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NOTE

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Slaughterhouse survey for detection of bovine viral diarrhea infection among beef cattle in Kyushu, Japan

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ABSTRACT. Bovine viral diarrhea virus (BVDV) footprint has spread across the globe and is responsible for one of the most economically important diseases in cattle. In Japan, some regional surveillance and preventive measures to control bovine viral diarrhea (BVD) have been implemented. However, BVDV infection is poorly understood in cattle industries, and there is no systematic BVD surveillance system and control program. Kyushu is the center for raising beef cattle in Japan. Therefore, this study aimed to determine the BVDV infection using a slaughterhouse survey among beef cattle in Kyushu, Japan. A total of 1,075 blood samples were collected at two regional slaughterhouses in Miyazaki prefecture from December 2015 to June 2016. Antigen ELISA was used for detection of BVDV antigen in blood samples. Two samples showed positive results (2/1,075; 0.18%). BVDV RNA was extracted from positive blood samples; the sequence was determined and analyzed by the neighbor-joining method for construction of the phylogenetic tree. Phylogenetic analysis based on the 5′-UTR revealed that the two positive samples were grouped into the same subtype BVDV-1b in the BVDV-1 genotype, but the infected cattle belonged to two different farms. In conclusion, this is the first study to identify the presence of BVDV in a slaughterhouse survey in Kyushu. These findings suggest that a slaughterhouse survey is a useful tool for developing a surveillance system for monitoring infectious diseases in cattle.

KEY WORDS: bovine viral diarrhea virus, monitoring, screening, slaughterhouse survey, surveillance

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Bovine viral diarrhea (BVD) is a widespread infection of cattle caused by Bovine Viral Diarrhea Virus (BVDV). BVDV belongs to the genus *Pestivirus* in the family *Flaviviridae* [2] and is further sub-divided into two genotypes, BVDV type 1 and BVDV type 2. These are identified as independent species within this genus and are further classified into biotypes, i.e. cytopathogenic (CP) and non-cytopathogenic (NCP). The biotype is based on phenotypic changes produced in cell culture systems *in vitro* and differences in genetic characteristics [3]. BVDV is considered one of the most important infectious agents in the productive cattle industries owing to its negative economic impact on dairy and beef production as well as cattle breeding [4, 8]. BVDV infection

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has been a target of many epidemiological surveys for control and/or eradication programs for decades [13]. Some countries have achieved eradication or significant reduction in the prevalence of BVD using control measures, such as test and slaughter of persistently infected (PI) animals [9, 17, 20]. In Japan, the BVD outbreak has been reported since the 1960s and categorized as a notifiable disease in 1988 [16]. Although systematic control programs for BVD are implemented in European countries, there is no national control program in Japan. Only recently, a survey of BVD was performed in Hokkaido, Japan [1]. Also, efforts are now being made for genetic and antigenic characterization of BVDVs isolated in Japan to study the latest outbreaks and for the control of BVD [1, 14, 15]. A pilot survey conducted in Kanto and western regions of Japan showed that 7.6% of farms surveyed had PI animals and 0.12% of the population was PI [10].

Kyushu island is a center of the beef cattle industry with the highest number of beef cattle farms in Japan. Out of the total beef cattle population in Japan, approximately 35.8% (901,100 heads) are located in the Kyushu region (Ministry of Agriculture and Forestry and Fisheries, 2018). However, the prevalence of BVDV infection in the beef cattle population in the Kyushu region is not well-studied. The aim of this study was to determine the existence and identify the type of BVDV infection in beef cattle in the Kyushu region using a slaughterhouse survey method.

Sampling was carried out in breeding and fattening beef cattle to collect blood samples at two different slaughterhouses in Kyushu, Japan. The beef cattle were defined as male and female Japanese black cattle, Holstein and cross breeding. The sampling period was from December 2015 to September 2016. The numbers of whole blood samples collected from slaughterhouse A and slaughterhouse B were 535 and 540, respectively. Blood discharged from the neck during the process of slaughtering and collected into blood tubes by veterinarians was used as samples. Blood samples were centrifuged at 1,500 × *g* for 5 min to obtain serum and subsequently stored at −20°C. The information of all the slaughtered beef cattle, including sex, age, breed, and birth and growth place, was obtained from the meat inspection office of each slaughterhouse. Serum was tested with antigen-capture ELISA using a BVDV Ag/Serum Plus IDEXX kit (IDEXX, Westbrook, ME, U.S.A.) according to the manufacturer's protocol. The reaction was monitored using iMark, Microplate Reader (Bio-Rad, Hercules, CA, U.S.A.). Viral RNA was extracted from 150 μl of BVDV Ag-ELISA positive bovine sera using the NucleoSpin® RNA virus kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's instructions. The extracted viral RNA was quantified using NanoDrop 8,000 (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and stored at −80°C until further use. The highly conserved 5′-untranslated region (5′-UTR) sequence was amplified by RT-PCR for detection and further characterization of BVDV. A primer pair capable to amplifying 5′-UTR of BVDV, sense primer 324 (position 108–128 of NADL strain) and antisense primer 326 (position 395–375 of NADL strain) were used as described previously [23]. The amplification of viral RNA prior to sequencing was done using the one-step RT-PCR Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The amplified product was subsequently sequenced. Sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). To 15 μl of the mixture containing 10.2 μl RNase free H_2O, 3.5 μl 5 × buffer, and 1 μl Big Dye reagent of the sequencing reaction, 5 μl of purified cDNA was added. Aliquots of 0.8 μl (10 μM) of each forward and reverse BVDV primers 324 (5′-ATG CCC TTA GTA GGA CTA GCA-3′) and 326 (5′-TCA ACT CCA TGT GCC ATG TAC-3′) were added separately in two master mixes. The mix was subjected to thermal cycling for amplification. Subsequently, unincorporated dye terminators were removed by ethanol precipitation. The sequencing reaction product was sequenced from the 5′ and the 3′ directions utilizing capillary electrophoresis on the 3730 Genetic Analyzer. Phylogenetic reconstructions for genetic typing of the viral samples were compiled using a 288-nucleotide region of the 5′-UTR (based on nucleotides position from 108 to 395 of the BVDV, NADL genome). The phylogenetic analysis was performed with the maximum likelihood method, and evolutionary distances were calculated using the Kimura 2-parameter method with 1,000 replicates in boot strap on ClustalW [21]. 5′-UTR sequences in FASTA format were imported into MEGA X software version 7 and sequence alignments performed using ClustalW. The sequences of BVDV subtypes strains used for analysis are shown in Table 1. Further, nucleotide sequence comparison of strains isolated in the current study was performed using 5′-UTR corresponding to 108–395 nucleotides. BVDV-1 IS27CP/01, which was isolated in Japan [15], was used as a reference strain.

One sample was found to be positive in each slaughterhouse using BVDV Antigen ELISA. The proportion of BVDV-positive samples in slaughterhouse A and slaughterhouse B was 0.18% (1/535) (95% CI: 0.01–1.20) and 0.18% (1/540) (95% CI: 0.01–1.19), respectively. The overall proportion of BVDV antigen-positive samples was 0.18% (2/1,075) (95% CI; 0.03–0.75). Phylogenetic tree analysis of the two sequences isolated from samples was performed using ClustalW. The sequences belonged to genotype BVDV-1 and grouped into BVDV-1b subtype (Fig. 1). Both samples clustered

Table 1. Summary of 26 bovine viral diarrhea virus (BVDV)-1 and BVDV-2 reference strains used as representatives from BVDV subtypes virus strains

Genotype	Subtype	Strain	Accession No.
BVDV-1	1a	Nose	AB019670
	1a	NADL	M31182
	1b	IW22/01/NCP	AB266473
	1b	Chiba-98	AB019686
	1b	IS27CP/01	AB359924
	1b	TK 161 NCP/99	AB042710
	1b	HKD 860 NCP/95	AB042686
	1b	CH0601	MH901223
	1b	71–25	KF205314
	1b	MS15.5	GU395540
	1b	7803/1b	KY499100
	1b	131-OS/14	KX853087
	1b	214	MF120594
	1b	CN10a@10	MG434586
	1b	SC	KX280711
	1j	KS86-1ncp	AB078950
BVDV-2	2a	KZ-91 CP	AB003619
	2b	Hokudai-Lab/09	AB567658

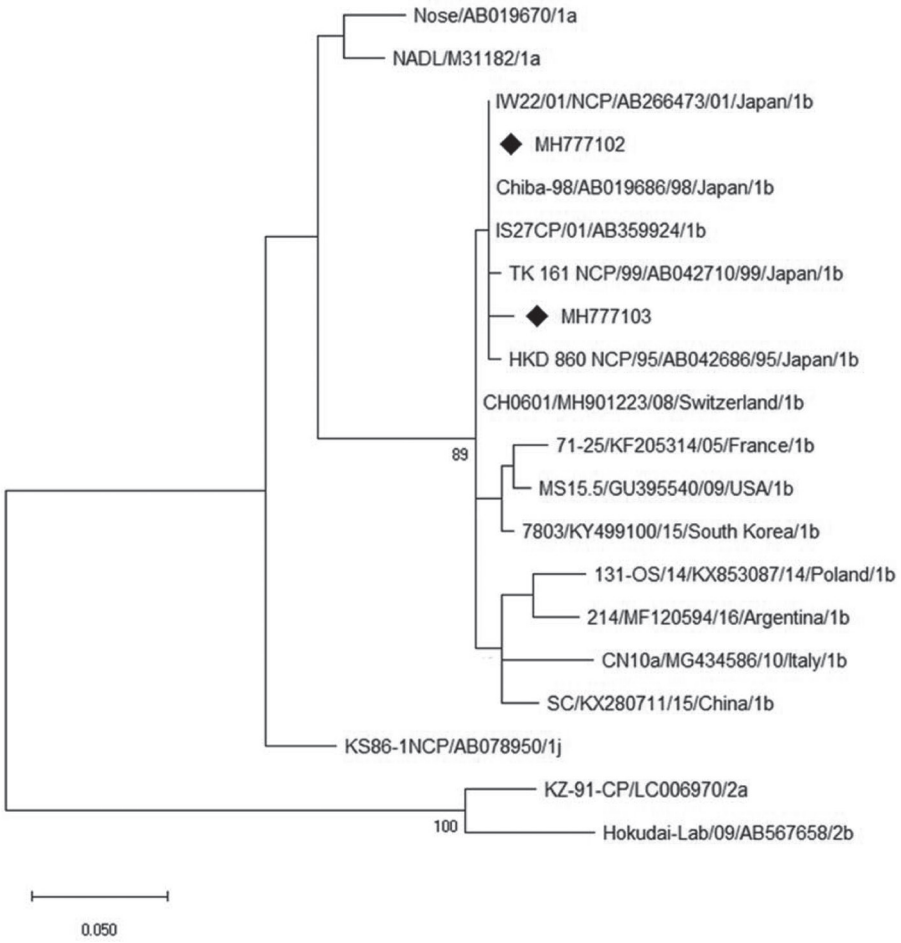

Fig. 1. Phylogenetic tree of the 5′-UTR was created using the nucleotide sequences of the two bovine viral diarrhea virus (BVDV)-positive samples identified in this study, along with nucleotide sequences of 18 BVDV strains representative of BVDV-1, BVDV-2 obtained from the NCBI GenBank. The accession Numbers of sequences are as follows: Nose, AB019670; NADL, M31182; IW22/01/NCP, AB266473; Chiba-98, AB019686; IS27CP/01, AB359924; TK 161 NCP/99, AB042710; HKD 860 NCP/95, AB042686; CH0601, MH901223; 71-25, KF205314; MS15.5, GU395540; 7803/1b, KY499100; 131-OS/14, KX853087; 214, MF120594; CN10a@10, MG434586; SC, KX280711; KS86-1ncp, AB078950; KZ-91 CP, AB003619; Hokudai-Lab/09, AB567658. The slaughterhouse isolates analyzed in this work are labeled with diamonds.

Table 2. History information of bovine viral diarrhea virus (BVDV) ELISA-positive cattle

Slaughterhouse	Production area	Born place	species	Age/month	Sex	Body condition
A	Kyushu	Hokkaido Prefecture	Cross breed	26	Female	Healthy
B	Kyushu	Kumamoto Prefecture	Japanese Black cattle	30	Castrated male	Healthy

with the IS27CP/01 reference sequence as shown by a high bootstrap value. It also revealed minor changes in genomic sequences. The history of slaughtered cattle that were found to be BVDV positive is as follows: 26-month-old F1 cow and a 30-month-old castrated male Japanese black cow raised in Miyazaki prefecture (Table 2).

This study aimed to detect the existence of BVDV infection in slaughtered beef cattle in Kyushu using a slaughterhouse survey. A total of 1,075 blood samples were collected and sera were obtained. Subsequently, bovine sera were analyzed using Ag-BVDV ELISA. Two samples were found to be positive in two slaughterhouses. The proportion was 0.18% (95% CI: 0.03–0.75) and both viruses clustered into BVDV-1b subtype.

Some countries have established BVD disease surveillance by epidemiological practices in order to predict, observe, and minimize the economic impacts caused by a disease outbreak in epidemic or/and pandemic situations. European countries initiated this systematic strategy as the general model of BVDV control [9, 18]. The strategy involves biosecurity, elimination of viral spreading, and active monitoring without initial screening for the BVDV antibody. Control of BVD is dependent on the identification and elimination of PI animals in a controlled manner [12]. Several infectious diseases control strategies have been proposed based on epidemiological knowledge and were introduced for designing and implementing effective regional or wider

control actions. In Belgium, a BVD control program based on detection of PI animals has been implemented [11]. Farm biosecurity and continuous monitoring have played a major role in BVDV eradication programs which prevented infection to free herds [13]. The regulatory monitoring is focused towards preventing the incidence of new BVDV infection in the herd by direct observation of previously infected animals or through examination of new dams at the time of purchase. [9]. The strategy for BVD control in Japan is focused on early detection of PI animals and vaccination to prevent the emergence of PI animals [19].

Some countries, under systematic monitoring and surveillance of animal diseases, have implemented sample collection at slaughterhouses as sampling and information sources to detect BVDV prevalence [6, 7]. Similarly, we have used slaughterhouses to acquire blood samples and cattle information because of the difficulties in on-farm sample collection, such as beef herd distribution and restricted role of farm biosecurity in the Kyushu region. These samples were utilized to detect BVDV infection and characterize isolates involved, which would help in identifying a possible route of viral spread in the region. Ear-tag number recording of slaughtered cattle, which provides access to life histories of the animals, played an important role in this study. Access to large sample size and easy blood collection at slaughterhouse also played an important role in the usefulness of this survey method. Furthermore, slaughterhouse sampling can reduce the manpower required and other costs. This sampling method presents a cost-effective opportunity to raise the awareness of BVDV eradication. Humans are not susceptible to BVDV and BVD is not considered to be a human health hazard. Therefore, in Japan, slaughterhouse food is not tested for BVD [22]. The key measures for effective control of BVD are early detection and culling of PI animals as soon as possible and vaccination of pregnant cows. Although (Ministry of Agriculture, Forestry and Fisheries (MAFF) highlights these important points, there are significant regional differences in the prevalence of BVD in Japan. Consequently, the countermeasures required for prevention of the disease are also varied for different regions. There are many regions like Kyushu Island, Japan, where the invasion of BVDV remains to be ascertained. The slaughterhouse sampling method is effective at screening for BVDV infection and surveying the prevalence of BVDV occurrence in unmapped regions.

Several studies have recommended retesting of BVDV Ag-positive animals after two or three weeks to identify the BVDV infection type. However, this has proven to be impractical as resampling blood serum was usually not possible, since the cattle were slaughtered by then. Recently, a simple immunoperoxidase method which uses hair follicles (HF-IPO) has been developed for the detection of PI animals [5]. The HF-IPO method can detect PI animals quickly and inexpensively with equal sensitivity to virus isolation (VI) and RT-PCR. This method would be useful for the slaughterhouse survey to detect PI animals. Also, BVDV Ag/Serum Plus IDEXX kit using ear-notch samples is useful and cost-effective for screening of neonatal calves for PI animals. The antigen ELISA BVDVs are genetically classified into two types and further sub-divided into at least 17 subtypes: BVDV-1 (1a–1q); and 3 subtypes BVDV-2 (2a–2c). BVDV sequences in this study were classified as BVDV-1 subtype 1b, which is very close to IS27CP/01 strain. Previous studies have reported IS27 CP/01 as the predominant strain in dairy cattle populations in Hokkaido, Japan [1]. The two BVDV-infected animals from beef cattle in Miyazaki were genetically analyzed and found to have BVDV-1b strains which caused a recent epidemic in Japan. Cattle ear-tag number recording at the time of slaughtering was used to obtain information about the individual animal. The information available showed that the BVDV-positive cattle were born in Hokkaido and Kumamoto prefecture, and had passed through some farms and livestock markets across the country.

The scope of this survey was limited by the selection of sampling animals; only cattle of an age suitable for slaughter were surveyed. Another major limitation of this method is the inability to distinguish between persistent and transient infection because BVD tests require collecting blood samples again two or three weeks later to confirm infection types. Furthermore, there is insufficient information regarding BVDV vaccination status of animals.

In conclusion, BVDV was found in both fattening and breeding beef cattle examined in two regional slaughterhouses in Kyushu, Japan. This was confirmed by the positive ELISA results for 0.18% of the tested animals and phylogenetic analysis results for BVDV-1 genotype. Moreover, this study shows that in spite of its limitations, slaughterhouse survey could be a useful method for the detection of infectious diseases in cattle.

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