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Research Article

Novel Subgenotypes of Bovine Viral Diarrhea Virus based on 5' UTR Molecular Epidemiology in Cattle from Huhhot of Inner Mongolia Autonomous Region, China

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Abstract

Bovine viral diarrhea (BVD) causes high economic losses in the cattle population worldwide. Here, we present the results of an epidemiological survey for Bovine viral diarrhea virus (BVDV) in Northwest of China. In this study, a total of 167 samples were collected from the bovine farms and cattle slaughters for epidemiology nearby Huhhot in Inner Mongolia Autonomous Region during 2017 and 2018. Positive BVDV isolates were genotyped based on a comparison of gene sequences from their 5'untranslated regions (5'UTR). Results indicated that, out of 167 samples, 68 (40.72%) were BVDV-RNA-positive. 28 of them were sequenced and analyzed with 5'UTR and used for constructing phylogenetic tree. Phylogenetic analysis based on 5'UTR revealed that13 of the BVDV isolates belong to BVDV-1 and 15 belong to BVDV-2 genotype. Interesting, two novel BVDV-1 subgenotypes and one novel BVDV-2 subgenotype were found in present study. Therefore, the result of this study will be useful to understand epidemiology in Inner Mongolia Autonomous Region and allow producers to better protect their livestock.

Keywords: Bovine viral diarrhea viruses, Epidemiology, 5'UTR, Novel Genotype

Importance:

An epidemiological survey indicated two novel BVDV-1 subgenotypes and one novel BVDV-2 subgenotypewere found based on 5'UTR phylogenetic tree in Northwest of China.

Introduction

BVDV can cause bovine respiratory, gastroenteric and reproductive clinical consequences, and still lead to the birth of immunotolerant. It is the main viral infectious disease of cattle, which is a worldwide epidemic disease. Bovine viral diarrhea virus (BVDV) is a member of the genus Pestivirus. According to the genetic characteristics of the genome, BVDV is divided into two genotypes, BVDV-1 and BVDV-2. At present, BVDV-1 strains have further been divided into 21 subtypes (1a-1u) (Tajima et al., 2001; Vilcek et al., 2001; Yamamoto et al., 2008; Gong et al., 2013; Deng M. et al., 2015). To date, the BVDV circulating in the Chinese cattle population is mainly BVDV-1b, -1c, -1m, -1p (Zhong et al., 2011). According to the difference of secondary structure of 5' -UTR sequence, BVDV-2 is divided into 4 subtypes, BVDV-2 a, 2 b, 2 c and 2 d (Giangaspero et al., 2008).

Bovine viral diarrhea virus infection in pregnant animals can also result in the birth of a persistently infected (PI) calf. PI animals are the main source of virus transmission to susceptible animals. It is an obstacle for the elimination of the virus in a susceptible population. The combined economic impact of BVDV has been estimated at a 20 to 57 million dollar loss per million calving's in the USA. It also caused a huge economic loss from BVDV in China. 5'-UTR, Npro, and E2 genes were also used for genetic typing of the pestiviruses (Vilcek et al., 2001; Vijayaraghavan et al., 2012).

Variability of BVDV is distinct. A growing number of BVDV-1 and BVDV-2 subgenotypes based on phylogenetic analysis indicated the BVDV is genetically highly heterogeneous. The highest number of various BVDV subgenotypes has been documented in European countries (Yesilbag et al., 2017). Different genomic regions, i.e., 5'UTR (Beer et al., 2002; Becher et al., 2003; O'Brien et al., 2017), Npro (Maya et al., 2016; O'Brien et al., 2017), E2 (Couvreur et al., 2002; Yilmaz et al., 2012), have been used for genotyping and classification of BVDV. Partial 5'UTR sequences have been most frequently used for phylogenetic analyses and

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genotyping of BVDV isolates. A 288bp size of 5'UTR was used for epidemiology of BVDV in present study.

The epidemiology and genetic variations analysis of BVDV can implicate in disease control as diagnostics and vaccines that work well against homologous strains can be less efficacious for genetically-distinct viruses.

Materials and methods

Sample collection and processing

The 167 whole blood, lung tissue and intestinal contents samples were collected from the cattle of six bovine slaughters and calf and bovine of 26 farms, which were healthy or showed minor respiratory disease, in Huhhot and its surrounding rural breeding area of Inner Mongolia Autonomous Region, China during 2017 and 2018. Blood samples were collected for diagnostic purposes by venipuncture into silicon-coated vacutainer tubes and were immediately transported to the laboratory and stored at -20^{O}C in tubes appropriate for freezing. Other tissue samples were homogenized in 2 ml of PBS and centrifuged at a 2,000×g for 3 min to remove the suspended solids. The supernatants were stored at −80^{O}C until testing.

RNA extraction and RT-PCR

Total RNA was extracted from whole blood or tissues using the TRIzol reagent (Life Technologies, U.S.) according to the manufacturer's instructions. Viral cDNA was constructed using reverse transcriptase (M-MLV, Invitrogen) with 10 μL of RNA. The polymerase chain reaction (PCR) amplification was performed with 5 μL of cDNA as the template using previous detection primers BVP1 (5'TAGCCATGCCCTTAGTAGGAC-3') and BVP2 (5'CTCCATGTGCCATGTACAGCA-3') (Vilcek et al., 2003) flank a 288-bp DNA fragment for BVDV 5'UTR with DNA polymerase Pfu (NEB, US). The PCR amplification was performed for 35 cycles: denaturation at 94°C for 30 s, annealed at 57°C for 30 s, and elongation at 72°C for 30 s. The samples were then incubated for an additional 10 min at 72°C and cooled to 4°C until further processing. Five microliters of the PCR products were analyzed on agarose gel (1.5%) electrophoresis at 120 V for 20 min.

Sequencing and Phylogenetic analysis of nucleotides

Twenty-eight isolates were randomly chosen from RNA positive samples for sequencing within the 5'UTR of the genome. The amplicons were purified using an Omega gel extraction kit (Omega, U.S.) and sequenced. These nucleotide sequences were assembled and proof read used the SeqMan program of Lasergene package (DNASTAR Inc., USA). For phylogenetic analysis, a total of 79 related reference sequences of 5'UTR (Listed in Table 1) were retrieved from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank) and field strains in present study were also included for comparison (28 strains). Some of reference strains are reported as classified subgenotypes. A phylogenetic tree was constructed by the UPGMA method with MEGA7.0. The evolutionary distances were computed using the Maximum Composite Likelihood method. The GenBank accession numbers for the sequences of 5'UTR genes of the 28 BVDV isolates in present paper are listed in Table 1.

Table 1: BVDV isolates and reference strains described in this study

No	Strain	Country - year of isolation	5'UTR reference	E2 reference	Genotype	Host
1	NM1	China-2017	MK204893		BVDV-1	Cattle
2	NM2	China-2017	MK204906		BVDV-2	Cattle
3	NM4	China-2017	MK204907		BVDV-2	Cattle
4	NM5	China-2017	MK204894		BVDV-1	Cattle
5	NM6	China-2017	MK204895		BVDV-1	Cattle
6	NM7	China-2017	MK204908		BVDV-2	Cattle
7	NM15	China-2017	MK204909		BVDV-2	Cattle
8	NM19	China-2017	MK204896		BVDV-1	Cattle
9	NM21	China-2017	MK204897		BVDV-1	Cattle
10	NM23	China-2017	MK204910		BVDV-2	Cattle
11	NM24	China-2017	MK204911		BVDV-2	Cattle
12	NM25	China-2017	MK204912		BVDV-2	Cattle
13	NM29	China-2017	MK204913		BVDV-2	Cattle
14	NM40	China-2017	MK204898		BVDV-1	Cattle
15	NM42	China-2017	MK204899		BVDV-1	Cattle
16	NM44	China-2017	MK204914		BVDV-2	Cattle
17	NM45	China-2017	MK204900		BVDV-1	Cattle
18	NM46	China-2017	MK204901		BVDV-1	Cattle
19	NM51	China-2017	MK204902		BVDV-1	Cattle
20	NM57	China-2017	MK204915		BVDV-2	Cattle
21	NM100	China-2017	MK204903		BVDV-1	Cattle
22	NM109	China-2017	MK204916		BVDV-2	Cattle
23	NM123	China-2017	MK204917		BVDV-2	Cattle
24	NM125	China-2017	MK204918		BVDV-2	Cattle

25	NM135	China-2017	MK204904		BVDV-1	Cattle
26	NM157	China-2017	MK204919		BVDV-2	Cattle
27	NMy1	China-2017	MK204920		BVDV-2	Cattle
28	810763	China-2018	MK204905		BVDV-1	Cattle
29	BVDV1/Serbia/2008	Serbia/2008	KY941186		BVDV-1	cattle
30	BVDV1/WB4/Serbia/2017	Serbia/2017	KY941185		BVDV-1	wild boar
31	04'-81	South Korea/2009	GQ985460		BVDV-1a	calf
32	UEL7-BR/11	Brazil/2011	KJ188147		BVDV-1a	Bovine
33	435FaUY/032014	Uruguay/2014	KT833794		BVDV-1a	Bovine
34	181	Argentina/2016	MF120592		BVDV-1b	fetal bovine serum
35	43865	South Korea/2009	GQ985459		BVDV-1b	calf
36	SC	China/2016	KX280711		BVDV-1b	calf
37	Bega-like	Australia/2012	KF896608		BVDV-1c	Bovine
38	Crookwell	Australia/1989	JQ743606		BVDV-1c	Bovine
39	Grafton	Australia/1989	JQ743607		BVDV-1c	Bovine
40	Mogilla	Australia/1987	JQ743605		BVDV-1c	Bovine
41	Bov_preto	Brazil/2015	KU564958		BVDV -1d	Bos taurus
42	fecal	China/2015	MF166858		BVDV -1d	yak
43	ELV_ca_10	Denmark/2010	JX966090		BVDV -1d	cattle
44	MSGOCAE110	Iraq/2016	MF347404		BVDV-1e	calf
45	MSKOCOE13	Iraq/2016	MF347405		BVDV-1e	Cow
46	CN5a@09	Italy/2016	KX766447		BVDV-1f	cattle
47	TO2a@11	Italy/2016	KX766452		BVDV-1f	cattle
48	MRI2569	South Korea/2006	LT902694		BVDV-1g	Bos taurus
49	48/08	Poland/2008	JN715036		BVDV-1g	cattle
50	L-AT	Austria/1998	FJ493483		BVDV-1g	cattle
51	BG9a@02	Italy/2002	MG434576		BVDV-1h	Bovine
52	UM/126/07	Italy/2007	LT631725		BVDV-1h	Bos taurus
53	436FaUY/052014	Uruguay/2014	KT833795		BVDV-1i	bovine
54	MRI2021	United Kingdom/2017	LT902249		BVDV-1i	Bos taurus
55	A469	Chile/2010	GU987129		BVDV-1j	Vicugna pacos
56	LL795	Chile/2010	GU987133		BVDV-1j	Lama glama
57	SuwaCp	Switzerland/1993	KC853441		BVDV-1k	Bovine
58	71-03	France/2005	KF205294		BVDV-1l	Bos taurus
59	TR84	Turkey/2012	MH753470		BVDV-1l	Bos taurus
60	XC	China/2015	MH166806		BVDV-1m	Bos taurus
61	KB01	South Korea/2007	GQ495676		BVDV-1n	Cattle
62	Shitara/02/06	Japan/2006	LC089876		BVDV-1n	Bos taurus
63	IS26/01ncp	Japan/2001	LC089875		BVDV-1o	Bos taurus
64	HY-3	China/2016	KY865366		BVDV-1o	dairy cattle
65	TJ06	China/2006	GU120246		BVDV-1p	Cattle
66	BJ0701	China/2007	GU120247		BVDV-1p	Cattle
67	SD0803	China/2008	JN400273		BVDV-1q	Pig
	VE/245/12	Italy/2010	LM994671		BVDV-1r	cattle
	UM/136/08	Italy/2008	LN515612		BVDV-1s	cattle
	SI/207/12	Italy/2012	LN515611		BVDV-1t	
68	08Q723	South Korea/2012	JQ418632		BVDV-2a	cattle
69	29342	South Korea/2009	GQ985458		BVDV-2a	calf
70	HB-1511	China/2015	KX096718		BVDV-2a	cattle
71	NY-93	USA/1993	KR093034		BVDV-2a	cattle
72	10-636	Argentina/2010	MH294527		BVDV-2b	Bos taurus
73	MSGPCAB233	Iraq/2017	MF491397		BVDV-2b	calf

74	MSGLCAB226	Iraq/2017	MF491396		BVDV-2b	calf
75	439RvUY/082014	Uruguay/2014	KT833799		BVDV-2b	Bovine
76	4p	Brazil/2015	MG436782		BVDV-2b	Homo sapiens
77	Bov/Ita/124.15-28	Italy/2015	KX890138		BVDV-2c	Bos taurus
78	Bov/Ita/856.14-578	Italy/2014	KX350085		BVDV-2c	Bos taurus
79	Bov/Ita/124.15-7	Italy/2015	KX350081		BVDV-2c	Bos taurus
80	354	Argentina/2000	AF244959		BVDV-2d	Bovine
81	BJ6(09)	China/2009	GU385894		BVDV-2	Bovine
82	Bangwa	South Korea/2008	GQ985457		BVDV-2	calf
83	IT-1732	Italy/2001	AJ416018		BVDV-2	Bovine
84	TR-2006-3	Turkey/2006	EU542423		BVDV-2	cattle
85	M10 3432	USA/2010	JN377413		BVDV-2	Bovine
86	890	USA/1994	NC_039237		BVDV-2	Bovine
87	Ind 5197	India/2007	EF547201		BVDV-2	Goat
88	58	USA/2007	FJ431191		BVDV-2	Goat
89	73	USA/2007	FJ431194		BVDV-2	Goat
90	0602ja	South Korea/2006	DQ973184		BVDV-2	Goat
91	LN01	China/2009	KC176779		BVDV-2	cattle
92	BVDV2/Serbia/2008	Serbia/2008	KY941187		BVDV-2	cattle
93	58-10	France/2005	KF205291		BVDV-2	Bos taurus
94	BVDV2-125c	USA/2013	KC596020		BVDV-2	Bos taurus
95	XJ-04	China/2004	FJ527854		BVDV-2	cattle
96	76/08	Argentina/2008	JX848364		BVDV-2	Bovine
97	106	Argentina/2014	MF120586		BVDV-2	Bovine
98	101	Argentina/2014	MF120585		BVDV-2	Bovine
99	Buffalo 17 band A	Argentina/2005	FM165309		BVDV-2	Buffalo
100	HI4463	Germany/2003	AY379546		BVDV-2	Not mentioned
101	13148906	USA/2006	FJ387325		BVDV-2	Bos taurus
102	86275	Argentina/2008	JX679696		BVDV-2	Bovine
103	LV-96	USA/2001	AF410787		BVDV-2	Not mentioned
104	Glessen 6	Germany/2003	AY379547		BVDV-2	Not mentioned
105	VS-6	USA/2001	AF410789		BVDV-2	Not mentioned
106	UEL12-BR/17	Brazil/2017	MG004720		BVDV-2	Bovine

Notes: The bold letters are strains isolated in present study.

Results

RT-PCR survey of clinical samples

RT-PCR assays were performed to determine the BVDV species with 5'UTR specific primers responsible for the infection. Of the 167 samples, 68 (40.72%) were BVDV-RNA-positive by RT-PCR. Of the products 28 were sequenced and blasted in NCBI GenBank. Thirteen BVDV-1 isolates (47.83%) were genotyped. Fifteen (52.17%) were classified as BVDV-2 in all the sequenced28 antigen positive samples. The electrophoresis results of RT-PCR products of some samples were shown in figure 1a. A summary of the isolates and reference strains is presented in Table 1. The 5'UTR genes of these isolates were used for further phylogenetic analysis.

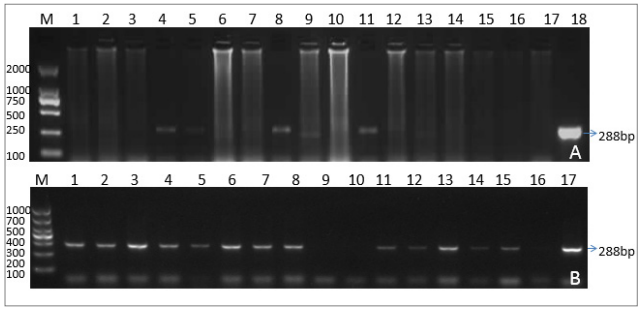
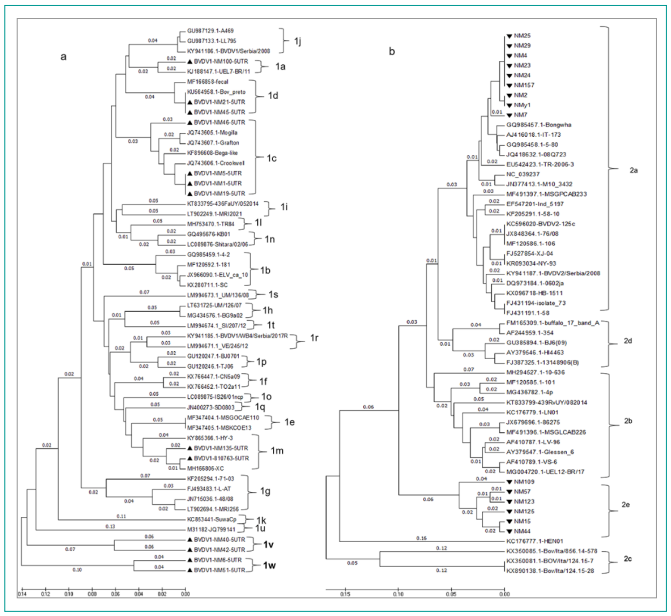


Figure1: RT-PCR detection of clinical samples by BVDV 5'UTR special primers. M, DL1000 Marker; 1-15, Clinical samples collected after 5 passages; 16, Negative control; 17, Positive control.

Subgenotypes based on phylogenetic analysis of 5'UTR of BVDV-1 in Inner Mongolia

The nucleotide sequences of field viruses and a number of known

reference strains representative of all known species and subtypes of BVDV were aligned and phylogenetic trees were constructed. It displayed phylogenetic trees for a selection of the 5'UTR in Fig. 2. The specified fragments of 5'UTR region (288bp) from cDNA preparations were detected and sequenced. Phylogenetic analysis was performed based on the 288bp fragments of 13 BVDV-1 strains of 68 BVDV-positive samples collected from cattle's in Inner Mongolia between 2017 and 2018. Thirty-six B reference strains from GenBank were used. BVDV-1 isolates analyzed in this work were deposited in GenBank under following accession numbers: MK204893-MK204905 (Listed in Table 1). The topology of the tree (Figure 2a) showed that all 49 BVDV-1 strains belonged to 20 distinct subgenotypes, namely BVDV-1a (n=2),BVDV-1b (n=4), BVDV-1c (n=8), BVDV-1d (n=4), BVDV-1e (n=2), BVDV-1f (n=2), BVDV-1g (n=4), BVDV-1h (n=2), BVDV-1i (n=2), BVDV-1j (n=3), BVDV-1k (n=1), BVDV-1l (n=1), BVDV-1m (n=4), BVDV-1n (n=2), BVDV-1o (n=1), BVDV-1p (n=2), BVDV-1q (n=1), BVDV-1r (n=2), BVDV-1s(n=1), BVDV-1t(n=1), BVDV-1u(n=1), BVDV-1v(n=2), BVDV-1w(n=2). The subgenotypes BVDV-1m, -1n, -1o, -1p, and -1q had been detected exclusively in Asia. Similarly, BVDV -1f, -1g,-1h, -1k, -1l, -1r, 1s, and -1t have not been reported to occur in countries outside Europe(Giammarioli et al., 2008; Yilmaz et al., 2012; Factor et al., 2016; Gomez-Romero et al., 2017; Silveira et al., 2017; Yesilbag et al., 2017). However, phylogenetic analysis clustered the 13 BVDV-1 isolates into six subgenotypes in present study: BVDV-1a, BVDV-1c, BVDV-1d, BVDV-1m, and two potentially novel subgenotypes, tentatively designated as 'BVDV-1v' and 'BVDV-1w' in present study (Figure 2).



UEL-BR/11 isolated from Brazil, which was belong to BVDV-1a. There are 4 strains: NM1, NM5, NM19 and NM46 in the BVDV-1c group, which also including 4 strains from Australia. The NM21 and NM45 were classified as BVDV-1d. ELV_ca_10 strain from Denmark belonging to BVDV-1d in a previous work was not clustered into BVDV-1d but BVDV-1b in present work. 810763 and NM135 strains isolated from bovine herd located west of Inner Mongolia were classified as BVDV-1m. The first BVDV-1m strain, ZM-95, was initially isolated from swine herds in Inner Mongolia in 1995(Wang X, 1996). Six BVDV-1m isolates from Beijing were detected in 2015(Weng et al., 2015). Accumulating evidence is indicating that BVDV-1m also is a predominant subgenotype in cattle herd (Deng Y. et al., 2012; Gao et al., 2013).

Interesting, the other four strains NM6, NM51, NM40 and NM42 were shown clustering into two novel distinct phylogenetic groups from BVDV-1a~1u. Here, we first tentatively named them as 'BVDV-1v' and 'BVDV-1w'. When NM6 is blasted with other strains in GenBank, the homology is up to 90%. There is highest identity between NM6 and MF-2, a BVDV-1c strain from China. Though NM51 is belong to same cluster with NM6, it has an 84% identity with BJ09_21 strain, which is also from China. NM40 only has a homology up to 82% with other strains in GenBank, which are belong to BVDV-1d and BVDV-1a when blasting. NM40 is likely origin from has most similar with AL3a@10, a BVDV-1d isolate from Italy. We deduced NM40 are likely origin from recombination with BVDV-1a and BVDV-1d.

Subgenotypes based on phylogenetic analysis of 5'UTR of BVDV-2 in Inner Mongolia

The 15 positive samples belonged to BVDV2, which were divided into 2 different subgenotypes (Figure 2b). Phylogenetic analysis results showed that 60% (9/15) of the sequences (NM2, NM4, NM7, NM23, NM24, NM25, NM29, NM157, NMy1) were typed into a single subgroup BVDV-2a. Specially, other 6 strains (NM15, NM44, NM57, NM109, NM123, NM125) were characterized as a new subgroup that is different from BVDV-2a~BVDV-2d. These six isolates could not be assigned to any known BVDV-2 subgenotype. We appointed it as 'BVDV-2e'. Subgenotypes BVDV-2b, -2c, and -2d strains, which have been reported in South America, were not detected in current study. The BVDV-2a subgenotype appears to be more common than the other subgenotypes worldwide.

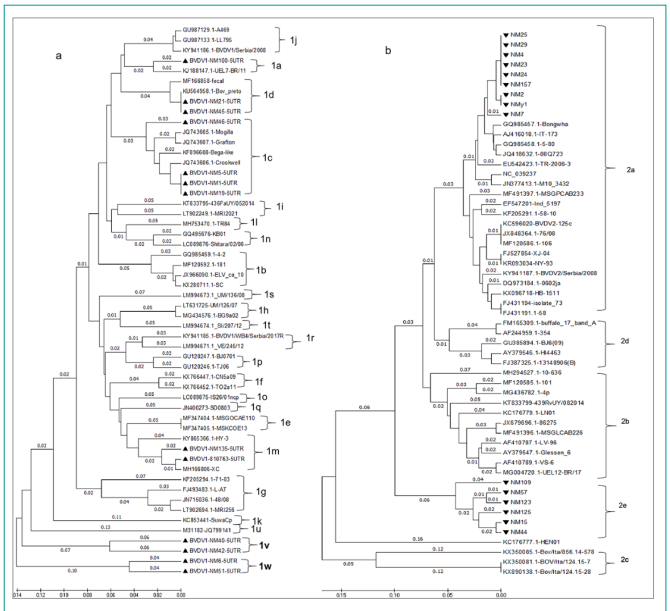


Figure 2: Phylogenetic tree based on the 5'UTR sequences of reference BVDV strains and isolates from Huhhot, China. Evolutionary analyses were conducted in MEGA7, supported by 1,000 bootstrap replications.

a. Phylogenetic tree showing the genetic relationship between Bovine viral diarrhea virus 1 isolates based on analysis of 49 nucleotides derived from the 5'UTR. The tree contains 13 BVDV-1 isolates sequenced in this study plus 36 reference strains (shown in Table 2). The studied strains were labeled with a symbol (▲).

b. The phylogenetic tree was generated based on comparison of nucleotide sequences of the 5'UTR of 15 BVDV-2 isolates with 39 reference sequences (shown in Table 2) downloaded from the GenBank database. The 15 BVDV-2 isolates are indicated in symbol (▼). Numbers at the phylogenetic branches indicate branch lengths (next to the branches) in the same units. The sequences for reference strains were listed with strain name and GenBank no.

Discussion

Were detected more than other 4 groups 1a, 1m, 1v and 1w.

Deng et al. investigated BVDV genotypes in four bovine species to found BVDV-1b, BVDV-1m and a new cluster BVDV-1u were dominant subtypes in China (Deng M. et al., 2015). However, they collected the samples from east but not west of Inner Mongolia. We mainly located in west of Inner Mongolia to supervise the prevalence of BVDV. Importantly, we found not only the subgenotypes BVDV-1m but also two novel clusters BVDV-1v and BVDV1w. It indicated that more complicated BVDV strains were spread in west of Inner Mongolia.

Infection with BVDV-2 was first described in North America in the early of 1990s (Corapi et al., 1989). BVDV-2 was found in cattle in Xinjiang Autonomous Region and in Qinghai province of China (Gong et al., 2014). The BVDV-2a subgenotype appears to be more common than the other subgenotypes worldwide (Giammarioli et al., 2008; Oguzoglu et al., 2010; Behera et al., 2011; Han et al., 2016; Gomez-Romero et al., 2017). A recent phylogenetic analysis of BVDV in Mongolia revealed BVDV-1a and BVDV-2a were dominant genotype (Ochirkhuu et al., 2016). Though Inner Mongolia of China locates nearby Mongolia, the BVDV genotype based on epidemiological studies showed more subgenotypes appeared in Inner Mongolia of China. Two BVDV-2 subgenotypes were found in present study.

Here, we present the results of an epidemiological survey for Bovine viral diarrhea virus (BVDV) in Inner Mongolia, China, especially in Huhhot and surrounding areas of Inner Mongolia Autonomous Region. Our investigation describes the genetic diversity of BVDV from cattle in Huhhot of Inner Mongolia. It provides important information for the clinician and diagnostician responsible for diagnosis of BVDV.

To summarize, the presence of novel subgenotypes BVDV-1v and BVDV-1w and BVDV-2e were described in China dairy cattle for the first time. Further studies are required to investigate the prevalence of BVDV infection in a larger cattle population as well as the role in various clinical conditions and economical losses. The presence of new species is important for the evaluation of current diagnostic protocols and for the development of control programs, such as vaccination. Furthermore, it underlines the necessity to quarantine and test imported cattle thoroughly before introduction into local herds.

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Author contributions:

FXW, YJW contributed to the design of the work; JHF, WDGW, KY, XPL, ZPM, MJ HZ, and YY performed the experiments in the study. FXW, YML, YMS, HW, CYL, BWZ, HZZ, and QJS analyzed the data. FXW, YJW wrote the manuscript. All authors read and approved the final manuscript.

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