RESEARCH ARTICLE

Antibody detection and genotyping of bovine viral diarrhea virus in the dairy farms in Ningxia, China

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Bovine viral diarrhea/mucosal disease (BVD/MD) is a viral infectious disease which brings serious harm to the cows. There were only reports on bovine viral diarrhea virus (BVDV) infected dairy cows in Ningxia Province, China with no BVDV typing being reported. The purpose of this study is to evaluate the prevalence and distribution of BVD in Ningxia and to analyze the diversity of the subtype genes of BVDV. The enzyme-linked immunosorbent assay (ELISA) was used to detect 1,890 blood samples from 13 dairy farms in 7 cities of Ningxia. The N-gene of 49 antigen positive samples were amplified by reverse transcription polymerase chain reaction (RT-PCR). Mega 7.0 software was used to construct phylogenetic tree and analyze the classification status of the virus strains. The antibody positive rate remained in the range of 88.15 - 100% with an average rate of 92.59%. The antigen positive rate was from 1.11 to 4.44%. The confirmed subtypes of BVDV include BVDV-1a, BVDV-1d, BVDV-1m, BVDV-1q, and BVDV-2a, which agree with the commonly seen subtypes in China. However, our study identified BVDV-1a, BVDV-1m, and BVDV-2a virus strains for the first time in Ningxia, which indicate the complexity of genetic diversity of BVDV in Ningxia. In conclusion, we conducted the first genotyping of BVDV in Ningxia, which sets a solid foundation for further study of the law of change and emergence mechanism of the subtypes of BVDV virus strains in Ningxia and China.

Keywords: bovine viral diarrhea virus; Ningxia; antibody; antigen; genotyping.

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Introduction

Bovine viral diarrhea/mucosal disease (BVD/MD) is a viral infectious disease which brings serious harm to the health of cows. There are mainly two statuses of the cows affected by bovine viral diarrhea virus (BVDV), which are transient infection (TI) and persistent infection (PI). BVD can bring terrible influence on the ability of producing and breeding of cows. It can cause abortion, infant deformity, diarrhea, and immunosuppression. The fetus may survive if its mother is infected by BVDV at a certain stage and becomes persistently infected cattle. Such cattle show immune tolerance to BVDV and carries the virus through its life, which becomes a main source of infection [1].

BVDV is a type of Pestivirus of Flaviviridae. There is no difference among the serotypes. According to the antigenicity and genomic differences, it can be classified into BVDV-1, BVDV-2, BVDV-3

(Hobi-like pestivirus), and other gene-types [2, 3]. Each gene-type can be further divided into different subtypes including BVDV-1a to BVDV-1u, BVDV-2a to BVDV-2d, *etc*. [4]. BVDV can also be classified into cytopathic (CP) and noncytopathic (NCP) based on the difference of lesions after vaccination [5]. Both CP and NCP can cause acute infection, while only NCP-type BVDV can infect fetus of 40-120 days-old through the placenta of cows in pregnancy and cause persistent infection [6].

BVD has been reported in most places in China except for Yunnan, Guizhou, Chongqing, Guangdong, Hainan, Hong Kong, and Taiwan. The commonly seen virus strains include BVDV-1a to BVDV-1d, BVDV-1m to BVDV-1q, BVDV-1u, BVDV-2a, BVDV-2b, and BVDV-3 [7-12]. There were only case reports of BVDV infected dairy cows in Ningxia Province, China without the reports of BVDV typing. This study investigated BVDV in dairy farms in Ningxia Province, China with serological testing and genotyping followed by genetic polymorphism analysis and evolutional investigation of BVDV in this district.

Materials and methods

Sample collection

Total 1,890 serum samples were collected from 13 dairy farms of 7 cities including Pingluo, Dawukou, Helan, Yinchuan, Lingwu, Qingtongxia, and Wuzhong in Ningxia Province, China during May 2018 to May 2019. The blood samples were taken from tail vein of newborn calf or bred cattle within 1-year-old. The serum samples were separated by using Eppendorf 5417R centrifuge (Eppendorf, Hamburg, Germany) at 3,000 rpm for 15 mins at 4℃ and were stored at -80°C.

Antibody detection

The BVDV antibody of all samples were tested by using Bovine Viral Diarrhea Virus Antibody Test Kit (IDEXX, Montpelier, France) with three repeats for each tested sample. Briefly, 100 µL of sample was added to each well on the reaction plate with 25 µL of two-well negative control and 25 µL of two-well positive control. The absorbance of each reaction well at the wavelength of 450 nm was detected by using iMark™ Microplate Absorbance Reader (Bio-Rad, Hercules, California, USA). The following S/P (sample/positive control) equation was applied to calculate the corrected optical density (COD) of each sample well.

$$S/P = \frac{A_{450} \text{ of sample} - A_{450} \text{ of negative control}}{A_{450} \text{ of positive control} - A_{450} \text{ of negative control}}$$

The serum was positive to antibody when the value of S/P was beyond 0.300. The samples which were positive to antibody were used for antigen detection.

Antigen detection

All samples were tested for BVDV antigen by using Bovine Viral Diarrhea Virus Antigen Test Kit (IDEXX, Montpelier, France). Briefly, 50 µL of antibody was added to each reaction well including 50 µL of two-well negative control, 50 µL of two-well positive control, and 50 µL of whole blood. The absorbance of 450 nm was obtained through iMark™ Microplate Absorbance Reader (Bio-Rad, Hercules, California, USA). The corrected optical density (COD) of each sample well was calculated by using the following S-N (sample – negative control) equation.

$$S\text{-}N = A_{450} \text{ of sample} - A_{450} \text{ of negative control}$$

The serum was positive to antigen when the value of S/P is beyond 0.300. Cattle with serum positive to antigen were isolated for 2 weeks before aseptic collection of anticoagulants for recheck. After rechecking, cattle with serum still positive to antigen were determined as persistent infection cattle.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from 100 µL of serum which were positive to antigen by using RNAiso Plus Kit (Takara, Osaka, Japan). The N-terminal

coding region (773 bp) was amplified by using One-step RNA RT-PCR Kit (Takara, Osaka, Japan) with the primers of NproF (5'-TCT CTG CTG TAC ATG GCA CAT G-3') and NproR (5'-TTG TTR TGG TAC ARR CCG TC-3') (R stands for A or G). The reaction was carried out as 5.0 µL of RNA (150 µg/mL), 0.5 µL of NproF primer (20 µmol/L), 0.5 µL of NproR primer (20 µmol/L), 12.5 µL of 2×PCR Buffer, 5.5 µL of H_2O, and 1 µL of One-step RT-PCR Taq (Takara, Osaka, Japan) under the condition of 50°C for 30 mins, followed by 35 cycles of 94°C for 2 mins, 55°C for 30s, and 72°C for 50s, and then, 72°C for 10 mins. 5 µL of PCR product was examined by 0.8% agarose gel electrophoresis. The gel picture was documented by using Gel Doc XR+ Gel Imaging Scanner (Bio-Rad, Hercules, California, USA).

Target fragment cloning and sequence determination

Both M13F (5'-GTA AAA CGA CGG CCA G-3') and M13R (5'-CAG GAA ACA GCT ATG AC-3') primers were used for amplification and verification of the positive segment based on the genomic information of similar BVDV strain documented in GenBank (www.ncbi.nlm.nih.gov/genbank). The amplified DNA segment was inserted into pGEM T-easy (Tiangen, Beijing, China) according to the manufacturer's instruction, and then, was transformed into DH5α competent cell (Tiangen, Peking, China) by using heat shock transformation technique. Colony PCR was employed for preliminary identification by using M13F and M13R primers. The plasmid DNA of identified virus strain was extracted by using a plasmid extraction kit (Tiangen, Beijing, China), and was sequenced by Sangon Biotech (Shanghai, China).

Target genome sequence analysis

The viral genome sequence was collected by splicing with SeqMan software (Madison, Wisconsin, USA). The gene structure of the virus strain was analyzed based on the sequence of reference strain SD-15 of BVDV (GenBank accession number: Q01499) in the National Center for Biotechnology Information (NCBI) database. BioEdit software (Madison, Wisconsin, USA) was applied to analyze the homology sequences between the obtained virus genome and reference strains. Recombination site was analyzed by using reorganized detection software Simplot (Johns Hopkins University, Baltimore, Maryland, USA) and RDP4 (University of Manchester, Manchester, UK). Neighbor-Joining with Mega 7.0 software (Madison, Wisconsin, USA) was used to construct a phylogenetic tree and analyze the classification status of the virus strain.

Statistical analysis

All data were subjected to statistical analysis for the interpretation of the results with IBM SPSS Statistics 26 software (IBM Corporation, New York, NY, USA). Difference analysis was performed by the Chi-square test.

Results

Antibody detection

The results showed that the antibody positive rate over all tested samples was from 88.15% to 100% with an average of 92.59%. The antibody positive rates in different locations including Pingluo, Dawukou, Helan, Yinchuan, Lingwu, Qingtongxia, and Wuzhong were 88.15% (238/270), 88.89% (240/270), 89.63% (242/270), 87.04% (242/270), 97.41% (263/270), 97.04% (262/270), and 100% (270/270), respectively. The highest antibody positive rate was found in Wuzhong with the very significant differences comparing to the other locations ($P < 0.01$). However, there were no significant differences of antibody positive rate among the other locations ($P > 0.05$).

Antigen detection

There were 49 BVDV antigen positive samples. The antigen positive rate was from 1.11% to 4.44% with an average of 2.59%. The antigen positive rates in Pingluo, Dawukou, Helan, Yinchuan, Lingwu, Qingtonxia, and Wuzhong were 1.11% (3/270), 2.59% (7/270), 1.11% (3/270), 85% (5/270), 3.70% (10/270), 3.33% (9/270), and 4.44% (12/270), respectively. The

Table 1. The main subtypes of BVDV from different cities of Ningxia, China.

Cities	Name	Subtypes of BVDV	Number of BVDV subtypes
Pingluo	PL-1	BVDV-1m	
	PL-2	BVDV-1m	1
	PL-3	BVDV-1m	
Dawukou	DWK-1	BVDV-1m	
	DWK-2	BVDV-1m	
	DWK-3	BVDV-1m	
	DWK-4	BVDV-2a	2
	DWK-5	BVDV-2a	
	DWK-6	BVDV-2a	
	DWK-7	BVDV-1m	
Helan	HL-1	BVDV-2a	
	HL-2	BVDV-2a	1
	HL-3	BVDV-2a	
Yinchuan	YC-1	BVDV-1m	
	YC-2	BVDV-1m	
	YC-3	BVDV-1m	3
	YC-4	BVDV-1a	
	YC-5	BVDV-1q	
Lingwu	LW-1	BVDV-1d	
	LW-2	BVDV-1d	
	LW-3	BVDV-1d	
	LW-4	BVDV-1d	
	LW-5	BVDV-1d	1
	LW-6	BVDV-1d	
	LW-7	BVDV-1d	
	LW-8	BVDV-1d	
	LW-9	BVDV-1d	
	LW-10	BVDV-1d	
Qingtongxia	QTX-1	BVDV-1a	
	QTX-2	BVDV-1m	
	QTX-3	BVDV-1d	
	QTX-4	BVDV-1d	
	QTX-5	BVDV-1d	4
	QTX-6	BVDV-1q	
	QTX-7	BVDV-1m	
	QTX-8	BVDV-1q	
	QTX-9	BVDV-1q	
Wuzhong	WZ-1	BVDV-1d	
	WZ-2	BVDV-1d	
	WZ-3	BVDV-1d	
	WZ-4	BVDV-1a	
	WZ-5	BVDV-1a	
	WZ-6	BVDV-1d	2
	WZ-7	BVDV-1d	
	WZ-8	BVDV-1d	
	WZ-9	BVDV-1d	
	WZ-10	BVDV-1d	
	WZ-11	BVDV-1d	
	WZ-12	BVDV-1d	

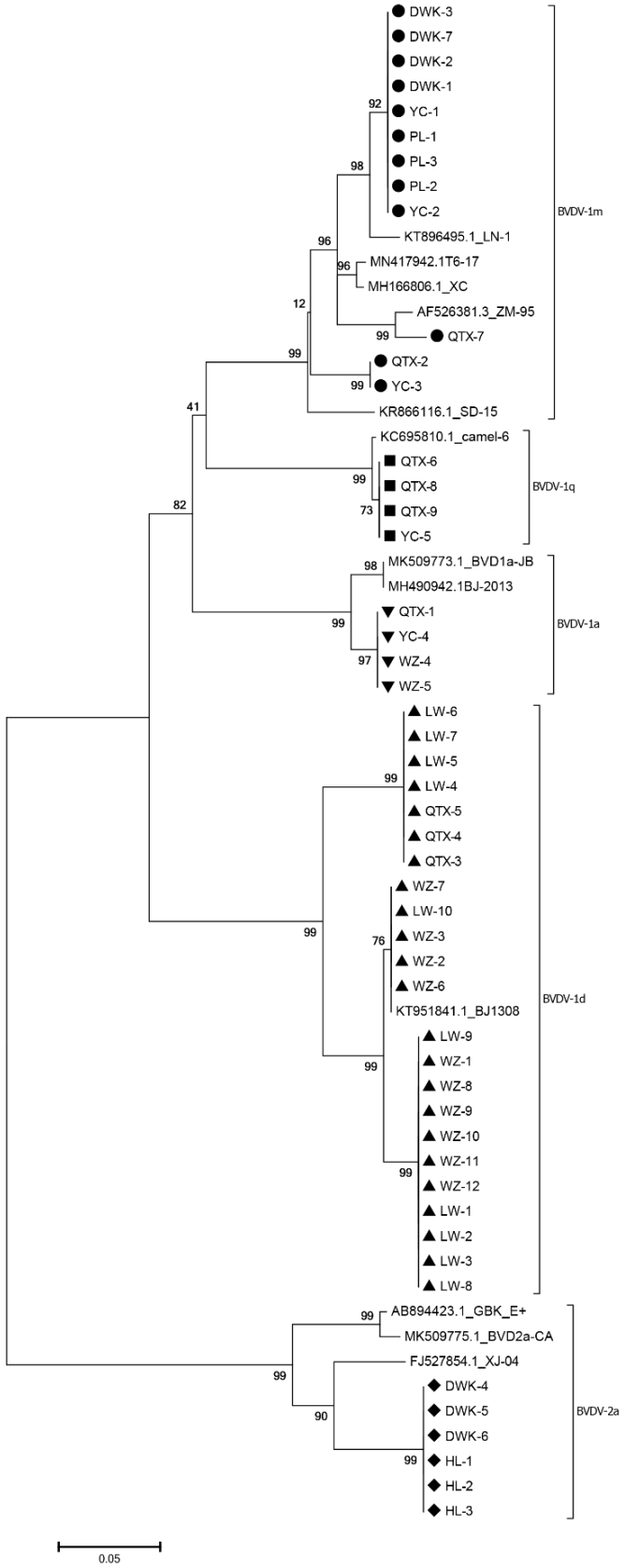

Figure 1. Phylogenetic tree of N-gene.

antigen positive rate in Wuzhong was the highest one while the antigen positive rates in Pingluo and Helan were the lowest. The results showed that there were significant differences of antigen positive rates in Pingluo and Helan comparing to that in Lingwu and Wuzhong ($P < 0.05$). The names of 49 antigen positive samples were listed in Table 1.

Genetic analysis of BVDV

A phylogenetic tree was constructed according to the sequence of BVDV-1 and BVDV-2 subtypes documented in GenBank by analyzing the obtained N-gene (Figure 1). The main subtypes of BVDV in dairy farms in Ningxia, China include BVDV-1a, BVDV-1d, BVDV-1m, BVDV-1q, and BVDV-2a.

Discussion

There was a large variation between different strains of BVDV, but there was no difference in serotypes. The host of persistent infection was not only an important source of infection of BVDV by excreting a large amount of toxins throughout the life and posing a serious threat to other animals, but also the main source of virus mutation. Therefore, the identification and elimination of persistently infected cattle was of great significance in the prevention and control of BVD.

This study evaluated BVDV infection in intensive dairy farming sites located in 7 different cities. Totally 1,890 samples were screened by BVDV antibody and antigen ELISA tests. The antibody positive rate was from 88.15% to 100% with an average of 92.59%. However, the antigen positive rate was from 1.11% to 4.44% with an average of 2.59%. The highest antibody positive rate was found in Wuzhong with the very significant antibody positive rate difference comparing to that in Pingluo, Dawukou, Helan, Yinchuan, Lingwu, and Qingtognxia. However, there was no significant difference of antibody positive rate among the other 6 cities. The antigen positive rate in Wuzhong was the highest one (4.44%) while the antigen positive rate in Pingluo and Helan were the lowest (1.11%). There were significant differences of antigen positive rates in Pingluo and Helan comparing to that in Lingwu and Wuzhong ($P < 0.05$), which were in accordance with the results found by Guo, *et al*. [13] and indicated that antibody test might no longer reflect the epidemic situation of BVDV. Antigen test to diagnose and eliminate the disease was the key to prevent and treat the disease.

BVDV genotyping is a test that can directly reflect the epidemic situation in a certain area. Initially, it is divided into different genotypes according to the antigenic difference between the strains. Later, the researchers found that this antigenic difference of the BVDV strain had a certain correlation with difference of the genome. At present, it can be divided into three genotypes, namely BVDV-1, BVDV-2, and BVDV-3. Each subtype is further divided into different genotypes [4, 8]. The main BVDV subtypes isolated and identified from dairy farms in Ningxia, China through this study included BVDV-1a, BVDV-1d, BVDV-1m, BVDV-1q, and BVDV-2a. The subtypes obtained were consistent with the popular BVDV subtypes in China. However, this study identified BVDV-1a, BVDV-1m, and BVDV-2a strains in Ningxia for the first time, indicating a more complex BVDV genetic diversity in Ningxia, China.

Currently, BVDV-1m is the main BVDV subtype that is prevalent in cattle and pig herds in China [14, 15]. It has also been reported in sheep flocks in Jiangsu Province, China [16] and Bactrian camels in Northwest of China [17]. However, few genetic information of this subtype strain was related to Ningxia. Homologous recombination plays an important role in the evolution of RNA viruses, which is conducive to repair viral genome damage and increase the adaptability of the virus to the host and the environment. Studies have shown that BVDV has genetic recombination of different genotype strains [18, 19]. However, no information about the same subtype of BVDV recombination was reported. In this study, the N-

gene of the type 2 strain was highly consistent with the Xinjiang 2004 strain (XJ-04) in China and could be subdivided into BVDV-2a gene subtypes. It is worth to note that there is a big difference between the type-2 strain and the antigen gene E2 of the existing vaccine strains in China. Therefore, in addition to formulating BVD vaccine immune roots in high-yielding dairy cow bases, attention should be paid to the identification and purification of type-2 strains that continue to infect cattle.

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