Survey on vertical infection of bovine viral diarrhea virus from fetal bovine sera in the field

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ABSTRACT. Bovine viral diarrhea virus (BVDV) isolation and antibody survey were performed using 2,758 fetal bovine sera (FBS) collected from slaughterhouses in New Zealand, Australia and the Dominican Republic, and then sent to Japan to manufacture commercial serum for cell culture use. FBS in the Dominican Republic were pooled for each several individuals, and those collected in other countries were separated according to each individual and subjected to the tests. BVDV was isolated from 25 (0.91%) FBS, and the BVDV antibody was detected in 44 (1.60%) FBS. The survey on 139 sets of paired sera of a dam and her fetus revealed that neither the BVDV antibody nor BVDV was detected in all FBS from BVDV antibody-positive dams.
KEY WORDS: bovine viral diarrhea virus, cattle, fetal bovine serum, infectious disease, vertical infection

Bovine viral diarrhea-mucosal disease is one of the important epidemics causing economic losses to the world's cattle industry [7–10], and persistently infected cattle are the most important source of infection [1, 11, 22]. Bovine viral diarrhea virus (BVDV) has been isolated from cattle showing a variety of clinical signs, such as transient fever, diarrhea, respiratory symptoms, mucosal disease and abortion [1, 13]. However, there are cattle which show no clinical symptoms, even if persistently infected with the virus. BVDV easily causes *in utero* infection and induces fetus immune tolerance and persistent infection, particularly during a specific period of pregnancy [5, 13, 15, 16]. On the other hand, BVDV is known to be infectious to many cultivated cells originating from various animal species, though cattle are the natural host of BVDV. Many cultured cells have reportedly been contaminated with BVDV [4, 20, 25], as fetal bovine serum (FBS) is widely used for cell cultures, and sera from persistently infected fetuses are pooled with non-contaminated FBS [2, 3]. Therefore, recognizing the *in utero* infection of BVDV is important not only to prevent BVDV infection in the field but also to produce FBS for use in cell cultures.

Approximately 2,700 FBS used in the present study were collected from fetuses in a slaughterhouse in New Zealand (NZ), Australia and the Dominican Republic, and then transferred to Japan to manufacture commercial serum for cell culture use. These sera were subjected to examinations of BVDV isolation and antibody detection, in order to remove inappropriate sera prior to production. The virus isolation was performed using primary bovine fetal muscle (BFM) cells [22], which were suspended in a culture medium of Eagle MEM containing 25% of each serum sample and cultivated at 37°C in a tissue culture plate. After being cultivated for five days, followed by the collection of culture fluids, these cells were infected with BVDV strain Nose (1,000 $TCID_{50}/0.1$ ml) [12]. A sample was judged to be free from BVDV, if CPE induced by Nose strain was found in the BFM cells after four days. If CPE was not detected, RNA was extracted from the collected culture fluids and subjected to RT-PCR for detection of the BVDV genome. Complementary DNA was synthesized using a PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan) as per the manufacturer's instructions. Polymerase chain reaction (PCR) was performed as previously described using primers specific to the highly conserved 5′ untranslated region of pestiviruses [27]. The sequences of primers are as follows: forward primer 324, 5′-ATG CCC WTA GTA GGA CTA GCA-3′ and reverse primer 326, 5′-TCA ACT CCA TGT GCC ATG TAC-3′. BVDV strains Nose, KS86-1-CP and KZ-91-CP were used as positive controls [12, 18, 24]. The FBS in which BVDV-specific fragments were amplified from collected culture fluids were treated as BVDV isolation positive.

Virus neutralization (VN) tests were conducted using 96 well plates as previously described using BVDV strain Nose and strain KS86-1-CP as genotype 1 antigens and strain KZ-91-CP as genotype 2 antigen [12, 18, 22, 24]. Briefly, 100 $TCID_{50}/0.05$ ml of virus in Eagle MEM were added to each serial twofold dilution of serum in 0.05 ml of Eagle MEM containing 20% of BVDV antibody-free FBS. After incubation for 60 min at 37°C, 0.1 ml of BFM cell suspension (1.0 × 10⁵ cells/ml) in Eagle MEM were mixed and incubated at 37°C for 5 days. VN antibody titers were determined as the reciprocals of the highest serum dilutions that neutral-

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Table 1. Survey on vertical infection of BVDV in fetal bovine sera

Year	Country	Sample numbers	Positive fetal bovine serum numbers				
			Antibody				BVDV isolation
			Total	Nose	KS86-1-CP	KZ-91-CP	
2009	New Zealand	139[a)]	0	0	0	0	0
2010	New Zealand	250	2	2	0	0	4
2011	New Zealand	124	5	5	3	0	3
2011	Australia	724[b)]	21	21	15	12	8
2012	New Zealand	121	1	1	1	0	1
2014	Dominican Republic	1400[c)]	15	15	12	4	9
Total		2758	44	44	31	16	25
			(1.60%)	(1.60%)	(1.12%)	(0.53%)	(0.91%)

a) All dams, except one, were BVDV antibody positive. b) Four individual sera were mixed and tested. c) Seven or eight sera were mixed and tested.

ized virus infectivity. Serum samples that had antibody titers 4 or more to one of BVDV strain were treated as BVDV antibody positive, because there are many antigenic differences in BVDV and some of them are poorly neutralized by heterologous sera [18], but most of them cross react even between genotypes 1 and 2 [17, 19].

On one farm in New Zealand where cattle were fed for FBS production, serum samples were collected from all dams and oxen beforehand, and then, sera were collected from their fetuses after 6 months. All oxen (13) were BVDV antibody positive, and only 5 among 172 dams were BVDV antibody negative. At the time when pregnant cows were sent to the slaughterhouse, a total of 139 of cows that had maintained pregnancy were antibody positive, except for one. All FBS collected from their fetuses (139) were both negative for BVDV isolation and the BVDV antibody.

Other FBS collected from fetuses in slaughterhouses in New Zealand, Australia and the Dominican Republic from 2010 to 2014 lacked information about the dams, except for not being vaccinated with BVDV. FBS samples collected in New Zealand (495) were separated according to each individual and subjected to the tests. A total of 724 FBS collected in Australia were pooled for each four individuals, and in cases of BVDV isolation or a positive BVDV antibody, each of the 4 pooled sera was inspected. A total of 1,440 FBS collected in the Dominican Republic were pooled for each 7 or 8 individuals and then tested. Any case of becoming positive in BVDV isolation or for the BVDV antibody was calculated as one positive sample for virus isolation or the antibody, without further investigation being conducted. It can't be excluded that plural positive individuals existed in the pooled sera. However, since both rates of the BVDV isolation and antibody-positive are approximately 1%, the possibility would happen very low. Table 1 lists the numbers of FBS having antibody to each BVDV strain or infectious BVDV in each country and the years when collected.

As a result, 44 (1.60%) FBS were antibody positive, and 25 (0.91%) FBS were positive for BVDV isolation (Table 1). Antibody to Nose strain was detected in all BVDV antibody positive FBS, and the titers were more than 64 in most of
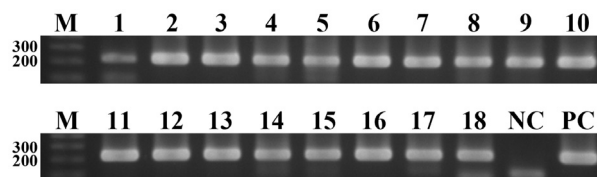

Fig. 1. Nested PCR analysis of bovine viral diarrhea virus (BVDV) isolates. A 221 bp products specific to genotype 1 were amplified from each isolate. Lane M: 100 bp molecular weight marker. Lanes 1 to 6: Isolates in New Zealand. Lanes 7 to 12: Isolates in Australia. Lanes 13 to 18: Isolates from Dominican Republic. Lane NC: Distilled water. Lane PC: BVDV strain Nose (genotype 1).

them. Antibody to KS86-1-CP was detected in 31 FBS, and the titers were lower than those to strain Nose, except for 2 sera. Antibody positive sera to strain KZ-91-CP were only 16, and the titer was less than 32 in most of them, but their antibody titers to strain Nose were more than 128. Therefore, BVDV genotype 2 infection was not prevalent in the areas where FBS were collected in present studies. It does not completely deny that some FBS had antibody to a certain BVDV strain which does not cross react with the antigens used in present studies, but such possibility would be very low.

To clarify the genotype of the prevalent BVDV strains, total 18 isolates (6 in New Zealand, 7 in Australia and 5 in Dominica Republic), which kept in freezer, were subjected to determining their genotypes by PCR using specific primers to genotype 1 and 2, respectively [14]. The sequences of primes are as follows: forward primer B_3, 5′- GGT AGC AAC AGT GGT GAG-3′ and reverse primer B_4, 5′- GTA GCA ATA CAG TGG GCC -3′ for the BVDV type 1, and forward primer B_5, 5′- ACT AGC GGT AGC AGT GAG -3′ and reverse primer B_4, 5′- CTA GCG GAA TAG CAG GTC-3′ for the BVDV type 2. A 221 bp product specific to genotype 1 was amplified from each isolate (Fig. 1), but the products specific to genotype 2 were not amplified. As a result, all 18 isolates were classified as the genotype 1.

The present study showed that the ratio of BVDV posi-

tive FBS (having the BVDV antibody or being contaminated with infectious BVDV) is rather low. However, the antibody titers to BVDV were usually very high (more than 1:256) when cattle were infected with the virus. BVDV is stable and maintains infectivity after heat inactivation at 56°C for 30 min. In the production of FBS for biological research, more than 100 fetus sera are usually pooled and sterilized by filtration. Therefore, it is not easy to obtain FBS free from the BVDV antibody and infectious BVDV.

Embryonic death and abortion were caused by BVDV infection in early pregnancy stage [1, 13], and these phenomena are not given consideration in present studies. Since such infertility usually happens during less than 80 days of gestation, they would happen approximately 1% of the dams as the gestational period of the cow is approximately 280 days and the possibility of BVDV infection is equally possible in anytime. Approximately 3% of dams would be suffered from BVDV infection in the field as they are not vaccinated.

Surveys on persistently BVDV infected cattle have previously been reported using cattle or newborn calves in the field, particularly when an epidemic of the disease breaks out [6, 21–23, 26]. However, as far as we know, the present study is the first report regarding a BVDV survey on *in utero* infection using more than 2,500 fetal calf sera. At present, whether viremia detected in FBS is temporary or persistent has yet to be elucidated, and whether these fetuses are immunotolerant against BVDV also remains unknown. However, persistently BVDV-infected calves born at the rate of 1% in the field would prove to be a sufficient source of BVDV infection. Thus, the vertical infection of BVDV is an important problem that must be controlled. In the present study, all FBS obtained from BVDV antibody-positive dams were BVDV antibody and isolation negative. Therefore, an appropriate vaccination will be possible to eradicate BVDV infection.

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