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Prevalence of HoBi-like viruses in Japan between 2012 and 2017 based on virological methods and serology

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Abstract

The purpose of this study was to investigate the prevalence of HoBi-like viruses in Japan and evaluate the immune response induced by bovine viral diarrhea virus (BVDV) vaccines available in Japan. No HoBi-like viruses were detected by RT-PCR and virus isolation in cattle sera collected in Japan between 2012 and 2017. Nevertheless, neutralizing antibody titers against HoBi-like viruses ranged from < 2 to 4,096, demonstrating cross-reactivity to HoBi-like viruses in cattle infected (naturally or vaccinated) with BVDV-1 and BVDV-2. These results suggest that continuous vaccination with both BVDV-1 and BVDV-2 contributes to the control of HoBi-like viruses in Japan.

Key Words: cross-reactivity, HoBi-like virus, vaccination

The genus *Pestivirus* of the family *Flaviviridae* comprises four recognized species: bovine viral diarrhea virus (BVDV)-1, BVDV-2, classical swine fever virus and border disease virus[19)]. In addition to the recognized species, putative and unclassified species, including Giraffe virus, Pronghorn virus, Bungowannah virus and HoBi-like virus (also referred to as BVDV-3 or atypical bovine pestivirus)[5,8)], have been identified recently. HoBi-like viruses have been isolated from fetal bovine sera (FBS) of South American origin or found as contaminants

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in laboratory-cultured cells worldwide[5]. Genetic similarities of HoBi-like viruses isolated in Italy and South America suggest that HoBi-like viruses have been introduced into European countries via contaminated vaccines or FBS[5,10]. These facts pose a serious concern about the safety of biological products (e.g., vaccines) manufactured using raw materials of animal origin, particularly FBS.

HoBi-like viruses were also isolated from cattle and/or water buffaloes in South America, Southeast Asia and Europe[5,8]. When cattle were infected with HoBi-like viruses either naturally or experimentally, the animals exhibited manifestations same as those of BVDV infection, ranging from subclinical to overt diseases, including growth retardation, gastroenteric disease, respiratory disease, reproductive disorders, increased mortality among young stock and persistent infection[5,7,8]. Mucosal disease-like syndrome in persistently infected (PI) cattle was also reported to have been caused by HoBi-like viruses in Italy and Brazil[5,9], and a pair of cytopathogenic (cp) and noncytopathogenic (ncp) viruses was isolated in Italian case[9]. Therefore, cases of HoBi-like virus infection may be misdiagnosed with other infections, including BVDV infection, based on only these manifestations.

Infections of pestiviruses are diagnosed by the detection of viruses, viral genes and/or specific antibodies. The genome of pestiviruses comprises positive-sense single-stranded RNA of approximately 12.3 kb in length. The single open reading frame, flanked by the 5´ and 3´ untranslated regions (UTRs), encodes nonstructural and structural polyproteins (N^{pro}, C, E^{rns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B)[20]. The 5´UTR, which is well-conserved among pestiviruses, is the major target for the genetic detection and classification of pestiviruses[21], but panpestivirus RT-PCR developed by Vilcek *et al.*[21] may fail to detect HoBi-like viruses due to the presence of the nucleotide mismatches between viral and primer sequences[2,18]. This fact posed a concern with respect to the diagnostic methods for the detection of pestiviruses, including HoBi-like viruses. Reactivity of monoclonal antibodies against BVDV-1 and BVDV-2 with HoBi-like viruses indicated that antigen/antibody detection methods for extant BVDVs, including virus isolation, antigen-capture ELISA and virus neutralization tests, could be useful for the detection of HoBi-like virus infections[7].

Antigenically and genetically, HoBi-like viruses are related to BVDVs; however, rabbit hyperimmune sera against BVDV-1 or BVDV-2 showed limited cross-reactivity with HoBi-like viruses[18]. Evaluating the cross-antibody responses between BVDVs and HoBi-like viruses in ruminants, the sera of animals immunized with BVDV-1 and BVDV-2 demonstrated low neutralizing antibody titers against HoBi-like viruses[3,11]. Limited cross-neutralization responses of extant BVDVs against HoBi-like viruses raised the question on the efficacy of available BVDV vaccines. Meanwhile, modified live vaccine (MLV) or killed vaccine (KV) containing BVDV-1 and BVDV-2 induced neutralizing antibodies against HoBi-like viruses in 34%–68% of vaccinated cattle, and the 2–4-fold differences in geometric mean titers (GMTs) of neutralizing antibodies were observed between BVDV-1/BVDV-2 and HoBi-like viruses[4]. A seroprevalence study using 2,000 cattle sera collected in the US showed a positive rate of 84.9% for neutralizing antibodies against HoBi-like viruses (with 13.8% demonstrating titers of 1,024 or above), with no evidence of HoBi-like viruses in the country[6]. These results indicated the cross-reactivity of BVDV-1 and BVDV-2 against HoBi-like viruses in cattle. BVDV vaccines are used worldwide to control BVDV infection, except in the regions where BVDV has been eradicated. To control HoBi-like viruses, the efficacy of current BVDV vaccines should be evaluated for all products.

In Japan, there is no report on the isolation of HoBi-like viruses to date and the prevalence of HoBi-like viruses remains unclear. Although MLV containing both BVDV-1 and BVDV-2 or BVDV-1 alone and KV containing both BVDV-1

Table 1. Neutralizing antibody titers of antisera against BVDV-1, BVDV-2 and HoBi-like viruses

Antisera against[a]	Virus strain		
	BVDV-1 (Nose strain)	BVDV-2 (KZ-91-NCP strain)	HoBi-like virus (D32/00_'HoBi' strain)
BVDV-1 (Nose strain)	1,024	16	32
BVDV-2 (KZ-91-CP strain)	32	256	8
HoBi-like virus (D32/00_'HoBi' strain)	4	8	256

[a] Titers against homologous strains are shown in grey

and BVDV-2 are approved by the Minister of Agriculture, Forestry and Fisheries, the cross-reactivity of these vaccines against HoBi-like viruses has not yet been evaluated. In the present study, we investigated HoBi-like virus infections in cattle in Japan by the detection of HoBi-like viruses and neutralizing antibodies against HoBi-like viruses from serum samples. We also evaluated the cross-reactivity to HoBi-like viruses in cattle immunized with currently available BVDV vaccines.

Ruminant antisera against BVDV-1, BVDV-2 and HoBi-like viruses used in the present study were obtained by immunization of each viral strains (Table 1) to cows or sheep. Details of immunization protocol can be obtained on request. In addition, a total of 495 cattle serum samples were collected between 2012 and 2017 from four prefectures in Japan as follows: 200 samples from Hokkaido between 2012 and 2014, 64 samples from Hokkaido in 2016, 137 samples from Yamanashi in 2016–2017, 22 samples from Toyama in 2017 and 72 samples from Nagasaki in 2015. Sera collected between 2015 and 2017 included those from cattle immunized with one dose of MLV containing BVDV-1 alone, one dose of MLV containing BVDV-1 and BVDV-2, one dose each of MLV and KV containing BVDV-1 and BVDV-2 or three doses or more of KV containing BVDV-1 and BVDV-2, or from unimmunized cattle against BVDVs. All the vaccines contained other viral antigens, including bovine herpesvirus 1, bovine parainfluenza virus 3 and bovine respiratory syncytial virus. These sera were collected within 1 year since the last BVDV vaccination. All sera were stored at −20°C and heat inactivated at 56°C for 30 min before use in the neutralization assay. Bovine fetal muscle (BFM) and bovine testicle (BT) cells used in the present study were confirmed to be free from BVDVs, and FBS (Japan Bioserum, Hiroshima, Japan) supplemented in cell culture media was confirmed to be free from BVDVs and anti-BVDV neutralizing antibodies[16] as well as HoBi-like viruses by RT-PCR[2].

Neutralizing antibody titers of ruminant antisera were measured against cp BVDV-1 Nose[15], ncp BVDV-2 KZ-91-NCP[17] and ncp HoBi-like D32/00_'HoBi' strains[18] in BFM cells as previously reported[1]. Neutralizing antibody titers of anti-BVDV-1, -BVDV-2 and -HoBi-like virus sera against homologous species were 1,024, 256 and 256, respectively (Table 1). The 32- to 64-fold differences in neutralizing antibody titers were observed between BVDV-1/BVDV-2 and HoBi-like viruses (Table 1).

Total RNA was extracted from pooled samples (5 sera/sample) derived from 183 sera collected in 2012–2014 and from 295 individual sera collected in 2015–2017 using QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) with a robotic workstation QIAcube (QIAGEN) in accordance with the manufacturer's instructions. Panpestivirus and HoBi-like specific RT-PCR

Table 2. Neutralizing antibody titers of cattle sera collected in Hokkaido between 2012 and 2014

District	Number of samples	GMT[a] (range) of neutralizing antibodies		
		anti-BVDV-1 (Nose strain)	anti-BVDV-2 (KZ-91-NCP strain)	anti-HoBi-like virus (D32/00_'HoBi' strain)
1	26	498.5 (<2–4,096)*	81.4 (<2–4,096)	176.3 (<2–4,096)**
2	23	124.2 (<2–2,048)	17.5 (<2–4,096)	32.0 (<2–512)
Total	49	259.6 (<2–4,096)	39.6 (<2–4,096)	79.1 (<2–4,096)

[a] Geometric Mean Titer, *$P < 0.05$, **$P < 0.01$ (both vs. District 2)

was performed using 324–326[21] and N2–R5[2] primers, respectively, with PrimeScript One Step RT-PCR Kit Ver. 2 (TaKaRa BIO, Shiga, Japan) following the manufacturer's protocol. Virus isolation using cattle sera collected in 2015–2017 was performed using an immunoperoxidase monolayer assay (IPMA). In brief, 50 µl of each sample was inoculated into 24-well microtiter plates with BT cell suspension, and the cells were incubated at 37°C in 5% CO_2 for 5 days. Following the observation of CPE, ncp pestiviruses were detected using an immunoperoxidase staining technique with a pestivirus NS3 specific monoclonal antibody (46/1)[14]. Neither genes nor infectious viruses were detected in any of the samples (Supplementary Table 1).

Neutralizing antibody titers of 49 cattle sera collected in two districts of Hokkaido between 2012 and 2014 (vaccination history unknown) were measured as shown above. Statistical analysis was performed using the Mann–Whitney's U-test using Microsoft Excel (Microsoft Corp, Redmond, WA, USA) with Statcel4 add-ins (OMS, Tokyo, Japan). $P < 0.05$ was considered significant. Neutralizing antibody titers against HoBi-like viruses ranged from < 2 to 4,096. These titers were equal or less than those against BVDV-1 and/or BVDV-2. GMTs of neutralizing antibodies against BVDV-1, BVDV-2 and HoBi-like viruses were 259.6, 39.6 and 79.1, respectively (Table 2). Neutralizing antibody titers against BVDV-1 and HoBi-like viruses were significantly higher in District 1 than District 2 ($P < 0.05$ and $P < 0.01$, respectively). The results of individual cattle are provided in Supplementary Table 2.

Neutralizing antibodies in cattle sera with/without BVDV vaccination history were evaluated against cp BVDV-1 Nose[15], cp BVDV-2 KZ-91-CP[17] and ncp HoBi-like D32/00_'HoBi' strains[18] in BT cells as previously reported[16] based on the method of potency testing in quality control tests of BVDV vaccines in Japan with modifications. In brief, a 50 µl volume of 2-fold diluted serum sample and an equal volume of viruses containing 200 $TCID_{50}$/0.1 ml were added to 96-well microtiter plates. After incubation at 37°C for 1 hr, BT cells were added and incubated at 37°C in 5% CO_2 for 5 days. Viruses were detected by observing CPE or IPMA for cp and ncp viruses, respectively. In wells where virus growth was inhibited, these were designated as antibody positive, and titers were recorded as the reciprocal of the highest serum dilution that caused 50% neutralization. Statistical analysis was performed using the Kruskal–Wallis nonparametric test followed by the Steel–Dwass nonparametric multiple comparison test using Microsoft Excel with Statcel4 add-ins. $P < 0.05$ was considered significant. Neutralizing antibody titers against HoBi-like viruses in cattle sera without BVDV vaccination history were < 2 or 2 (Table 3). In cattle immunized with one dose of MLV containing BVDV-1 alone, neutralizing antibody titers against BVDV-1 (GMT = 329.4)

Table 3. Neutralizing antibody titers of cattle sera with/without vaccination history

Vaccination history	Number of samples	GMT[a] (range) of neutralizing antibodies		
		anti-BVDV-1 (Nose strain)	anti-BVDV-2 (KZ-91-CP strain)	anti-HoBi-like virus (D32/00_'HoBi' strain)
BVDV-1 MLV[b] (one dose)	22	329.4 (32–2,048)	6.6 (<2–64)	5.8 (<2–32)
BVDV-1, 2 MLV (one dose)	20	174.9 (2–2,048)	374.8 (64–2,048)	34.3 (<2–512)
BVDV-1, 2 MLV (one dose) + BVDV-1, 2 KV[c] (one dose)	20	115.4 (2–2,048)	326.3 (64–1,024)	42.2 (4–128)
BVDV-1, 2 KV (three doses or more)	24[d]	209.1 (4–2,048)	128.0 (8–1,024)	30.2 (2–256)
none	46	1.0 (<2)	1.0 (<2)	1.0 (<2–2)

[a] Geometric Mean Titer, [b] Modified live vaccine, [c] Killed vaccine
[d] Includes 4 samples from cattle boostered with one dose of BVDV-1 MLV

were higher than those against BVDV-2 (GMT = 6.6) and HoBi-like viruses (GMT = 5.8). GMTs of neutralizing antibody titers against BVDV-1 and BVDV-2 in cattle immunized with MLV, KV or a combination of MLV and KV, all of which contained both BVDV-1 and BVDV-2, were high and ranged from 115.4 to 374.8. Although GMTs of neutralizing antibodies (30.2–42.2) in these cattle against HoBi-like viruses were lower than those against BVDV-1 and BVDV-2, these titers were 5 to 7 times higher than those induced by one dose of MLV containing BVDV-1 alone ($P < 0.01$). The results of individual cattle are provided in Supplementary Table 3.

A previous study reported that rabbit hyperimmune sera against BVDV-1 or BVDV-2 demonstrated limited cross-neutralization responses against HoBi-like viruses[18]. In the present study, we examined the cross-reactivity of ruminant antisera immunized with BVDV-1 or BVDV-2 isolated in Japan or a prototype of HoBi-like viruses. As predicted, antisera demonstrated low cross-reactivity against heterologous species, resulting in differences in neutralizing antibody titers of not less than 32 times between BVDV-1/BVDV-2 and HoBi-like virus (Table 1). These results support a previous report that despite antigenic similarities, HoBi-like viruses are antigenically distinct from both BVDV species[3].

Cross-neutralization assays in cattle sera collected in Hokkaido between 2012 and 2014 indicated cross-reactivity of BVDV-1 and BVDV-2 against HoBi-like viruses (Table 2). A difference in GMTs of neutralizing antibodies against three species between two districts of Hokkaido may be caused by differences in prevalence of BVDVs and BVDV vaccination history in the region.

To investigate the cause of high antibody titers against HoBi-like viruses in cattle, serum samples from cattle with/without BVDV vaccination history were evaluated by cross-neutralization assays, suggesting that cattle immunized with MLV and/or KV containing both BVDV-1 and BVDV-2 demonstrated higher titers of neutralizing antibodies against HoBi-like viruses than those immunized with MLV containing BVDV-1 alone, although titers were 3–10 times lower than those of homologous species (Table 3). GMTs of cattle sera collected in 2012–2014 against HoBi-like viruses (Table 2) were equivalent to or higher than those of sera vaccinated with BVDV-1 and BVDV-2 (Table 3). These high titers were considered to be induced by vaccination and/or natural infection with both BVDV-1 and BVDV-2. In the US, the difference in GMTs of neutralizing antibodies against HoBi-like viruses in cattle

immunized with MLV/KV containing both BVDV species was within 2–4 times compared with those against BVDV-1 and BVDV-2[4]. Significantly higher neutralizing antibody titers were demonstrated against HoBi-like viruses in cattle vaccinated with MLV compared with KV, suggesting that MLV is more effective in preventing HoBi-like virus infections[4]. Neutralizing antibody titers of the MLV group in the present study were higher than those of the KV group, but the differences were not statistically significant. In the US study, positive rates of neutralizing antibodies against HoBi-like viruses in vaccinated cattle were 34%–68% when titers of 10 or above were designated as antibody positive[4]. Our study shows that 80%–90% of cattle vaccinated with BVDV-1 and BVDV-2 demonstrated neutralizing antibody titers of 16 or above to HoBi-like viruses (Supplementary Table 3); therefore, BVDV strains used in Japanese vaccines are indicated to induce neutralizing antibodies against HoBi-like viruses more efficiently. The control of BVDVs in Japan is based on the slaughter of PI animals and vaccination, suggesting that these strategies are effective in controlling diseases caused by HoBi-like viruses if vaccines containing BVDV-1 and BVDV-2 are used. However, additional studies are required to evaluate whether these vaccines can prevent transplacental infection in pregnant cows.

Although the areas for sample collection and the number of samples were limited in the present study, the results of virus detection showed no evidence of HoBi-like viruses in Japan. Same as BVDVs, HoBi-like viruses are one of the risk factors for the contamination of biological products (e.g., antisera and vaccines) via raw materials of animal origin[3,16]. A previous study indicated that a possible introduction of HoBi-like viruses into European countries was caused via biological products[5,10]. In Japan, FBS used for the production of live veterinary vaccines and final containers of bovine live vaccines have been tested under relevant regulations for BVDVs using the *in vitro* assays since the 1980s; these assays include the detection of extraneous ncp BVDVs based on the interference of over-infected cp BVDV in cells infected with ncp BVDVs[13]. HoBi-like viruses can be detected by this interference assay (data not shown); thus, the current regulation on veterinary vaccines seems to prevent the introduction of HoBi-like viruses into Japan. Continuous monitoring of HoBi-like viruses in the field and biological products is needed, although there is no evidence of the presence of HoBi-like viruses in Japan.

Cattle in Japan induce neutralizing antibodies against HoBi-like viruses with no evidence of HoBi-like virus prevalence in the field. Higher antibody titers are demonstrated in cattle vaccinated with BVDV-1 and BVDV-2. These results indicate that relatively high titers of neutralizing antibodies against HoBi-like viruses are induced when cattle are infected (naturally or vaccinated) with both BVDV-1 and BVDV-2 without infection with HoBi-like viruses. Our results also indicate that diagnostic laboratories should consider possible cross-reaction against HoBi-like viruses by extant BVDVs in the field. Recent studies revealed the antigenic diversity of Brazilian isolates of HoBi-like viruses[12]; therefore, further studies are needed to investigate the cross-reactivity of vaccines against antigenically distinct HoBi-like viruses.

In conclusion, our results present no evidence of HoBi-like viruses in Japan, and cross-reactivity against HoBi-like viruses was seen in cattle immunized within 1 year with Japanese vaccines containing both BVDV-1 and BVDV-2. Continuous vaccination with both BVDV-1 and BVDV-2 may contribute to the control of HoBi-like viruses in Japan, though it is important to prevent the introduction of HoBi-like viruses into Japan.

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Supplemental data

Supplemental data associated with this article can be found, in the online version, at http://dx.doi.org/10.14943/jjvr.66.4.317

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