

Genetic and Pathobiological Characterization of Bovine Viral Diarrhea Viruses Recently Isolated from Cattle in Japan

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ABSTRACT. The 475 strains of bovine viral diarrhea virus (BVDV) isolated from cattle in 12 prefectures of Japan in the last 7 years were phylogenetically classified as BVDV-1 or BVDV-2 on the basis of the nucleotide sequence of the 5'-untranslated region. BVDV-1 strains were further subtyped as 1a (101 strains), 1b (163), 1c (128), 1j (3), and So CP/75-like (1), and all of the 79 BVDV-2 strains belonged to subtype 2a. These 2a BVDVs contain two isolates that had high nucleotide identities with those of highly pathogenic BVDV-2 strains reported in North America (Pellerin *et al.*, 1994). However, acute infection with severe mortality like North American outbreak was not observed and most of the present BVDV-2 strains were isolated from persistently infected (PI) cattle showing mild or no clinical sign. Moreover, it was revealed that 61.5% of the 39 PI cattle with cytopathogenic BVDVs did not show typical mucosal disease and 54.6% of the 405 PI animals only with non-cytopathogenic BVDVs were apparently healthy. The present results indicate that the prevention of the infection with an appropriate vaccine and active surveillance covering healthy cattle are required for the control of BVD.

KEY WORDS: bovine viral diarrhea virus, molecular epidemiology, pathogenicity, phylogenetic analysis.

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Bovine viral diarrhea virus (BVDV) belongs to the genus *Pestivirus* with classical swine fever virus and border disease virus in the family *Flaviviridae*. The genome of BVDV consists of a single-stranded, positive-sense RNA approximately 12.5 kb in length. On the basis of the nucleotide sequence of the 5'-untranslated region (5'-UTR) or the E2 gene, BVDV strains are phylogenetically categorized into genotype 1 (BVDV-1) and genotype 2 (BVDV-2) [8, 35]. BVDV-1 strains can be further divided into at least 11 genetic subgroups [22, 42], and are distributed worldwide including in Japan [23, 30, 36]. BVDV-2 strains were first reported in 1994 and are now known to be highly pathogenic, causing hemorrhagic disease [9, 27, 29]. BVDV-2 strains can be further divided into at least 2 genetic subgroups [2, 14, 34].

Bovine viral diarrhea (BVD), a disease with fever, diarrhea, or erosion of the digestive organs, persists in many countries of the world inflicting heavy economical damage to the livestock business [1]. When healthy cattle are infected with BVDV, temporary fevers, respiratory symptoms, or diarrhea are observed, and most of the cattle are cured and build a lifelong immunity. However, if pregnant cattle become infected with a non-cytopathogenic (ncp) BVDV, the virus can easily cross the placenta and infect the fetus *in utero*. Symptoms of fetuses vary depending on age; in the case of an infection with ncp virus during 80 to 120 fetal days, immunologic tolerance for BVDV develops. Such fetuses are born as persistently infected (PI) calves.

calves appear healthy but produce and discharge BVDV, infecting the herd [36]. Cattle persistently infected with BVDV may succumb to fatal mucosal disease. In such cases, both ncp and cytopathogenic (cp) BVDV have been isolated [5].

In Japan, outbreaks of BVD have been reported since the 1960s [24], and antigenic and genetic analyses of BVDV have been carried out [16, 21, 23, 30, 33]. In the present study, genetic and pathobiological analyses of BVDVs isolated recently in Japan were performed to characterize the latest outbreaks for the control of BVD.

MATERIALS AND METHODS

Viruses and Cell Cultures: The 397 BVDV isolates were dispensed from the livestock hygiene centers of Hokkaido Prefecture. A total of 78 isolates were provided respectively from the prefectural livestock hygiene centers of Akita (8 isolates), Yamagata (3), Chiba (2), Tokyo (10), Shizuoka (11), Aichi (28), Shiga (6), Kumamoto (2), Kagoshima (2), and Okinawa (2), and the Gunma Prefecture livestock health laboratory (4). These viruses were isolated from the serum, buffy coat, pleural effusion, fecal specimen, or emulsion of an organ (lung, liver, kidney, spleen, heart, or brain) of cattle or aborted fetuses suspected of having a BVDV infection. Forty of the 475 strains were cp BVDV and the others were ncp BVDV. All the viruses were grown in Madin-Darby bovine kidney (MDBK) cells in minimum essential medium (MEM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% horse serum (Invitrogen, Carlsbad, CA, U.S.A.). The detection of the viral antigen was performed by immunostaining [26].

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Sequencing of 5'-UTR and the phylogenetic analysis: Viral RNA was extracted from the supernatant of inoculated MDBK cells using TRI Reagent LS (Sigma-Aldrich, St. Louis, MO, U.S.A.) according to the manufacturer's protocol. RT-PCR amplification of the 5'-UTR was carried out using Ready-To-Go RT-PCR Beads (GE Healthcare, Piscataway, NJ, U.S.A.) with the primers 324 and 326 [41]. The RT-PCR products were purified with a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany). The purified products were used as templates in sequencing reactions (Dye Terminator Cycle Sequencing Chemistry Protocol; Beckman Coulter, Fullerton, CA, U.S.A.) primed with RT-PCR primers and analyzed on a Multi Capillary DNA Analysis System CEQ2000 (Beckman). The sequences were first aligned by multiple sequence alignment using GENETYX-MAC Version 13 (GENETYX, Tokyo, Japan). A phylogenetic analysis was then carried out using neighbor-joining trees with 1,000 replicates in a bootstrap analysis on ClustalW [37]. TreeView version 1.6 [25] was used for displaying trees. From the present results, individual isolates were classified into genetic subtypes based on the methods of Vilcek *et al.* [39, 40]. The nucle-

otide sequences of the following 19 strains were used as references for each subtype; Nose (AB019670), and NADL (M31182) as 1a; Osloss (M96687) as 1b; Bega (AF049221) as 1c; F (AF298065) as 1d; 20-V661-2 (AF298058) as 1e; W (AF298073) as 1f; L (AF298069) as 1g; G (AF298066) as 1h; 23/15 (AF298059) as 1i; M065B/93 (U97409) as 1j; Rebe (AF299317) as 1k; So CP/75 (AB042661) as So CP/75-like; KZ-91 CP (AB003619), 890 (U18059), 5521-95 (AF039174), and 502643-02 (AY161304) as 2a; 34B (AF244952) and Soldan (U94914) as 2b [6, 8, 12, 17, 19, 21, 23, 28, 30, 38, 40, 42, 43].

RESULTS

Genotyping of the isolates: A phylogenetic analysis of the 5'-UTR sequences revealed that the BVDV strains isolated from cattle in Japan were BVDV-1 subtypes of 1a, 1b, 1c, 1j, and So CP/75-like, and BVDV-2 subtype 2a (Table 1). Distribution rates of subtype 1a (101 isolates), 1b (163), 1c (128), 1j (3), So CP/75-like (1), and 2a (79) were 21.3%, 34.3%, 26.9%, 0.6%, 0.2%, and 16.6%, respectively. This distribution was also observed in each prefecture. Mean-

Table 1. Identification of genotypes of BVDVs isolated recently in Japan

Year of isolation	Prefecture	No. of isolates	Genotype					
			BVDV-1					BVDV-2
			1a	1b	1c	1j	So CP/75-like	2a
2000	Hokkaido	3	2	1	0	0	0	0
2001	Hokkaido	31	5	14	4	2	0	6
2002	Hokkaido	74	12	26	19	0	0	17
	Gunma	1	0	0	0	0	0	1
	Tokyo	3	2	1	0	0	0	0
	Shizuoka	3	0	3	0	0	0	0
2003	Hokkaido	91	11	34	42	0	0	4
	Gunma	1	0	0	0	0	0	1
	Tokyo	1	1	0	0	0	0	0
	Aichi	3	1	0	2	0	0	0
	Kumamoto	2	0	0	0	0	0	2
2004	Hokkaido	93	24	29	32	0	0	8
	Yamagata	1	1	0	0	0	0	0
	Tokyo	5	1	2	2	0	0	0
	Aichi	7	1	2	4	0	0	0
	Shiga	2	2	0	0	0	0	0
	Kagoshima	2	0	2	0	0	0	0
2005	Hokkaido	70	11	22	15	0	0	22
	Yamagata	2	0	0	2	0	0	0
	Akita	8	7	1	0	0	0	0
	Chiba	2	2	0	0	0	0	0
	Tokyo	1	1	0	0	0	0	0
	Shizuoka	3	2	1	0	0	0	0
	Aichi	11	2	2	1	0	0	6
	Shiga	4	1	1	0	1	0	1
	Okinawa	1	0	0	1	0	0	0
2006	Hokkaido	35	3	20	4	0	0	8
	Gunma	2	1	0	0	0	0	1
	Shizuoka	5	3	2	0	0	0	0
	Aichi	7	4	0	0	0	1	2
	Okinawa	1	1	0	0	0	0	0
Total		475	101	163	128	3	1	79

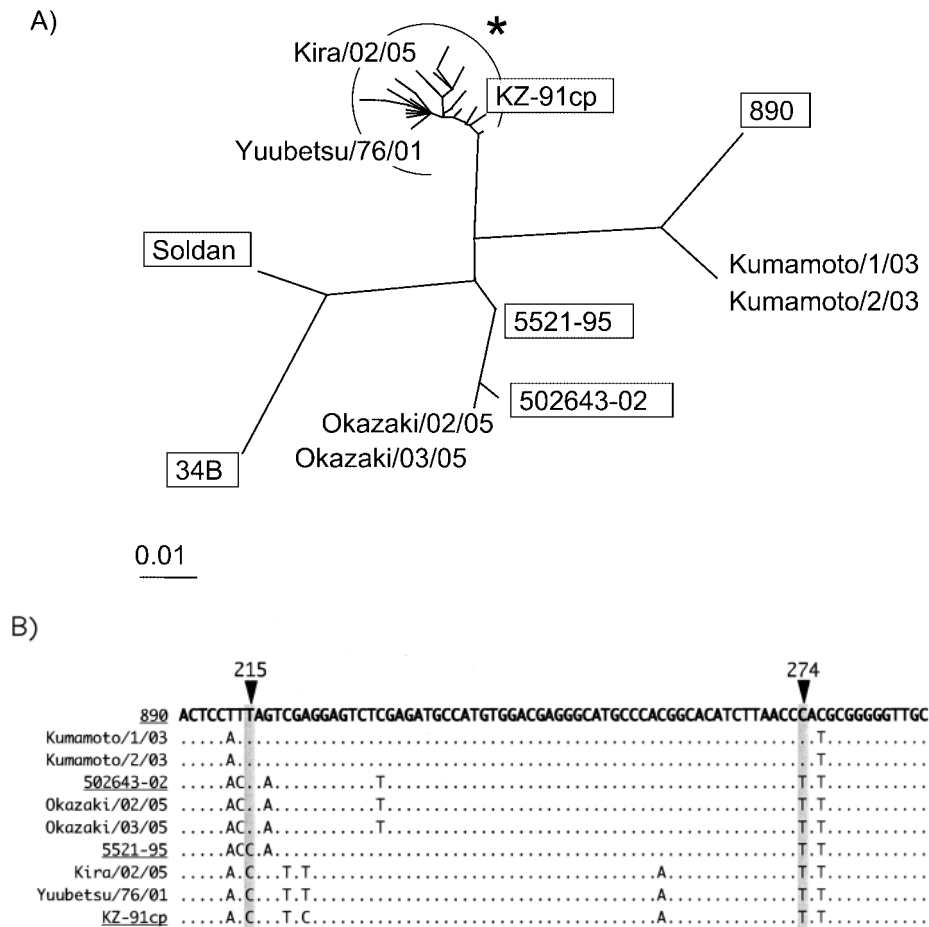


Fig. 1. Comparative analysis of Japanese BVDV-2a isolates and BVDV-2 reference strains. A) Phylogenetic analysis of BVDV-2 isolates (79 isolates) based on the 5'-UTR sequence. Six reference strains are framed. The 75 isolates represented by Kira/02/05 and Yuubetsu/76/01 and reference strain KZ-91cp were grouped in a circle (asterisk). The other 4 strains belonged to two different lineages. B) Partial 5'-UTR sequences of the BVDV-2a isolates. Two nucleotide points (highlighted) are the virulence markers of highly pathogenic BVDV-2 strains reported by Topliff and Kelling [38], and nucleotide numbers on the virulence markers originate from the sequence of strain 890. Dots indicate identical nucleotides to those of the strain 890 gene. Reference strains are underlined.

Table 2. Classification of clinical signs of cattle affected with BVDVs

Cytopathogenicity of isolated virus (number of cattle)	Acute infection			Persistent infection									No
	Hemorrhagic syndrome	Diarrhea	Fever	Mucosal disease	Diarrhea	Poor development	Death	Pneumonia	Neurologic symptom	Abortion or aborted fetus	Asthenia	No sign	Infomation
BVDV-1													
Cp ^{a)} and ncp ^{b)} (10)	0	0	0	4	4	0	0	0	0	0	0	1	1
Cp (24)	0	0	0	10	11	0	1	0	0	1	1	0	0
Ncp (363)	1	2	1	23	55	36	2	9	8	17	4	188	17
BVDV-2													
Cp and ncp (2)	0	0	0	0	0	0	0	0	0	0	1	1	0
Cp (4)	0	0	0	1	3	0	0	0	0	0	0	0	0
Ncp (72)	0	0	1	3	11	9	1	0	0	6	0	33	8

a) Cytopathogenic.

b) Non-cytopathogenic

while, BVDV-1j strains were isolated sporadically in 2001 in Hokkaido Prefecture, in 2005 in Shiga Prefecture, and So CP/75-like strain was isolated in 2006 in Aichi Prefecture.

Comparative analysis of the BVDV-2a isolates with BVDV-2 reference strains: For the genetic characterization of the 79 subtype 2a strains in detail, a phylogenetic analysis of these isolates and reference BVDV-2 strains was performed (Fig. 1A). Seventy-five isolates were grouped into the same cluster as reference strain KZ-91cp which was a BVDV-2a strain isolated in Japan in 1991 [21]. Okazaki/02/05 and Okazaki/03/05 were closely related to reference strains with low virulence; strain 5521-95 [38], and strain 502643-02 [43]. Kumamoto/1/03 and Kumamoto/2/03 were grouped into the same cluster as strain 890, a highly pathogenic BVDV-2 isolated in the U.S.A. [28]. As virulence markers of BVDV-2 viruses, the 215th nucleotide, thymine, and the 274th nucleotide, cytosine, in the 5'-UTR of strain 890 were reported [38]. A sequence alignment of this region among 4 reference strains and the present 6 isolates is shown in Fig. 1B. Both nucleotides of Kumamoto/1/03 and Kumamoto/2/03 were identical to those of the highly pathogenic strain 890. The 215th nucleotides of Okazaki/02/05 and Okazaki/03/05 were thymine. On the other hand, both nucleotides of the other 75 isolates were the same as those of the reference strain KZ-91cp. Cattle from which BVDV-2 strains were isolated did not show hemorrhagic syndrome, but exhibited clinical signs similar to those of animals infected with BVDV-1.

Clinical signs of cattle infected with cp and ncp BVDVs: To investigate the relationship between the cytopathogenicity of the isolates and clinical signs of infected cattle, the present isolates were classified according to cytopathogenicity and recorded clinical signs (Table 2). Five strains were isolated from cattle in the acute phase. The others were from PI cattle. As mentioned above, acutely infected cattle from which 2a strain Betsukai/353/05 was isolated just showed a fever. One third of the 40 animals shed both cp and ncp viruses or cp virus only, exhibited signs typical of mucosal disease. The others showed no symptom or various symptoms such as diarrhea, death, abortion, and asthenia. In the case of the cattle persistently infected only with ncp virus, 45.4% (N=184) showed certain clinical signs, while 54.6% (N=221) did not show any clinical signs.

DISCUSSION

Studies on the molecular epidemiology of BVD by Nagai *et al.* [21, 23], Sakoda *et al.* [30], and Hayashi *et al.* [16] indicated that outbreaks had occurred due to infections with a variety of BVDV strains in Japan. By genetic subtyping based on the methods of Vilcek *et al.* [39, 40], subtype 1a' (eg. IS 7 NCP/97 [23]) and 1c (eg. KS86-1 ncp [23]) viruses in the above reports were classified to subtype 1c and 1j respectively [16, 21, 22, 30]. In the present study, 475 BVDV strains isolated from cattle in 12 prefectures in 2000 to 2006 were analyzed genetically and pathobiologically. It is confirmed that BVDVs of subtypes 1a, 1b, 1c and 2a are

dominant in Japan. The present results indicate the same tendency with the above epidemiological studies [16, 21, 22, 30]. It has been shown that a vaccine containing only BVDV-1 virus particles are sufficient to block the infection of BVDV-2 virus [32], but at the same time a BVDV-1 vaccine are not effective to prevent the placental infection [4]. These results indicate that the use of vaccine containing both BVDV-1 and BVDV-2 strains is recommended for the effective control of BVD in Japan. In addition, BVD outbreaks due to two minority subtypes BVDV-1j and So CP/75-like were detected. The latest report of the isolations of subtype 1j virus in Japan was in 1986 and 1987 as subtype 1c viruses [23]. Since then, no 1j virus has been isolated. The present 1j viruses were isolated from cattle in 2001 in Hokkaido Prefecture and 2005 in Shiga Prefecture. So CP/75 was isolated in 1975 in Kagoshima Prefecture and the 5'-UTR sequence of this virus was unique [23]. The present Shitara/02/06 isolated in Aichi Prefecture in 2006 was the first Japanese isolate that has a sequence homology in 5'-UTR with So CP/75. The present results indicate that BVDVs of the minority subtypes still circulate in Japan. The prevalence of BVDVs, thus, should be monitored continuously.

BVDV-2 strains were isolated from cattle showing signs of hemorrhagic syndrome such as hemoid diarrhea, nasal hemorrhage, mucosal petechia, and thrombopenia, in the late 1980s to early 1990s in North America, and have been designated highly pathogenic [7, 9, 10, 27, 29]. In Japan, BVDV-2a strains have been isolated only from PI cattle which showed similar clinical signs to those animals from which BVDV-1 strains were isolated [21, 23, 30]. In the present study, 79 of the 475 isolates were identified as BVDV-2a strains. The nucleotide sequences of the present isolates in Kumamoto were almost identical to those of the highly pathogenic BVDV-2 strain 890, with identical virulence markers (Fig. 1B). The present ncp BVDV-2 strains isolated from the cattle which did not show any symptoms of hemorrhagic syndrome. Although the present results indicate that the pathogenicity of BVDV-2 strains in Japan is not high, a few isolates had high nucleotide identities with the highly pathogenic strain in the 5'-UTR where the viral RNA genome constitutes the internal ribosomal entry site that initiates translation [20]. The virulence markers of BVDVs and clinical symptoms of the cattle should be monitored.

As shown in Table 2, 61.5% of the 39 cattle from which both cp and ncp viruses or only cp virus were isolated did not exhibit typical symptoms of mucosal disease, but did show ordinary signs of BVD such as diarrhea, death, abortion, and asthenia. Only two cattle from which both cp and ncp viruses were isolated did not show any clinical signs. These cattle might develop typical mucosal disease near future, since it was reported that cp virus was isolated from apparently healthy cattle a year before they developed mucosal disease [15]. Among the cattle persistently infected only with ncp virus, 54.6% (N=221) did not show any clinical signs, whereas the others showed ordinary clin-

ical signs. It has been thought that BVDV-1 viruses cause mild disease, but it was also reported that cattle from which BVDV-1 were isolated showed hemorrhagic syndrome [3]. In the present study, Nemuro-Shibetsu/462/06 (subtype 1b) was isolated from the cattle developed severe hemorrhagic syndrome and died. The relationships between subtypes and pathogenicity, or individual status of cattle (breed, age, or condition) and symptoms [13, 31] were not found in the present study (data not shown). To clarify the molecular basis of the pathogenicity of BVDV, consecutive monitoring of infections and the determination of virulence factors are required. And if one animal is diagnosed as persistently infected with BVDV, the rest of the herd must be checked for BVDV infection. Rapid antigen detection methods [11, 18] should be applied to the rapid diagnosis and stamping out of infected cattle for the control of BVD.

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