demonstrated those pathogenecity and antigenicity.

It was suggested that 3 strains of BVDV isolated from PI calves in Tochigi Prefecture in Japan belonged to BVDV 2. It was recognized lack of *Pst* I site on the 5'-UTR of genome of them. Inoculated with the 3 strains, the calves showed the mild decrease of platelet counts which was specific clinical sign of BVDV 2. It should be reported that the 3 strains were the first BVDV 2 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 1 and BVDV 2 in the antigenicity.

Efficacy of bovine viral diarrhea vaccine used in Japan against *Bovine viral diarrhea virus 2* strain 890

Antigenic heterogeneity in BVDV has been demonstrated, but their serotypes have not been established yet. Especially, BVDV 2 is antigenically distinct from BVDV 1. In previous chapter, it was reported that three strains of BVDV 2 isolated in Japan antigenically differed from BVDV 1 strain No. 12-43, which is the vaccine strain, used in Japan. Therefore, it was feared that BVDV 2, escaping neutralization by vaccine-induced antibody, might contribute to vaccine failure. The purpose of the study reported in this chapter was to determine the efficacy of a Japanese modified-live BVDV vaccine containing ncp biotype of BVDV 1 in protecting calves from diseases caused by a virulent strain of BVDV 2 strain 890 which is a representative strain of BVDV 2

The vaccinated calves did not develop any clinical sign and hematological change such as observed in unvaccinated calves after the challenge. Furthermore, the challenge virus was not recovered from the

vaccinated calves throughout the duration of the experiment, whereas it was recovered from all unvaccinated calves. The BVDV 1 vaccine used in Japan is efficacious against infection with BVDV 2 strain 890.

Verification of the active bovine viral diarrhea virus (BVDV) contaminated in bovine live viral vaccines by means of the detection of negative-sense RNA of BVDV with RT-PCR

Most of viral vaccine is made from vaccine strain cultured with biological material such as cells and fetal bovine serum. However, it means that the vaccines have some risks of contamination with extraneous infectious agent derived from biological materials. BVDV is well known as contaminant within bovine serum and cells. If extraneous active BVDV is present in a live viral vaccine made with biological materials contaminated with active BVDV, it would cause serious problems for animal hygiene.

Since the RT-PCR methods has been recognized to be a very sensitive for the detection of BVDV in serum, cell cultures, seed virus and vaccines, those RT-PCR methods are recognized to be powerful tool for detection of viral RNA. However, it is unfortunately impossible to determine whether the PCR products are amplified from RNA in active or inactive virus because of

the use of a random primer or a specific antisense primer. Therefore, the RT-PCR method was developed for the detection of an active BVDV by amplification of negative strand viral RNA replicated in cells infected with BVDV. The purpose of this study was to verify that extraneous active BVDV in a bovine live viral vaccine could be detected by the RT-PCR method.

The RT-PCR method was confirmed to be no less sensitive than the interference method. Moreover, it was also shown that all of the non-cytopathogenic BVDV strains examined in this study were detected by this method. Furthermore, ingredients in bovine live viral vaccines did not interfere with the RT-PCR method. The author concludes that the RT-PCR method is very useful for quality control of bovine live viral vaccines except for BVD live vaccine.

Conclusion

BVDV isolated causing hemorrhagic syndrome and acute severe BVD formed a new genetic group (genotype) of BVDV distinct from early strains. Variation between BVDV 1 and BVDV 2 has practical significance. Understanding for variation among BVDV is essential to developing a successful control of BVDV. Therefore, BVDV strains isolated in Japan were

segregated into BVDV 1 and BVDV 2 using genomic criteria, and demonstrated those pathogenecity and antigenicity .

Three BVDV strains belonged to BVDV 2. While the pathogenicity of these strains was weak, antigenicity of these strains differs from those of early strains. Therefore, the author examined protection by a Japanese BVDV 1 vaccine in calves against BVDV 2 strain 890. The vaccinated calves did not developclinical signs or fever nor have hematological change (decreased WBC) after challenge. The BVDV 1 vaccine is efficacious against infection with BVDV 2 strain 890.

The removal of PI animals is considered to be integral to an effective control strategy. Unfortunately, PI animal may be born from cow injected with a vaccine contaminated ncp BVDV. Accordingly, it is very important to examine for the presence of extraneous BVDV in a bovine viral vaccine. So, it is shown that extraneous active BVDV in a bovine live viral vaccine could be detected by the RT-PCR method. As expected, the RT-PCR method is very useful for quality control of bovine live viral vaccine except for BVD live vaccine.

The author concludes that the present works are possible to contribute to develop a successful control of BVDV 2 in Japan and the quality control of a BVD live vaccine, which is a very important tool for prevention of bovine infectious diseases.

SUMMARY IN JAPANESE

和文要旨

牛ウイルス性下痢症(BVD)は 1946 年にアメリカで初めて報告され、その後カナダで粘膜病を伴う重篤な BVD が報告された。BVD はその数年後に牛ウイルス性下痢-粘膜病として広く知られるようになった。現在、BVD は牛ウイルス性下痢ウイルス(BVDV)の感染による大変重要な疾病として世界中で報告され畜産経営に甚大な被害を及ぼしている。

BVDV は、フラビウイルス科ペスチウイルス属に属するプラス１本鎖 RNA ウイルスである。ペスチウイルス属は、BVDV１、BVDV２、ボーダー病ウイルスおよび豚コレラウイルスの４種からなり、その遺伝子の長さは約 12.3Kb である。 5'末端の非翻訳領域の塩基配列は属内で最も保存され、特異的であるため遺伝子検査に適している。

BVDV には２つのバイオタイプがあり、１つは培養細胞に細胞変性効果(CPE)を引き起こす細胞病原性株で、もう１つは CPE を引き起こさない非細胞病原性株である。この性状は病原性と関連し、非細胞病原性株のみが持続感染牛をつくると言われ、その持続感染牛に細胞病原性株が重感染すると粘膜病になるとされている。

1980 年代後半、北米で食用子牛に出血を主徴とする高病原性を呈する BVDV 感染が頻繁に発生した。その分離株について遺伝子の系統樹解析を行ったとこ

ろ、従来の野外流行株、ワクチン株、診断用抗原、若しくは研究材料として一般的に用いられていた株とは異なるグループにあることが判明し、このグループを新たな種、BVDV2 とし、既知の株が属するグループを BVDV1 と命名された。その後、BVDV2 の流行は北米だけにとどまらず欧州や南米にも拡がった。我が国においても早急に BVDV2 の浸潤状況を把握し、抗原性状、病原性について検討し、本疾病の防圧の対処する必要が求められた。そこで、我が国で分離された BVDV の遺伝子性状について検討し、BVDV2 の検出を試みた。

また、BVDV1 を製造用株とする現行ワクチンは BVDV2 感染を予防できないとする報告があったことから、ワクチンの製造用株に BVDV2 を加えることが言及されるようになった。そこで、著者は我が国で使われている BVDV1 を製造用株としているワクチンが BVDV2 感染症の予防に対して有効かどうかを明らかにする必要があると考えた。

一方、1970 年代から細胞培養に使用していた牛胎子血清に BVDV が混入するケースが増加傾向にあり、培地および培養細胞への汚染が目立つようになった。このため、BVDV が迷入していないワクチンの製造を確保するためには、ワクチンの各製造工程における適切な試験の実施と効果的な品質保証制度の開発が必要不可欠であると思われた。

上述のように、BVDV、とりわけ新たな BVDV2 は、家畜衛生およびワクチ

ンの品質管理においてたいへん重要な病原因子である。そこで、著者は、我が国における BVDV2 の存否、BVDV2 に対する現行ワクチンの効果、および RT-PCR による牛用ワクチン中に迷入する活性 BVDV の検出法を開発する必要があると考え以下のような研究を行なった。

本論文は３章から構成される。第１章では我が国で分離された BVDV を遺伝子型別した。第２章では、我が国で使用されている BVD ワクチンの BVDV2 感染に対する効果を評価した。第３章では、BVDV の複製過程で生成されるマイナス鎖 RNA を RT-PCR により検出する方法が、牛用生ウイルスワクチン中に迷入する活性 BVDV の検出に有用か検証した。

第１章　我が国で分離された牛ウイルス性下痢ウイルスの遺伝子型別

BVDV2 の 5'末端非翻訳領域には BVDV1 に比べ *Pst* I サイトがない。このことを指標に BVDV は容易に BVDV１と BVDV2 に型別できる。そこで、著者は我が国で分離された BVDV を BVDV1 若しくは BVDV2 に型別し、検出された BVDV2 の病原性および抗原性を明らかにした。

その結果、栃木県の持続感染牛から分離された３株の 5'末端非翻訳領域に *Pst* I サイトがなかったことから、これら３株は BVDV2 と型別された。これら３株を牛に実験感染させたところ，発熱およびロイコペニーと共に BVDV2 に特徴

的な血小板減少が認められた。以上の結果から、これらの３株は，我が国における初めての BVDV2 であることが判明した。また、実験感染牛の血清中和抗体価を測定したところ、ホモの株に対する抗体価と BVDV1 である既知株に対する抗体価に差が認められ，抗原性の相違が示唆された。

第２章　牛ウイルス性下痢ウイルス 2-890 株に対する我が国で使用されている牛ウイルス性下痢症ワクチンの有効性

前章で、著者は BVDV2 であることが明らかとなった 3 株の抗原性はワクチン株である№12-43 株のそれと異なることを示した。このことから、BVDV2 がワクチン抗体をすり抜けることで既存のワクチンが無効となることが危惧された。そこで、BVDV1 を製造用株として我が国で汎用されている BVD ワクチンが BVDV2 の代表株かつ強毒株である 890 株感染に対する有効性を調べた。ワクチン接種牛および未接種牛に BVDV2-890 株を攻撃したところ、ワクチン接種牛には未接種牛に診られた臨床症状や血液学的変化が観察されず、攻撃株も分離されなかった。この成績から我が国の BVDV ワクチンは BVDV2-890 株感染に対して有効であることが明らかとなった。

第３章　牛用ウイルスワクチンに迷入する活性牛ウイルス性下痢ウイルスの迅速検出法の検証

多くのウイルスワクチンは細胞や牛胎子血清などの生物由来原料を使って培養したウイルスをワクチン株として製造されている。しかし、それは生物由来原料を介して感染因子が迷入するリスクを常に持っているということを意味するものである。BVDV は牛血清や細胞に混入することがよく知られている。活性 BVDV に汚染された生物由来原料を使ってワクチンを製造した場合、それは家畜衛生上、非常に重篤な問題を生じることになるかもしれない。

従来用いられた RT-PCR は、血清や培養細胞、シードウイルス、ワクチンから BVDV を検出できる非常に感度のいい方法であるが、これまでの RT-PCR はランダムプライマー若しくは特異的なアンチセンスプライマーを用いるため、その混入している BVDV が活性を有しても有していなくても PCR 産物が増幅されてしまう。そこで、BVDV が感染細胞で複製する際に生じるマイナス鎖 RNA を増幅することで活性を有する BVDV のみを検出する新しい RT-PCR 法を開発し、この新しい RT-PCR 法を用いて牛用生ウイルスワクチン中に迷入する活性 BVDV を検出できるかどうかを検証した。

この RT-PCR 法は、動物用生物学的製剤基準において採用されている干渉法と同程度の感度であることが確認された。さらに、供試した全ての非細胞病原

性 BVDV を検出することができ、牛用ウイルス生ワクチン中に含まれる成分の干渉を受けなかった。本法は牛用ウイルス生ワクチンの品質管理法として有用であると考えられた。

著者が我が国で初めて BVDV2 と型別した 3 株の病原性は、北米で分離されたウイルスに比べると弱いものであったが、抗原性は既知の BVDV1 とは異なるものであった。そこで、著者は我が国の BVDV1 を製造用株とした現行ワクチンが BVDV2 感染を予防できるか調べるために、ワクチン接種牛および未接種牛に BVDV2-890 株を攻撃した。その結果、ワクチン接種牛には未接種牛に診られた臨床症状や血液学的変化が観察されず、攻撃株も分離されなかった。我が国の BVD の現行ワクチンは BVDV2-890 株感染に対して有効であった。

BVD をコントロールする上で最も効果的なことは持続感染牛の摘発淘汰である。非細胞病原性の BVDV が迷入したワクチンを妊娠牛に接種すると、不幸にも持続感染牛が生まれてくる恐れがある。したがって、牛用ウイルスワクチンに BVDV が迷入していないか調べることは大変重要なことである。そこで、著者は新しい RT-PCR 法を用いて牛用生ウイルスワクチン中に迷入した活性 BVDV を検出できるか検証したところ、期待通り、新しい RT-PCR 法は牛用生ウイルスワクチンの品質管理にたいへん有用であることを示した。

著者は、今般の研究成果が、我が国における BVDV2 感染症の防疫対策や牛の感染症対策において重要なツールである牛用ワクチンの品質管理に役立つものであるとの結論に至った。