Short Paper

Genetic characterization and clinical characteristics of bovine viral diarrhea viruses in cattle herds of Heilongjiang province, China

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Abstract

Background: Heilongjiang province is the main cattle-producing area in China, and molecular epidemiological studies of bovine viral diarrhea viruses (BVDV) in cattle have not been performed in the province. **Aims:** The objective of this research was to determine the genetic and clinical characteristics of BVDV in cattle. **Methods:** Fifty-three BVDV-positive clinical samples were collected from 22 cattle farms in Heilongjiang, and the 5´-untranslated region (5´-UTR) was used to carry out a phylogenetic analysis of the viruses. **Results:** The similarity of the 5´-UTR sequences among these BVDVs was 84.2%-100%, and the phylogenetic analysis showed that all viruses belong to the BVDV-1 species, which is classified into five subtypes: BVDV-1b (47.17%, n=25), 1c (15.09%, n=8), 1d (16.98%, n=9), 1 m (3.77%, n=2), and 1o (16.98%, n=9). The statistical results showed that the BVDV-1b subtype had a positive correlation with gastrointestinal disease ($P<0.05$; 95% CI: 1.19 to 3.34). There were up to three or four BVDV-1 subtypes in some dairy cattle farms, but farms with a single subtype were prevalent (5/10). **Conclusion:** BVDV-1b is predominant in cattle herds of Heilongjiang province, China, and shows a positive correlation with gastrointestinal disease. BVDV-1o was found for the first time in Chinese cattle, which increased the complex distribution of BVDV-1 subtypes in cattle herds of China.

Key words: BVDV, Cattle, China, Genetic evolution

Introduction

BVDV is a single linear positive-strand RNA virus of the genus *Pestivirus* in the *Flaviviridae* family. The genotyping of *pestiviruses* is primarily based on the 5´-untranslated region (5´-UTR), N-terminal autoprotease (N^{pro}), and envelope glycoprotein (E_2) genomic regions (Baule *et al*., 1997; Vilcek *et al*., 2001). Vilcek *et al*. (2001) demonstrated the highest degree of sequence conservation in the 5´-UTR genomic region, which is needed for meaningful genotyping results. The genus *Pestivirus* has three viral species that can infect bovines, *Pestivirus* A (BVDV-1), *Pestivirus* B (BVDV-2), and *Pestivirus* H (HoBi-like Pestivirus), according to the Flaviviridae Study Group of the International Committee on Taxonomy of Viruses (ICTV) (Simmonds *et al*., 2017; Smith *et al*., 2017). BVDV-1 is further classified into 21 subtypes, and BVDV-2 is classified into four subtypes (Vilcek *et al*., 2001; Flores, 2002; Luzzago *et al*., 2014; Yesilbag *et al*., 2014, 2017; Giammarioli *et al*., 2015; Smith *et al*., 2017).

BVDV infection may be subclinical or present as a mild, severe, or fatal disease. The disease may show a wide variety of clinical symptoms, including enteric disease, respiratory disease, abortion, or persistent infection, in all cattle breeds. Some authors have reported BVDV subtypes that may be related to particular symptoms in cattle; for example, some BVDV-1 strains could be involved in acute respiratory diseases of calves (Fulton *et al*., 2002), and some BVDV-2 strains could lead to hemorrhagic syndrome in adult animals and acute diarrhea in young cattle (Carman *et al*., 1998).

Heilongjiang province is the main area of cattle production and the largest settlement of dairy cows in China, which currently has 1.1 million dairy cows. According to a meta-analysis in Heilongjiang, the pooled seropositivity rate of BVDV is 37.1% (Ran *et al*., 2019). Molecular epidemiological studies have not yet been conducted in Heilongjiang, however, some studies on BVDV subtypes (BVDV-1a, b, c, d, m, p, q, u, and BVDV-2a) have been performed in other regions of China (Deng *et al*., 2015; Hou *et al*., 2019). This study has two main objectives:

(1) To investigate the genetic profile of BVDV strains in Heilongjiang

(2) To assess whether subtypes of BVDV are associated with specific clinical presentations

Materials and Methods

Sample collection

The survey was conducted in Heilongjiang province, China (125°03′-125°33′S, 46°42′-46°58′W). Samples were collected from 22 cattle farms in different districts in Heilongjiang province from 2010 to 2018 (Fig. 1). A total of 53 BVDV-positive samples were selected based on ELISA or PCR from different cattle and used to determine the viral subtypes (Supplementary Table 1 (ST1)). The hosts of all 53 BVDV-positive samples were classified into three clinical types: asymptomatic infection (SD), not showing any clinical symptoms; gastrointestinal disease (GD), characterized by diarrhea or oral mucosal ulcers; and respiratory disease (RD), characterized by bovine respiratory disease complex.

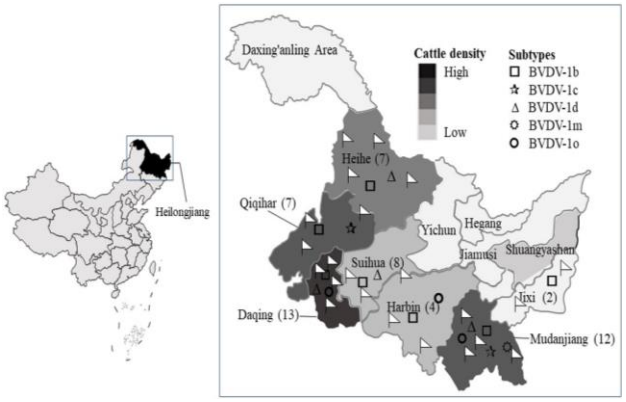

Fig. 1: Spatial distribution of sampled farms and BVDV subtypes. The numbers in the brackets represent the number of BVDV isolates collected from seven districts of Heilongjiang province, China. The symbols represent different BVDV subtypes. The triangle flags represent the spatial distribution of the sampled farms

For SD samples, 11,134 serum samples from weaned calves and all adult cows were collected from four dairy cattle farms and one beef cattle farm in Harbin, Qiqihar, Mudanjiang, and Jixi in Heilongjiang province. The BVDV eradication plan was enforced to control BVDV by removing Ag-positive cattle from herds at these cattle farms. BVDV antigen ELISA kits (IDEXX Laboratories, Inc., Liebefeld, Switzerland) were used to test all serum samples. The overall positive rate of BVDV antigens was 2.31% (258/11,134). Twenty-two BVDV Ag-positive serum samples were selected randomly from cattle without clinical symptoms. For GD samples, positive samples of 15 cattle were collected from dead or sick animals manifesting gastrointestinal symptoms, including diarrhea or mucosal disease, from four dairy cattle farms and one beef cattle farm. For RD samples, positive samples of 16 cattle were collected from dead or sick animals manifesting respiratory disease from seven dairy cattle farms and five beef cattle farms. All the GD and RD samples were tested by the PCR method. Relevant clinical information was provided by local veterinarians or farmers. All of the herds had not been vaccinated against BVDV.

Reverse transcription-polymerase chain reaction (RT-PCR) and sequencing

Total RNA was extracted from 200 µL of serum or contents of the intestinal tract or tissues using TIANamp virus RNA kits (Tiangen Biotech Co., Ltd., Beijing, China) and reverse transcribed by reverse transcription kits (Toyobo, Japan). Universal primers complementary to the 5´-UTR of the *Pestivirus* genome were used to amplify a 288-bp DNA fragment via PCR (Vilcek *et al*., 1994). Amplified target fragments were purified and cloned using pMD-18T vector kits (TaKaRa). The clones (three per sample) were sequenced by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China) using the M13+ and M13 sequencing primers with an automated 3730 ABI sequencer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis of the BVDVs

The nucleotide sequences of the BVDV 5´-UTR were proofread using the SeqMan II sequence editor, aligned using the Clustal W method of the MegAlign program of DNASTAR software, and phylogenetically analyzed via the Neighbor-Joining (NJ) method using MEGA X software (Kumar *et al*., 2018). Evolutionary distances were calculated using the Kimura 2-parameter method. The optimal tree is shown with the sum of branch length = 2.10. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The sequences corresponding to the 5´-UTR of BVDV in this study were submitted to GenBank. A total of 34 representative sequences of BVDV subtypes were also retrieved from GenBank (Supplementary Table 1 (ST1)).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5, and the differences between the groups (clinical symptoms, breeds, and BVDV-1 subtypes) were compared using Pearson's Chi-squared and Fisher's exact tests. Probability values $P<0.05$ were considered statistically significant (*), while $P<0.01$ was considered very significant (**).

Results

Phylogenetic analysis of the BVDVs

The 53 5´-UTR nucleotide sequences determined in this research were submitted to the GenBank database (Supplementary Table 1 (ST1)). Phylogenetic analysis showed that all 53 detected sequences were BVDV-1 and belonged to 5 subtypes (Fig. 2). The subtypes were BVDV-1b (47.17%, n=25), 1c (15.09%, n=8), 1d (16.98%, n=9), 1 m (3.77%, n=2), and 1o (16.98%, n=9) (Table 1). The alignment of the 5´-UTR sequences among the 53 BVDVs showed that the sequence similarity was 84.2%-100%, and the differences were mainly located in two variable regions that corresponded to nucleotides 208-223 and 256-320 in the NADL sequence. The differences were mainly deletions of either one or two nucleotides or nucleotide substitutions. The BVDV-1b subtypes shared a sequence similarity of 93.3%-98.9% with reference strain 4-2. BVDV-1c subtypes shared a sequence similarity of 96.8%-99.6% with the reference strain AQMZ02A/21/2. BVDV-1d subtypes shared a sequence similarity of 97.1%-98% with the reference strain Lamspringe-735. BVDV-1 m subtypes shared a sequence similarity of 95.1%-97.9% with reference strain ZM-95, and BVDV-1o subtypes shared a sequence similarity of 92.2%-98% with reference strain IS25CP01.

Association between BVDV-1 subtypes and clinical presentations in cattle

To investigate the association between BVDV-1 subtypes and symptoms of the infected host, a statistical analysis was performed. Two subtypes, a BVDV-1b-positive rate of 73.33% (11/15) and a BVDV-1o rate of 26.67% (4/15), were detected in the GD samples. Three subtypes, BVDV-1b, d, and o, were detected in the RD samples, with positive rates of 50% (8/16), 43.75% (7/16), and 6.25% (1/16), respectively. The prevalent subtypes were more heterogeneous in the SD samples, with 5 subtypes detected, BVDV-1b (27.27%), BVDV-1c (36.36%), BVDV-1d (9.09%), BVDV-1 m (9.09%), and BVDV-1o (18.18%), in 22 samples. Further analysis found that the BVDV-1b-positive rate was 73.33% in the GD samples, which was significantly higher than that (36.84%) of BVDV-1b in the SD and RD samples ($P<0.05$; 95% CI: 1.1878 to 3.3356; OR=4.7143), as

Table 1: BVDV-1 subtypes from cattle with different clinical symptoms

Subtypes	Percentage rate (Number)	Clinical symptoms		
		Gastrointestinal disease	Asymptomatic infection	Respiratory disease
BVDV-1b	47.17 (25/53)	73.33 (11/15)	27.27 (6/22)	50 (8/16)
BVDV-1c	15.09 (8/53)	0	36.36 (8/22)	0
BVDV-1d	16.98 (9/53)	0	9.09 (2/22)	43.75 (7/16)
BVDV-1m	3.77 (2/53)	0	9.09 (2/22)	0
BVDV-1o	16.98 (9/53)	26.67 (4/15)	18.18 (4/22)	6.25 (1/16)

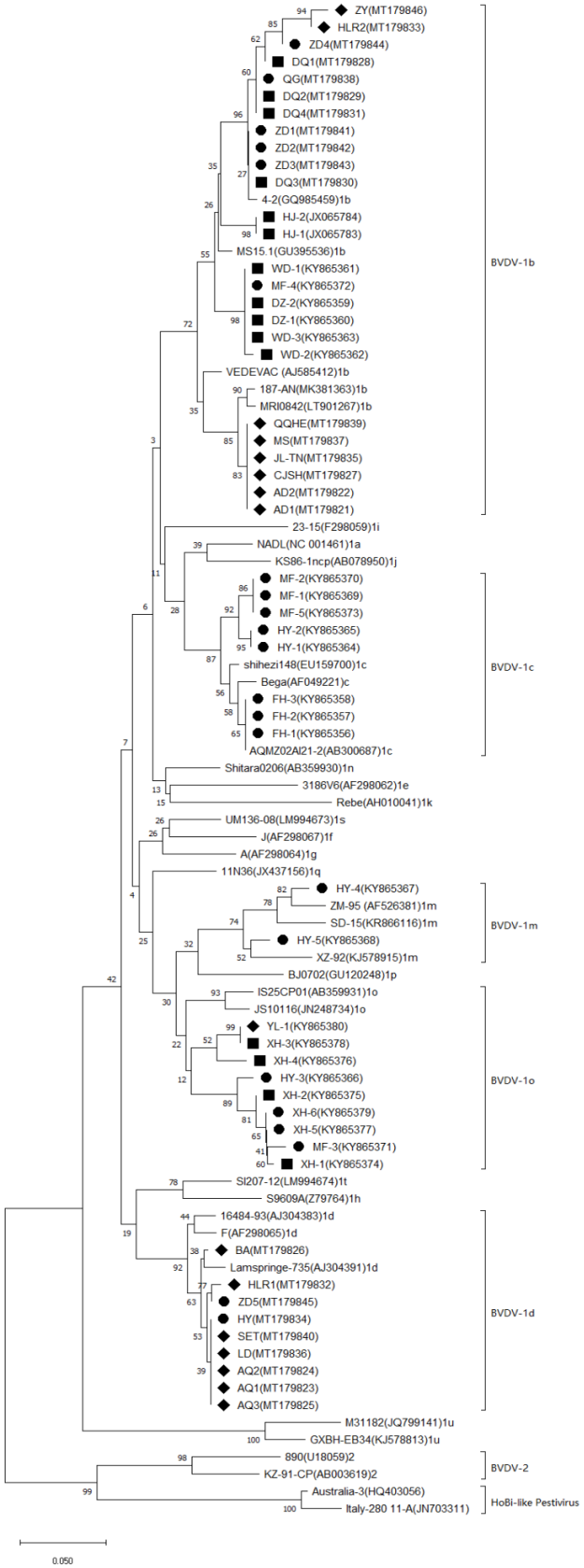

Fig. 2: The phylogenetic tree created using the 5´-UTR nucleotide sequences from 53 BVDV isolates and 34 BVDV reference strains retrieved from GenBank (Supplementary Table 1 (ST1)). ■ Indicates strains from samples with GD, ● Indicates isolates from samples with SD, and ◆ Indicates isolates from samples with RD

Table 2: Risk factor analysis for BVDV-1b subtypes

Variables	Number	BVDV-1b (%)	Odds ratio	P-value	95% confidence interval
GD	15	11 (73.33)	4.7143	0.0308 *	1.1878 to 3.3356
Asymptomatic infection and respiratory disease	38	14 (36.84)			

shown in Table 2.

The prevalence of BVDV-1 subtypes in the same herd in which more than two BVDV strains were detected was analyzed, which corresponded to 43 BVDV strains from 10 cattle farms. As Fig. 3 shows, three and four BVDV-1 subtypes coexisted in the HY and MF dairy cattle farms. However, farms with only one viral subtype were prevalent (5/10).

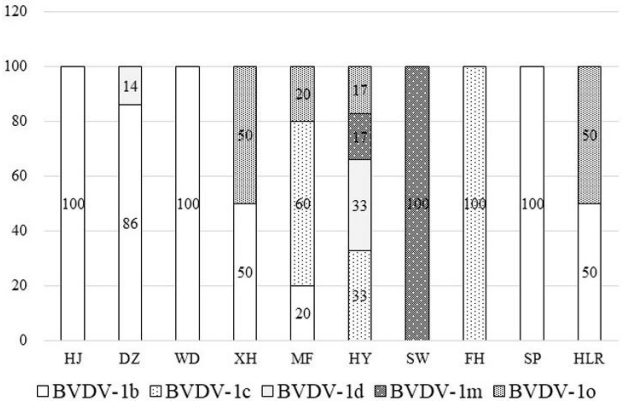

Fig. 3: Distribution (percentage) of BVDV-1 gene subtypes in cattle farms with more than two isolates. Vertical axis represents the percentage of BVDV-1 subtypes, and horizontal axis represents different cattle farms

Discussion

In this study, five BVDV-1 subtypes, BVDV-1b, 1c, 1d, 1 m, and 1o, were found to circulate in different districts of Heilongjiang province, China (Fig. 1). BVDV-1b was the most prevalent subtype in Heilongjiang province (Table 1), and it is also the most prevalent subtype worldwide (Yeşilbağ *et al*., 2017; Mirosław and Polak, 2019). BVDV-1a and c were the dominant subtypes in eastern China (Hou *et al*., 2019). To date, the BVDV-1o subtype has only been found in pigs of China (Deng *et al*., 2012). This study is the first detection of BVDV-1o in cattle herds in China. Before 2015, BVDV-1 m was considered a popular subtype in cattle from China; however, the positive rate was only 3.77% (2/53) in this study, which may have been due to geography (Deng *et al*., 2015). *Pestivirus* B and *Pestivirus* H were not detected in this work.

Both BVDV-1b and 1o can be detected in cattle with different clinical symptoms, and their positive rate is greater than that of other BVDV-1 subtypes in this research. The positive rate of BVDV-1b in GD was significantly higher than that in SD and RD ($P<0.05$), while the OR value was 4.71 more than 1, indicating a positive correlation between BVDV-1b and GD. BVDV-1b (8/15) and 1d (7/15) subtypes were detected in cattle of natural death with RD, which is consistent with previous reports (Baule *et al*., 1997; Obando *et al*., 1999). BVDV-1 infection is the most complicated, and five BVDV-1 subtypes have been detected in SD cattle herds. This may be due to persistently infected cattle that are not easy to find in the herd and have a long survival time compared to other symptomatic cattle. Genetic mutations of BVDV could be caused during the long-term struggle of BVDV with the host (Peterhans and Schweizer, 2013).

In this study, multiple BVDV-1 subtypes coexisted in the same dairy cattle farm, which increased the difficulty of controlling BVDV infection by vaccines. This study found that there was a history of frequently imported dairy cows from Australia, New Zealand, and other districts of China, which could explain the multiple BVDV-1 subtypes coexisting in some dairy cattle herds (MF and HY) so that different BVDV subtypes predominate in different geographic locations (Vilcek *et al*., 2005). These findings suggest that the genetic diversity of BVDV in the cattle herds of Heilongjiang province, China, may be caused by animal migration or virus strains introduced from other countries.

The results indicate that the BVDV-1b subtype was prevalent in Heilongjiang province, China, and the BVDV-1o subtype was detected for the first time in cattle in China. The distribution of BVDV-1 subtypes in the cattle herds was heterogeneous, and some herds can have multiple viral subtypes circulating in the cattle. The BVDV-1b subtype was positively correlated with gastrointestinal diseases (OR>1). In the future, we intend to expand the sampling area and analyze more samples to support our conclusions. In addition, continuous surveillance must be initiated to detect the emergence of novel species or subtypes of pestivirus in China.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Supporting Online Material

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