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BRIEF REPORT

Molecular detection and characterization of bovine viral diarrhea virus in Mongolian cattle and yaks

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Abstract Bovine viral diarrhea virus (BVDV) is classified into two species, namely, *Bovine viral diarrhea virus 1* and *Bovine viral diarrhea virus 2*, and affects cattle worldwide, resulting in significant economic loss. The prevalence of BVDV-1 and BVDV-2 infections and its genotypes in Mongolian animals has not been studied. In this study, we surveyed BVDV infection in dairy cattle and yaks from Bornuur and Bulgan counties by RT-PCR, and the average infection rate in the sampling sites was 15.8 % and 20.0 %, respectively. In addition, molecular features of the 5'-UTR region of the BVDV genome in Mongolian cattle and yaks were identified as belonging to the subtypes BVDV-1a and BVDV-2a, respectively. Determining the prevalence, geographical distribution, and molecular diversity of BVDV-1 and BVDV-2 in various host species in Mongolia is important for further studies and process control programs.

Bovine viral diarrhea virus (BVDV) is classified into two species, namely *Bovine viral diarrhea virus 1* and *Bovine viral diarrhea virus 2*, within the genus *Pestivirus* and family *Flaviviridae*, together with classical swine fever virus and border disease virus [12]. Furthermore, new bovine pestiviruses have been reported in several countries, including Thailand [13], Italy [3], Brazil [26], and China [17], and these are referred to as HoBi-like or atypical bovine pestivirus. However, these newly discovered viruses have not yet received official recognition. The diversity of BVDV includes both genetic and antigenic differences, which have an impact on both diagnostic testing and vaccination effects [8]. According to sequence comparison studies that were typically based on the 5'-UTR region, there are 21 distinct subtypes of BVDV-1 (1a–1u) [5], and two subtypes of BVDV-2 (2a-2b) have been recognized to date [22, 23, 33, 35]. Although some researchers, including Luzzago *et al.* [15], have suggested that BVDV-2 should be classified into three subtypes (2a-2c), this has not been universally adopted, and most researchers still use the former classification. Although the natural hosts of BVDV are cattle, this virus can infect both domestic and wild ruminants, including deer, pigs, sheep, goats, bison, and camelids. Transmission of BVDV occurs by direct and indirect routes. Of these routes, the most important source of BVDV infections are persistently infected (PI) hosts that shed large amount of virus throughout their lives, because the virus can be much more efficiently transmitted in these hosts than in non-PI animals [30]. BVDV is divided into a non-cytopathogenic (NCP) biotype and a cytopathogenic (CP) biotype based on their effects on cultured cells. The CP biotype induces apoptosis in cultured cells, whereas the NCP biotype does not; only the NCP biotype of BVDV induces persistent infection. The CP biotype of BVDV is relatively rare [11]. The clinical manifestations that are associated with BVDV infections are diverse, with the most common clinical signs in infected cattle being an affected respiratory system, abortion, and diarrhea. Moreover, these clinical signs of BVDV are more frequently reported with BVDV-2 than BVDV-1 [1]. BVDV is a widely distributed disease worldwide and has been reported in the cattle populations of many countries, including

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China [5, 17, 24], Korea [20], Japan [19], Thailand [10], Poland [23], Turkey [32], Brazil [6], Australia [16], and the United States of America [25]. However, studies regarding the prevalence and genotype diversity of this virus in animals in Mongolia remain rare. The aim of this study was to detect BVDV and determine its genotypes in different bovine species in Mongolia, including native cattle, dairy-breed cattle, and yaks.

During 2014, a total of 127 blood samples, including, 68 samples from yaks, 40 from native cattle, and 19 from dairy cattle, were collected from the Songinokhairkhan district of the city of Ulaanbaatar, Bulgan and Tsenkher counties of the province of Arkhangai, and Bornuur and Lun counties of the province of Tuv in Mongolia. The samples were collected from the jugular vein of each animal using a BD K3EDTA Vacutainer tube (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and were stored at 4 °C until RNA extraction. Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The synthesis of cDNA was performed with random hexamers in a total reaction volume of 20 μL. BVDV infections were detected using a reverse transcription polymerase chain reaction (RT-PCR) assay targeting the 5'-UTR region, and positive samples were characterized further. The oligonucleotide sequences of PCR primers used in this study were 324/5'-ATGCCCWTAGTAGGACTAGCA-3' and 326/5' TCAACTCCATGTGCCATGTAC-3', as described previously [28]. RT-PCR amplification was conducted in a total volume of 30 μL containing 1 μL (100 ng) of a cDNA sample that was added to 29 μL of a reaction mixture containing 0.15 μL of Ex Taq polymerase (Takara Bio Inc.,), 2.4 μL of dNTPs, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, 3 μL of Ex Taq buffer, and 21.45 μL of double-distilled water. PCR amplification was performed under the following thermal cycling conditions: initial denaturation at 94 °C for 5 min, followed by 50 cycles of denaturation at 98 °C for 15 s, annealing at 55 °C for 30 s, extension at 72 °C for 45 s, and a final synthesis at 72 °C for 1 min in a GeneAmp PCR System 9700 (Applied Biosystems, USA). The amplified PCR products were confirmed using a Mupid-exU Electrophoresis System (Takara Bio Inc.) on a 2.0 % agarose gel and were visualized under an ultraviolet light printgraph AE-6905CF (Atto, Tokyo, Japan). The β-actin gene was amplified as an internal control to confirm the presence of cDNA in the template [22]. The positive samples were subjected to sequence analysis. PCR products were extracted using a FastGene gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). The extracted PCR products were ligated into pGEM-T Easy Vector (Promega), and the plasmid was introduced into *Escherichia coli* strain DH5α (Takara Bio Inc.,), plated on Luria-Bertani (LB) agar (Invitrogen, Carlsbad, CA, USA), and cultured in LB broth (Invitrogen). Plasmid cDNA from the positive clones was extracted from the LB culture using a FastGene Plasmid Mini Kit (Nippon Genetics). The sequencing amplifications of the plasmids were performed using a GeneAmp PCR System 9700 (Applied Biosystems). The quality of the plasmids was assessed using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Finally, the nucleotide sequences of the amplified plasmids were determined using a CEQ8000 DNA analysis system (Beckman Coulter, Fullerton, CA, USA). All identified pathogens were initially analyzed using the Bio-Edit program and the Basic Local Alignment Search Tool (BLAST) application. Phylogenetic trees were constructed using the MEGA 6 program.

In this study, we collected 127 blood samples from animals that were representative of the Mongolian native cattle, dairy cattle, and yaks in five distinct areas of Mongolia. Eleven of the 127 samples were found to be BVDV positive. The infection rate of BVDV-positive samples was 15.8 % (3/19) in cattle from Bornuur County of Tuv Province and 20.0 % (8/40) in yaks from the Bulgan County of Arkhangai Province. In contrast, no positive samples were detected from Mongolian native cattle in Lun County of Tuv Province, Tsenkher County of Arkhangai Province, or yak samples from the Songinokhairkhan district of the city of Ulaanbaatar. The ages of the BVDV-positive animals ranged from 1 month to 17 years old, and all of were female cattle and yaks, except for one 2-year-old male yak (Table 1). Most of the animals did not exhibit any clinical symptoms at the time of sampling, and only the youngest yak's calf had diarrhea containing blood; however, the exact cause was unknown.

There are several tests for BVDV diagnosis, including virus isolation, RT-PCR, immunohistochemistry (IHC), and antigen enzyme-linked immunosorbent assay (Ag ELISA), but the tests require time and repeated sampling to clarify whether the infection is persistent. We could not conduct other diagnostic tests other than RT-PCR due to sample transportation problems between Mongolia and Japan, and also because of a lack of information about the prevalence of the infection in the country. The current study was primarily a molecular survey of BVDV infection in Mongolian animals together with a description of the host animals and their geographical location.

Epidemiological studies have shown that BVDV is globally distributed in the cattle populations of many countries. Of these, the highest prevalence is in China with overall infection rates 22.64 % for RT-PCR detection and 58.09 % for antibody detection in several ruminant species, including cattle, yaks, and water buffalo [5] and 23.1 %-33.6 % in pigs [4]. Thus, the results of these studies were consistent with our results showing that yaks are more

Table 1 BVDV detection in Mongolian cattle and yaks

Province	Province	Latitude/ Longitude	Positive/tested animals (%)	Breed	Positive sample ID	Age	Sex	Genotype	Accession number
Tuv	Bornuur	48.459978 / 106.256054	3/19 (15.8%)	Simmental	37	17 Y	F	1 a	LC099930
				Holstein	40	7 Y	F	1 a	LC099931
				Holstein	91	6 Y	F	2 a	LC099932
	Lun	47.867643 / 105.250982	0/20	Mongolian native	-	-	-	-	-
Arkhangai	Bulgan	47.319397/ 101.118807	8/40 (20.0%)	Yak	1	7 Y	F	2 a	LC099925
					2	8 Y	F	2 a	LC099926
					8	8 Y	F	2 a	LC099926
					10	8 Y	F	1 a	LC099927
					15	1 M	F	1 a	LC099927
					27	2 Y	M	1 a	LC099928
					40	5 Y	F	2 a	LC099929
					42	4 Y	F	2 a	LC099926
	Tsenkher	47.438997 / 101.752536	0/20	Mongolian native	-	-	-	-	-
Ulaanbaatar	Songinokhairkhan	48.122235 / 106.647677	0/28	Yak	-	-	-	-	-
Total			11/127 (8.7%)						

Y: years

M: months

susceptible to BVDV infection. The overall positive rate of BVDV infection in the two sampling areas in Mongolian cattle and yaks was 8.7 % (11/127), which appears to be lower than that in China. However, the infection rate in Mongolia is likely to be higher, and a more realistic rate could be obtained if a large number of cattle and yaks were screened for detection of virus infection, particularly among dairy-breed cattle in the country, because the number of samples from the infected areas was small. All of the positive samples were subjected to further sequence analysis targeting the 5'-UTR region. Out of 11 sequences, eight were identified as BVDV-1 or BVDV-2. The nucleotide sequences were 78.3 %–100 % identical to one another, with the sequences of BVDV-1 being 95.2 %–100 % identical and those of BVDV 2 being 91.1 %–100 % identical to one another. In phylogenetic analysis, four sequences (LC099927, LC099928, LC099930, LC099931) obtained from three yaks in Bulgan County of Arkhangai Province and two cattle in Bornuur County of Tuv Province were found to belong to BVDV-1a and were subclustered with Japanese isolates, with 97.9 %-98.95 % sequence identity (Fig. 1). In contrast, four other sequences (LC099925, LC099926, LC099929, LC099932) obtained from five yaks in Bulgan County of Arkhangai Province and one cattle in Bornuur County of Tuv Province clustered in BVDV-2a and branched with isolates from Germany, the USA, and Japan. Of these, two sequences (LC099929, LC099932) derived from each positive sampling site were 100 % identical to one other, whereas two other sequences (LC099925, LC099926) obtained from four yaks in Bulgan County of Arkhangai Province were quite divergent from the others (Fig. 1). In general, each of the sampling sites may have separately contracted the infectious diseases, because there was no correlation with animal movement between these areas. In addition, the habitats of dairy cattle and yaks are different in the country, with dairy cattle mostly distributed around the city of Ulaanbaatar, whereas yaks are only distributed in high mountainous areas, including Arkhangai Province, but both BVDV 1 and BVDV 2 were identified from each animal species in this study. This indicates that BVDV is likely widely prevalent in Mongolian livestock, including dairy cattle and yaks.

A recent phylogenetic analysis of BVDV revealed the existence of at least 21 subtypes within BVDV-1 (1a−1u) [5]. BVDV 1a has been reported in several countries, including Canada, France, Germany, New Zealand, Mozambique, Spain, Sweden, the UK, the USA [29], Australia (17), China [4], Korea [20], and Japan [18]. In

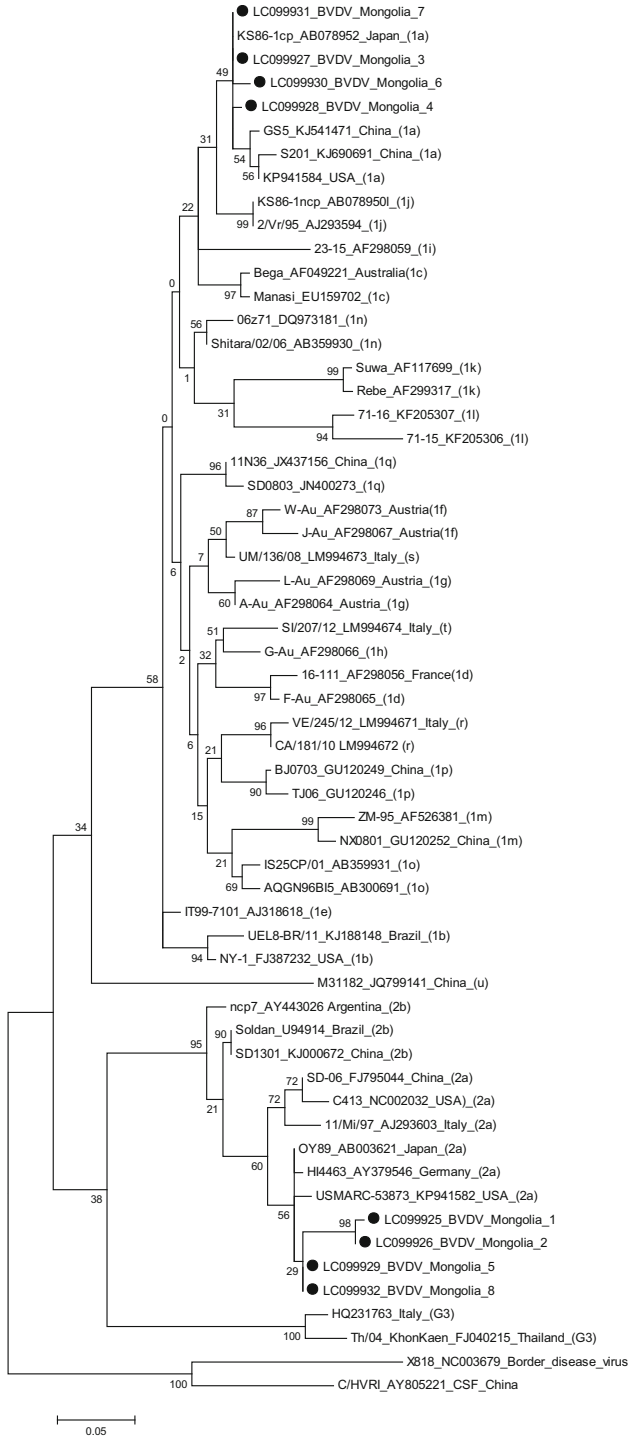

Fig. 1 Phylogenetic tree constructed by the maximum-likelihood method using 287- to 290-bp-long sequences from the 5'-UTR region of BVDV, supported by 1,000 bootstrap replications

contrast, two subtypes of BVDV-2 have been identified, namely, BVDV-2a [22, 35] and BVDV-2b [31]. Subtype 2a appears to be prevalent in the USA [7], Italy [14], Korea [20], Poland [23], Japan [15] and Turkey [21]. In contrast, 2b has been identified in China [31], Brazil [2], and Argentina [9]. However, some European countries have successfully implemented control and eradication programs for BVDV infections since the 1990s, and the Scandinavian countries are now considered free from the disease; however, the control program is still underway in some European countries [27]. There are three important aspects of BVDV prevention and control: 1) identifying and eliminating persistently infected animals; 2) enhancing immunity by vaccination, and 3) implementing biosecurity measures to prevent exposure of susceptible animals to BVDV [30]. The animal-origin product industry in Mongolia is considered one of the major sectors that influences the country's economic development. However, epidemiological studies of infectious diseases, including BVDV, have been limited in this country. We investigated the prevalence of BVDV infection and identified BVDV genotypes in Mongolian cattle and yaks, but further studies are required to determine the prevalence and genetic diversity of BVDV infections in a larger number of susceptible animals, including cattle and yaks, because the information gained would be important for processing control programs.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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