



# Prevalence of bovine viral diarrhea virus in dairy cattle herds in eastern China

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## Abstract

Bovine viral diarrhea virus (BVDV) is a worldwide spreading pestivirus affecting cattle and other ruminants; however, there have been few reports on epidemiologic investigation of BVDV in eastern China. In this study, bulk tank milk from 36 herds of dairy cattle in eastern China was submitted to serological investigations, 77.8% of herds was BVDV antibody positive. Individual animal status in two herds was further investigated collecting blood samples, the positive ratio was 49.74% and 24.64%, and the average positive ratio of calves, heifers, and lactating cows was 15.94%, 40.16%, and 41.7%, respectively. Moreover, clinical survey was carried out among 8170 dairy cattle from 36 herds, for diarrhea syndrome, respiratory problems and reproductive failure, and pathogens of all clinical cattle were further investigated. The results showed that BVDV was one of the main pathogen, which infected animals combining with various other viruses. Then, nine BVDV strains were isolated; phylogenetic analysis showed that BVDV subtypes currently circulating in eastern China were BVDV 1a and BVDV 1c. In addition, out of 377 cows tested, the 1.86% detected positive to the BVDV antigen. This study provided the foundation of further study on vaccination and control strategies of BVDV in eastern China.

**Keywords** Bovine viral diarrhea · Epidemiological investigation · Virus isolation · Genetic typing · Phylogenetic analysis · Persistent infection

## Introduction

Bovine viral diarrhea virus (BVDV) is a major pathogen causing bovine viral diarrhea (BVD) among cattle and other ruminants, leading to significant economic loss throughout the world (Brodersen 2014). BVDV is an enveloped, positive strand RNA virus, which belongs to the genus *Pestivirus* of

the family *Flaviviridae*. Two BVDV biotypes are distinguished: cytopathogenic (CP) and noncytopathogenic (NCP) viruses, according to their respective effects on cell culture (Ammari et al. 2010). NCP is the most common naturally occurring biotype, but cytopathogenic effects on cultured cells do not relate to virulence in vivo (Peterhans et al. 2010). The occurrence of CP BVDV in cattle persistently infected with NCP BVDV is directly correlated with induction of lethal mucosal disease (Hilbe et al. 2013; Kuwabara et al. 2007; Walz et al. 2010). In addition, BVDV is genetically divided into three types: type 1 (BVDV-1), type 2 (BVDV-2), HoBi-like viruses (BVDV-3), or “atypical pestiviruses”, and it is noted that only BVDV-1 and BVDV-2 are recognized species, and Hobi-like is a tentative species (Bauermann et al. 2013; Giammarioli et al. 2015b; Stahl et al. 2010). Recently, the virus isolates have been further grouped into different subgenotypes by phylogenetic analysis (Abe et al. 2016; Gomez-Romero et al. 2017; Otonel et al. 2014; Yesilbag et al. 2017; Yilmaz et al. 2012). So far, 21 subgenotypes (BVDV-1a to BVDV-1u) and BVDV-2 subtypes (2a-2d) have been reported worldwide (Deng et al. 2015; Giammarioli et al. 2015a; Xue et al. 2010; Yesilbag et al. 2014).

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BVDV is worldwide distributed, and its seroprevalence in cattle varies among countries. In Ethiopia, seroreaction to BVDV antigens was detected in 32.6% of the 1379 cattle and 69.8% of the 149 herds sampled (Aragaw et al. 2018). Within herd of Tamil Nadu, BVDV seroprevalence was 12–65% (Kumar et al. 2018). In suburb of Mashhad-Iran, the seroprevalence of BVDV infection in industrial dairy cattle herds was 72.25% (Talebkhani Garoussi et al. 2009). In Ireland, BVDV infection was detected in 98.7% of non-vaccinated herds (Cowley et al. 2012). In addition, BVDV-1b and BVDV-1e were the most prevalent subtypes circulating in Italy, whereas the BVDV-1a and BVDV-2a subtypes were predominant and widespread in Korea (Bazzocchi et al. 2017; Han et al. 2018). The prevalence of persistently infected cattle from selected, nonrandom sample in six large South African feedlots was 2.9% (Meiring et al. 2011), while prevalence of PI calves in Japan in a dairy herd was estimated about 7.0%, which was very high ratio compared to that in previous reports (Helal et al. 2012). In China, the pooled BVDV prevalence in dairy cattle in China was up to 53.0% according to the data obtained from systematic review and meta-analysis (Ran et al. 2018). Furthermore, the BVDV subtypes currently circulating in China were various (1a, 1b, 1c, 1d, 1m, 1o, 1p, and 1q) (Xue et al. 2010). However, previous studies are mainly conducted in northern and western China; it is less known about prevalence of BVDV and PI animal status among dairy cattle in eastern China recently.

In this study, bulk tank milk and blood samples were analyzed by blocking ELISA for monitoring the herd and individual infection status, and clinical manifestations were surveyed for the causative pathogens; then, viruses were isolated, prevalence of PI with BVDV was further investigated, and phylogenetic analysis was performed to identify the circulating BVDV subtypes currently. Collectively, this study has a better understanding of the epidemic of BVDV among dairy cattle in eastern China.

## Materials and methods

### Study area and sample collection

The study was performed on 36 dairy herds with size ranging from 30 to 700 animals in 5 provinces in eastern China (Shandong, Anhui, Hebei, Jiangsu, and Fujian) (Table 1). These herds were not vaccinated against BVDV. Generally, the age group “calf” was defined as  $\leq 12$  months old, while “lactating cows” were defined as  $> 30$  months old, according to the management adopted in the herds.

Bulk tank milk samples were collected from 36 dairy herds and centrifuged to be skimmed at  $1000\times g$ , 15 min  $4^{\circ}\text{C}$ . Three different layers appeared in the tube after the centrifugation, a middle layer corresponding to milk serum samples was

collected, and stored at  $-20^{\circ}\text{C}$  in sterile plastic tubes until its analysis with BVD/MD P80-ELISA test kit (LABORATORIOS HIPRA S.A. Spain).

To further investigate the individual status of herds identified as BVDV positive by analysis of bulk tank milk samples, 402 peripheral blood samples were collected from the two herds (herd number 4 and herd number 9) by jugular or coccygeal vein puncture. Samples were centrifuged at  $3000\times g$  for 10 min to obtain the sera and stored at  $-20^{\circ}\text{C}$  for further analysis. Then, the antibody against BVDV was detected with BVD/MD P80-ELISA test kit.

### Clinical surveys

Clinical manifestations were surveyed among 8170 individuals including calves, heifers, and lactating cows from 36 dairy cattle herds. Depression, anorexia, rapid respiration, excessive lacrimation, excessive nasal secretion, sialorrhea, and watery diarrhea were recorded. The reproductive disease was checked at the stage of gestation. The cattle with severe disease were dissected; their tissue samples were taken and stored at  $-70^{\circ}\text{C}$  for RT-PCR and virus isolation.

### RT-PCR and PCR

Total RNA and/or DNA was extracted from clinical samples or cell cultures using Viral RNA/DNA Extraction Kit Ver.4.0 (TaKaRa, Japan) as described by the manufacturer's instructions. For RNA viruses, cDNA was synthesized from 500 ng of total RNA using RNA reverse transcription kit. The PCR primer pairs used for detection of BVDV, infectious bovine rhinotracheitis virus (IBRV), bovine parainfluenza virus type 3 (BPIV3), bovine rotavirus (BRV), bovine coronavirus (BCoV), and bovine enterovirus (BEV) were previously described (Hou et al. 2018). To assess whether BVDV positive dairy cattle were PI animals, blood samples were collected again after 3 weeks and detected repeatedly with RT-PCR; it would be considered as the PI cattle once BVDV gene detection was positive.

### Cell culture and virus isolation

Madin-Darby bovine kidney (MDBK) cell culture and virus isolation used in this study were performed following the Agricultural Industry Criteria of the People's Republic of China (publication no. SN/T1129.2–2002). In brief, the feces and nasal swabs were collected and put into a tube containing 2 mL PBS (HyClone, USA) with 100 Units penicillin and streptomycin ( $100\text{ }\mu\text{g/mL}$ ). Some of the tissue samples (lung, kidney, spleen, intestinal canal, and aborted fetus) were prepared and treated by tissue homogenizer and supplemented with PBS. Blood samples were collected using an EDTA vacuum blood tube from the jugular vein. The collected tissue and

**Table 1** The BVDV prevalence of 36 herds in eastern China

Herd No.	Location	Size	%IN	Status	Herd No.	Location	Size	%IN	Status
1	Shandong	700	72.09549	P++	19	Anhui	377	86.6313	P++
2	Shandong	79	30.71618	N	20	Anhui	87	78.67374	P++
3	Shandong	168	66.04775	P++	21	Shandong	260	76.87003	P++
4	Anhui	195	89.82759	P++	22	Anhui	140	12.99735	N
5	Jiangsu	95	72.8382	P++	23	Shandong	261	84.50928	P++
6	Jiangsu	140	−39.3103	N	24	Jiangsu	176	60.84881	P++
7	Anhui	102	81.43236	P++	25	Fujian	186	80.90186	P++
8	Hebei	30	85.14589	P++	26	Fujian	140	63.92573	P++
9	Hebei	207	40.15915	P	27	Hebei	200	−5.99469	N
10	Hebei	306	85.35809	P++	28	Shandong	168	11.72414	N
11	Jiangsu	148	75.17241	P++	29	Shandong	182	82.81167	P++
12	Fujian	121	78.35544	P++	30	Shandong	326	83.23607	P++
13	Anhui	209	81.43236	P++	31	Shandong	525	13.74005	N
14	Fujian	119	79.31034	P++	32	Hebei	670	78.14324	P++
15	Anhui	68	−15.5438	N	33	Anhui	250	78.88594	P++
16	Hebei	323	76.12732	P++	34	Shandong	95	84.40318	P++
17	Shandong	102	68.48806	P++	35	Shandong	342	79.41645	P++
18	Shandong	86	26.68435	N	36	Jiangsu	587	51.51194	P

For tank of milk sample, according to the manufacturer, %IN value lower than 35 is negative (labeled N in the table) with prevalence of positive animals < 10%; %IN value higher or equal to 35 and lower than 60 indicates prevalence of positive animals between 10% and 30% (labeled P). At %IN value higher or equal to 60, prevalence of positive animals takes possession of more than 30% (labeled P++)

blood samples were repeatedly frozen and thawed for three times, and then, they were inoculated onto the MDBK cell monolayer for virus culture. The culture plates were incubated at 37 °C, with 5% CO<sub>2</sub> for 2 h. After centrifugation, the supernatants were discarded, and plates were rinsed twice with PBS (pH 7.2, 0.01 mol/L), and 1 mL DMEM (HyClone, USA) with 2% horse serum (Gibco, USA) was added. The infected MDBK cells were checked daily and appearance of cytopathic effects (CPE) was observed and recorded. If the CPE was not found, the cultures were frozen and thawed twice and the clarified supernatant was passaged five times in MDBK cells. Meanwhile, uninfected MDBK were included as negative controls.

### Electron microscopy

A total of 200-mL viral cell culture supernatant was harvested and stained with phosphotungstic acid (PTA), blotted dry, and the viruses were observed under transmission electron microscope (TEM, Tecnai G2 20ST) as previously described (Zheng et al. 2017).

### Phylogenetic analysis

For each isolated virus, 289 bp fragment of the 5'-UTR regions was amplified and sequenced. Sequences of other

strains were obtained from GenBank. Evolutionary analyses were conducted in MEGA version 5 (Tamura et al. 2011). The evolutionary history was inferred using the neighbor-joining method (Sun et al. 2012; He et al. 2016). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (50 replicates) is shown next to the branches. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site.

### Statistical analysis

All data were managed in Microsoft Excel spreadsheets. A Chi-square test was used to test the significance of overall seroprevalence and regional differences, with  $p < 0.05$  as the minimum level for statistical significance.

## Results

In order to investigate the prevalence of BVDV, 36 dairy cattle herds distributed in 5 provinces (Shandong, Anhui, Hebei, Jiangsu, and Fujian) in eastern China were selected since these areas were historically the major regions with dairy cattle production and trade. Firstly, we analyzed bulk tank milk samples from 36 dairy cattle herds with BVD/BD p80 blocking

ELISA. The results showed that 28 samples were positive (77.78%) (Table 1). Since no vaccine was used in these herds, it meant that there was a high likelihood of infection among herds in eastern China.

We next investigated seroprevalence of BVDV in individual status of two cattle farms (herd number 4 and herd number 9) with higher inhibitory rates (the higher prevalence of anti-BVDV antibodies). Blood samples from 402 dairy cattle in two herds were analyzed. A total of 49.74% of animals in herd number 4 and 24.64% of dairy cattle in herd number 9 were positive, and the average positive rate in two herds was 36.82% (Fig. 1a). Furthermore, the positive rate of BVDV infection of calves, heifers, and lactating cows was 15.94%, 40.16%, and 41.7%, respectively (Fig. 1b), and the rate of heifers and lactating cows was significantly higher than that of calves ( $p < 0.05$ ).

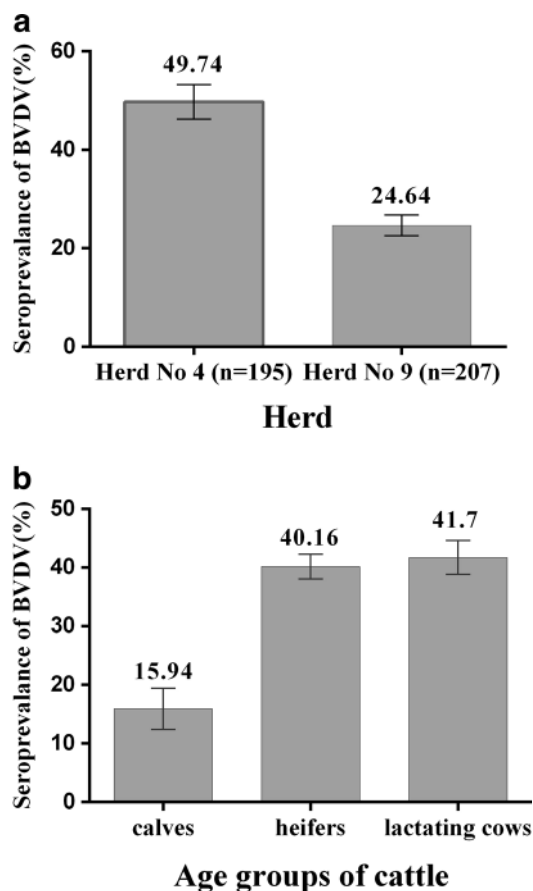
Clinical manifestations were investigated among 8170 individuals; there were 650, 407, and 122 dairy cattle with diarrhea syndrome, respiratory problems, and reproductive failure, respectively. The ratios of these diseases were 7.96%,

4.98%, and 1.49% (Fig. 2a). Viruses of gastrointestinal and respiratory diseases from all clinical manifestations were detected by PCR or RT-PCR (Hou et al. 2017). The ratio of BVDV infection alone was 6.5%, whereas the ratio of BVDV infection combined with BEV, IBRV, IBRV/BPIV-3, or BEV/BCov/BRV was 12.82%, 15.38%, 10.26%, and 2.5%, respectively (Fig. 2b). So, BVDV was the main pathogen, which infected most animals combined with other viruses.

Isolation of BVDV from RT-PCR positive clinical samples was performed on MDBK cells and identified after five passages. Nine BVDV strains were isolated, and named as 103, M7, 40, 457, TR-BP1, 1025, JPF, 0840, and JN1201, respectively. Only 457 generated obvious cell lesion and net among monolayer cells; the others had no cytopathic effect (Fig. 3a). The isolates were identified with RT-PCR (Fig. 3c) and followed sequencing analysis confirmation. Virus particles were further observed under the electron microscopy. The negatively stained virus displayed a typical BVDV morphology (Fig. 3b). The details including source, biotype, and genotype are shown in Table 2.

The 289 bp fragment of the 5'-UTR regions of each isolated virus was amplified and sequenced. Evolutionary analyses conducted in MEGA5 based on the sequence of the 5'-UTR showed that eight BVDV strains were BVDV-1, and BVDV 1a and 1c were the dominant genotypes. In addition, one BVDV-2 strain was isolated and identified (Fig. 4).

Blood samples were collected from 402 dairy cattle, and the results of PI cows screening by RT-PCR demonstrated that there were 43 animals infected with BVDV at the first time, the infective ratio was 10.7%. Blood samples from 43 positive animals were taken again 3 weeks later. Repeated detection of BVDV antigen with RT-PCR indicated that eight dairy cattle were finally considered as PI animals, the ratio of PI was 1.86%. All PI dairy cattle were eradicated from the herd.



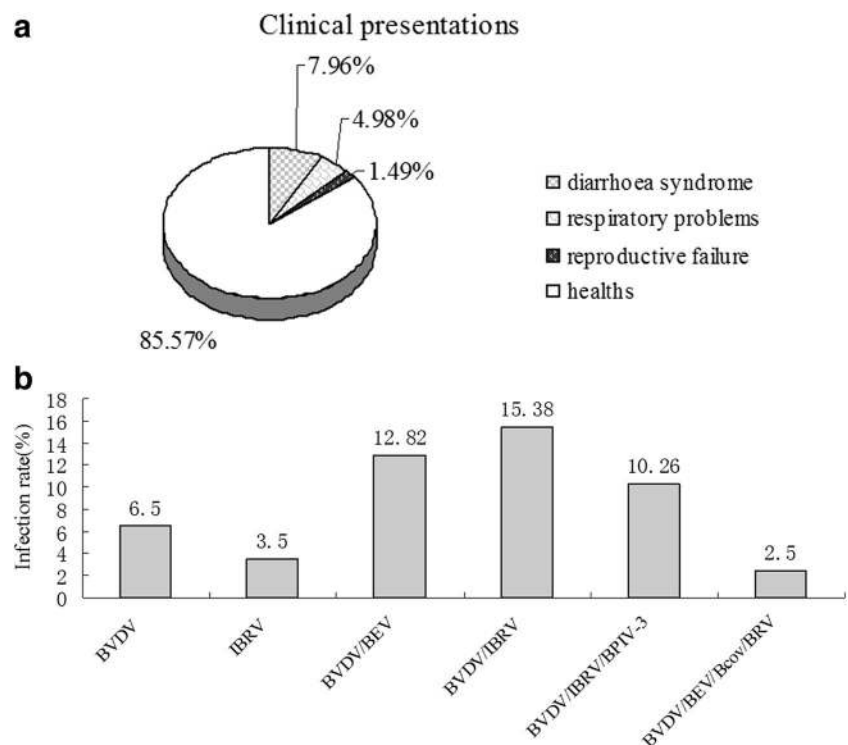
**Fig. 1** The seroprevalence of BVDV in individual animals. A total of 402 blood samples were collected from herd #4 and herd #19 including calves, heifers, and lactating cows, and antibody against BVDV was detected by BVD/MD P80-ELISA test kit. The seroprevalence of BVDV in two herds was analyzed (a), and rate of calves, heifers, and lactating cows was statistically analyzed, respectively (b)

## Discussion

BVDV infection has a global distribution and affects animal health and reproductive performance, resulting in significant economic losses. Recently, the prevalence study of BVDV in cows has been determined worldwide (Amelung et al. 2018; Aragaw et al. 2018; Evans et al. 2018; Khodakaram-Tafti and Farjanikish 2017; Kumar et al. 2018; Ochirkhuu et al. 2016). In China, the disease was firstly reported in the 1980s of the last century (Bachofen et al. 2013; Fulton 2009), and most of the investigations concerning BVDV infection were seldom published internationally (Deng et al. 2015). What is more, there was no further epidemiologic report on BVDV in detail from eastern China. Systematic epidemiologic investigation and genetic typing of BVDV in China will be useful for studies on vaccine and control strategies.



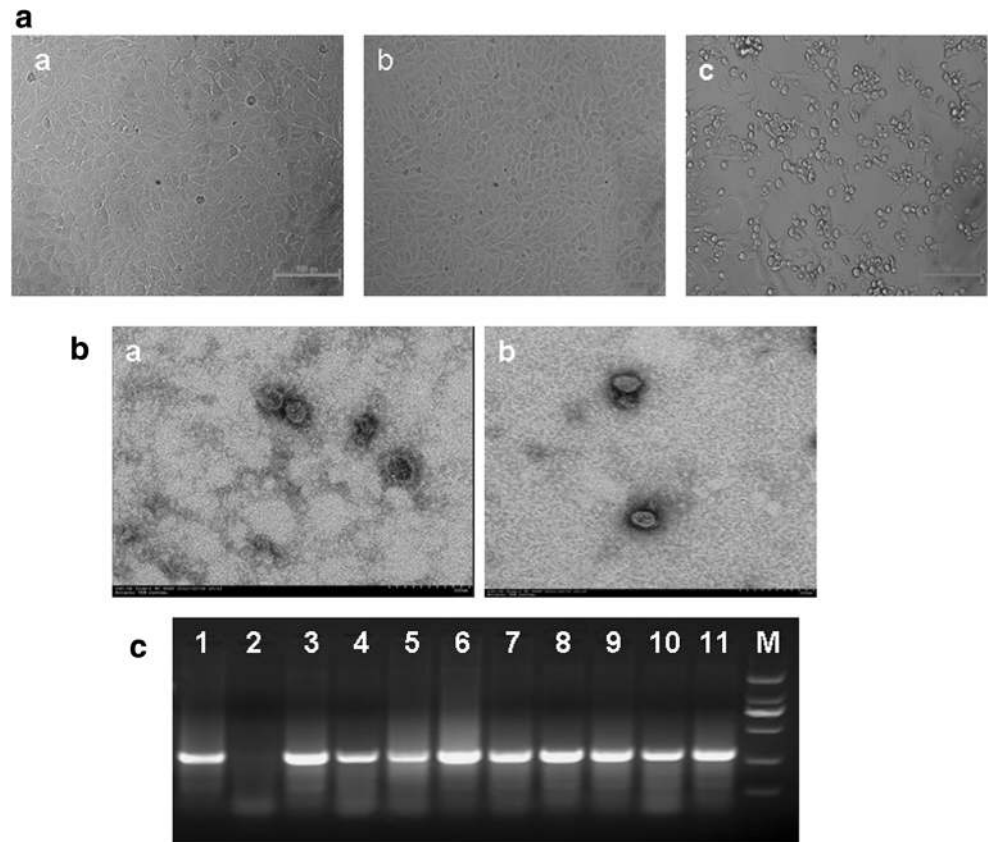
**Fig. 2** Clinical survey and pathogen analysis. Clinical manifestations among 8170 dairy cattle, including diarrhea syndrome, respiratory problems, and reproductive failure, were investigated. The ratios of these diseases were calculated (a). Viruses of gastrointestinal and respiratory diseases in blood samples from all clinical cattle were detected by PCR or RT-PCR. The ratio of each or combined pathogens was statistically analyzed (b)



In this study, bulk milk from 36 dairy cattle herds, which have no BVDV vaccination history and widely distributed in eastern China, was used to investigate epidemic of BVDV, as

detection of BVDV antibody of bulk milk was a fast and easy method for the investigation of BVDV dairy cattle herds (Hanon et al. 2017; Lanyon et al. 2014a, b; Velasova et al.

**Fig. 3** Isolation and identification of BVDV. Total nine strains were isolated, only SD-926 isolate generated cytopathic effects, the others had no cytopathic effect on cell (a), a: the MDBK cell as negative control, b: the NCP of SD-TR strain; c: the CPE of SD-926 strain. The isolate cultures were further checked under the electron microscopy, the negatively stained virus particles were 40–60 nm in diameter, and displayed a typical BVDV morphology (b), d: the NCP of SD-TR strain; e: the CPE of SD-926 strain. The isolates were finally confirmed by RT-PCR and sequencing analysis, agarose gel electrophoresis of PCR products was run following amplification of cDNA of BVDV (c), lane M: weight size marker (2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp), lane 1: positive control; lane 2: negative control, lanes 3–11: isolate SD-TR, SD-40, SD-0957, SD-103, SD-JPF, SDM7, SD-1037, SD0840, and SD926, respectively



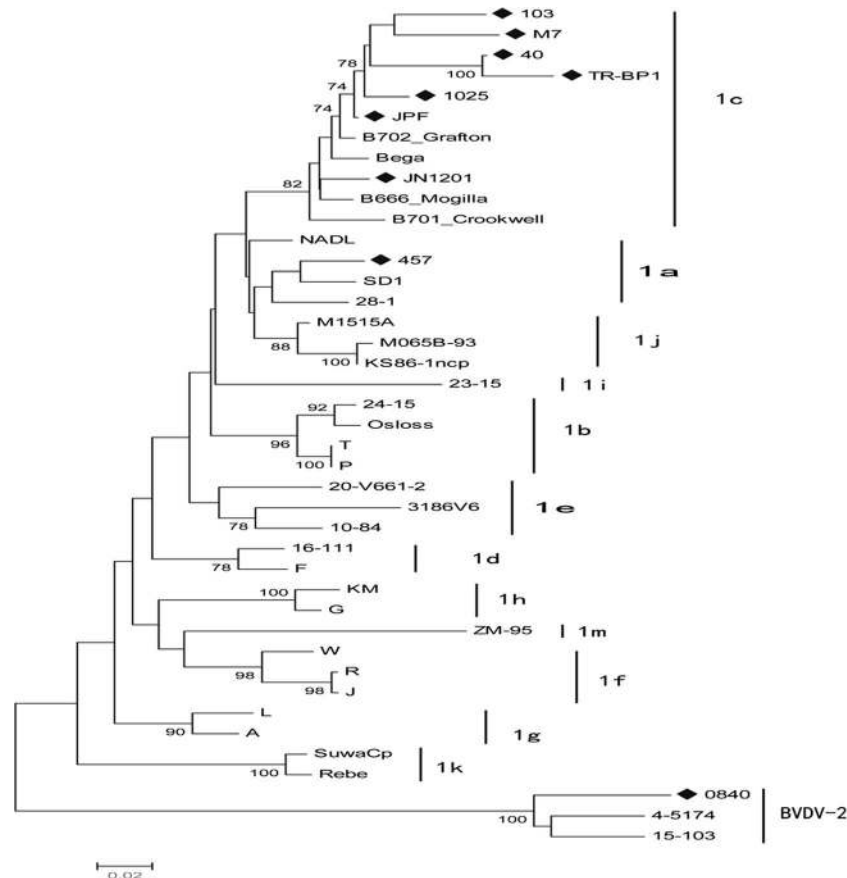
**Table 2** Biotype and subgenotype distribution of BVDV isolates

Name	Sample	Herd	Biotype	genotype
103	Nasal swabs	#4	NCP	BVDV-1c
M7	Feces samples of diarrhea cattle	#9	NCP	BVDV-1c
40	Kidney tissue samples	#12	NCP	BVDV-1c
TR-BP1	Feces samples of diarrhea cattle	#17	NCP	BVDV-1c
1025	Blood samples of respiratory problems cattle	#19	NCP	BVDV-1c
JPF	Intestinal canal tissue samples	#20	NCP	BVDV-1c
JN1201	Lung tissue samples	#9	NCP	BVDV-1c
0840	Aborted fetus	#29	NCP	BVDV-2
457	Feces samples of diarrhea cattle	#34	CP	BVDV-1a

2017). Among representative samples from dairy cattle herds, 77.78% of herds had infection of BVDV (Table 1). Moreover, the result of bulk milk will be negative according to Ab-ELISA manufacture's test procedure since prevalence of positive animals in one herd is less than 10%, so prevalence of BVDV positive herds should be more than 77.78%; this

implied that BVDV may widely spread in eastern China. Combined with the data from status of individual animals of two herds (Fig. 1b), it appeared that there was universal infection within one herd.

It is noteworthy that, although the positive rate is high, the majority of BVDV positive cases do not manifest clinically



**Fig. 4** Phylogenetic analysis of BVDV. Evolutionary analyses were conducted in MEGA5. The evolutionary history was inferred using the neighbor-joining method. The sequences of 103, M7, 40, TR-BP1, 1025, JPF, JN1201, 0840, and 457 isolates were determined in this study; sequences of other strains were obtained from GenBank. GenBank Accession number: NADL,M31182; SD1,M96751; 28-1,AF298061; Osloss,M96687; 24-15,AF298060; P,AF298070; T,AF298072; Bega,AF049221; B666 Mogilla, JQ743605; B701

Crookwell,JQ743606;B702; Grafton,JQ743607; 16-111,AF298056; F,AF298065; 10-84,AF298054; 20-V661-2,AF298058; 3186V6,AF298062; J,AF298067; R,AF298071; W,AF298073; A,AF298064; L,AF298069; G,AF298066; KW,AF298068; 23-15,AF298059; KS861-ncp, AB078950; M1515A,U97429; M065B,U97409; SuwaCp,AF117699; Rebe,AF299317; ZM-95,AF526381; 890,U18059; 15-103,AF298055; 4-5174,AF298063

apparent symptoms. In this study, based on statistical analysis of clinical cases among 8170 individuals from 36 dairy cattle herds, diarrhea syndrome took the largest proportion, and reproductive failure was infrequent (Fig. 2a). The pathogens showed that BVDV was the main pathogen, which infected dairy cattle combined with other viruses (Fig. 2b). The BVDV can induce immunosuppression, so BVDV infecting animals easily facilitate secondary infections by other pathogens (Peterhans and Schweizer 2013; Alkheraif et al. 2017; Darweesh et al. 2018).

Virus isolation in this study was performed because it is commonly considered to be the gold standard for diagnosis of BVDV (Lanyon et al. 2014a, b), and eight NCP and BVDV-1 strains as well as one CP and BVDV-2 strain were successfully isolated. Reportedly, the worst part of BVD disease in cattle is the persistence of infection caused by NCP biotype of BVDV, and our results further confirmed that most of BVDV field isolates from China were NCP. Moreover, it was showed that BVDV-1a, BVDV-1c, as well as BVDV-2 were circulating in eastern China based on the sequence of highly conserved 5'-UTR (Fig. 4). This study was potentially provided with additional background on the genetic heterogeneity that all BVDV isolates from cattle in China between 2005 and 2008 were of the BVDV type 1 genotypes (Xue et al. 2010). In addition, BVDV-2 genotype does exist in dairy herd although BVDV-1 was still main genotype in eastern China.

PI calves are probably one of the most important means by which BVDV infection can be maintained in dairy cattle herds (Wang et al. 2011; Amelung et al. 2018). Classically, the prevalence of PI animals ranges between 0.5% and 2% (Stott et al. 2012). In this study, we found that the ratio of PI animals was 1.86% among 377 cows, which indicates that the herds containing PI animals are at higher risk of BVDV infection in eastern China. Therefore, as the identification of potential source of contamination in dairy cattle herds has been of increasing concern, epidemiological studies and eradication or control programs may be considered essential for removal of persistently infected cattle in some regions.

Taken together, we determined the prevalence of BVDV in eastern China and found that BVDV was widely spread in herds, and NCP and BVDV1a and BVDV-1c were the dominant biotype and genotype of isolates. Moreover, high ratio of PI animals increased the risk of disease within feedlots. This study will lay the foundation of further research on vaccine and control strategies of BVDV in eastern China. However, the survey has been performed on a very limited number of samples in some cattle farms; systematic epidemiologic investigation of BVDV on a substantial number of samples in China is required.

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## Compliance with ethical standards

**Statement of animal rights** Experimental protocols for obtaining cattle acute-phase blood and tissue samples used in this study were carried out in strict accordance with China law on use of animal on research and all procedures were approved by the Shandong Normal University Animal Care and Use Committee.

**Conflict of interest** The authors declare that they have no conflict of interest.

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