

Detection of BVDV 1q in China: Genetic Characterization and Experimental Infection for the Investigation of It's Pathogenicity

Yanhua HE ^{1,2,a,†} Xusheng MA ^{3,†} Xin HUANG ² Jinliang SHENG ¹
Fagang ZHONG ² Xinxia ZHAO ¹ Yunfeng ZHANG ^{1,2} Chuangfu CHEN ^{1,b} 

[†] These authors contributed equally to this work

¹ College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang 832003, CHINA

² State Key Laboratory of Sheep Genetic Improvement and Healthy Production/Xinjiang Academy of Agricultural and Reclamation Sciences, Shihezi, Xinjiang 832000, CHINA

³ HouBo College of XinJiang Medical University, Kelemayi, Xinjiang 834000, CHINA

^a ORCID:0000-0002-9131-4456; ^b ORCID:0000-0001-5181-6518

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Abstract

Bovine viral diarrhoea virus (BVDV) is a pathogen that affects ruminants worldwide and is one of the most economically important diseases of cattle. Although BVDV infections have been increasingly reported in China, the pathogenesis and genetic characteristics of these BVDV isolates have not been thoroughly investigated. Here, we report the identification and characterization of a novel BVDV isolate, designated LC, which was isolated from the feces of a cattle with diarrhoea. The complete genome of isolate LC was 12.271 nucleotides and contained a 5'-UTR of 389 nucleotides, a 3'-UTR of 189 nucleotides, and a large ORF encoding a polyprotein consisting of 3898 amino acids. Genomic comparisons and phylogenetic analyses of the complete genomic sequence clearly showed that the isolate was a BVDV-1q subtype. Experimental infection of calves with isolate LC resulted in the development of clinical signs including elevated rectal temperatures, nasal discharge and decreased leucopenia. Viral antigen was detected in infected animal tissues using immunohistochemistry. This is the first report of the genomic sequence of a BVDV-1q virus isolated from cattle. The virus strain was moderately pathogenic in calves and could potentially be used as a BVDV challenge virus to evaluate the efficacy of BVDV vaccines.

Keywords: Bovine viral diarrhoea virus, BVDV, Genomics, Genotyping, Pathogenesis

Çin'de BVDV 1q'nun Tespiti: Patojenitesinin Araştırılması Amacıyla Genetik Karakterizasyon ve Deneysel Enfeksiyon

Öz

Siğir viral diyare virüsü (BVDV) dünya çapında ruminantları etkileyen ve siğirlerin ekonomik olarak en önemli hastalıklarından biri olan bir patojendir. BVDV enfeksiyonları Çin'de giderek daha fazla rapor edilmesine rağmen, BVDV izolatlarının patogenezi ve genetik özellikleri tam olarak araştırılmamıştır. Bu çalışmada, ishalleri dışkılarından izole edilen ve LC olarak adlandırılan yeni bir BVDV izolatının tanımlanması ve karakterizasyonu rapor edildi. İzolat LC'nin tam genomu, 5'-kodlanmayan bölgesinde 389 nükleotid ve 3'-kodlanmayan bölgesinde 189 nükleotid içeren 12.271 nükleotitten oluşmaktaydı ve 3898 amino asitten oluşan bir poliproteini kodlayan büyük bir ORF içeriyordu. Tüm genomik dizinin genomik karşılaştırmaları ve filogenetik analizleri, izolatın bir BVDV-1q alt tipi olduğunu açıkça göstermiştir. İzolat LC ile buzağuların deneysel enfeksiyonu, rektal sıcaklık artışı, burun akıntısı ve lökopeni gibi klinik bulguların gelişmesine neden oldu. Viral antijen, enfekte hayvan dokularında immünohistokimyasal olarak tespit edildi. Bu çalışma, siğirlardan izole edilmiş bir BVDV-1q virüsünün genomik dizisinin ilk bulgusudur. Virüs suşu buzağularda orta derecede patojenite göstermiştir ve BVDV aşılmasının etkinliğini değerlendirmek için potansiyel bir BVDV bağışıklık virüsü olarak kullanılabilir.

Anahtar sözcükler: Siğir viral diyare virüsü, BVDV, Genomik, Genotipleme, Patogenez

INTRODUCTION

Bovine viral diarrhoea virus (BVDV), the etiological agent of bovine viral diarrhoea/mucosal disease, is a prevalent

virus of economic importance in the cattle industry. BVDV-associated diseases range from clinically mild to severe and can involve the respiratory, enteric, reproductive, immune, and endocrine systems. BVDV belongs to the *Pestivirus*



İletişim (Correspondence)



+86-0993-2058002



cfchen301@yeah.net

genus of the *Flaviviridae* family, the same genus as classical swine fever virus (CSFV) and border disease virus (BDV) of sheep^[1,2].

The BVDV genome comprises single-stranded RNA of about 12.3~12.5 kb in length. The whole genome can be divided into the 5'-untranslated region (5'-UTR), a large open reading frame (ORF), and the 3'-untranslated region (3'-UTR). According to sequence comparisons of the BVDV 5'-UTR, BVDV was divided into two genotypes: bovine viral diarrhoea virus type I (BVDV-1) and bovine viral diarrhoea virus type II (BVDV-2)^[3]. At present, according to comparisons of the 5'-UTR, N^{pro}, and E2 sequences in the BVDV genome, BVDV-1 can be divided into 22 subtypes (1a-1v), and BVDV-2 can be divided into four subtypes^[4-7]. Because of the high mutation rate of RNA viruses and increasingly frequent international exchange and trade, new subtypes of BVDV are still emerging. Recently, an atypical bovine virulent BVDV-3 genotype was detected in bovine serum^[8], which can be divided into two genetic subtypes (*Brazilian* source and *Thai* source), but the viral typing of BVDV-3 has not been confirmed according to the international classification of viruses. A comparative analysis of the 5'-UTR sequences of BVDV isolated from 2005 to 2013 in China was conducted. Most of the endemic strains in China were reported to be BVDV-1, and the main subtypes were BVDV-1b, BVDV-1m, and BVDV-1q^[9-11].

According to the results of a BVDV epidemiological survey in China, more than 46.7% of cattle farms tested positive for BVDV antigen, and the persistent infection rate of BVDV in cattle herds was 2.2%^[12-14]. In 2008-2010, in China's Xinjiang region, an epidemiological investigation of BVDV in district dairy farms revealed an average infection rate of 43.39%, with genotype BVDV1b being predominantly detected^[15]. In another investigation of epidemic strains in Xinjiang in 2015, 2 of the 21 strains analyzed belonged to the BVDV-1b subtype, 15 strains had high homology with strain SD0803 of porcine BVDV isolated from Shandong, belonging to BVDV-1q, and the other four strains belonged to BVDV-2^[6]. The BVDV-1q subtype strain may have become an epidemic strain of BVDV in Xinjiang. The pathogenicity of BVDV-2 strains has been widely reported^[16-18], but few studies have reported the pathogenicity of BVDV-1 strains.

In this study, one virus was isolated from fecal samples of cattle using MDBK cell cultures, and identified as a BVDV isolate by reverse transcriptase-polymerase chain reaction (RT-PCR) method and immunofluorescence assay. To investigate the genetic subgroup of the strain, the 5'-UTR and complete genome of the virus was sequenced and compared with other reference BVDV strains by phylogenetic analysis. The pathogenesis of the virus was evaluated by intranasally inoculating to susceptible calves to assess the potential endemic risk to the cattle herd in China. To understand the origin and evolution of BVDV strain and determine the molecular characters of the BVDV strains predominantly spread in China. The results

confirmed the existence of the BVDV type 1q in China, and it may be helpful in preventing the BVD in China and a challenge virus strain for efficacy evaluations of vaccines.

MATERIAL and METHODS

Herd(s) History

A disease characterized by severe diarrhea occurred on a cattle farm with 420 cattle in Xinjiang province. The sick cattle were observed to manifest pyrexia, anorexia in early days, and later oral mucous ulcer and severe diarrhoea containing mucous and haemorrhage excretions found.

Approximately 15% (63/420) of cattle showed clinical signs. Treatment of the sick cattle with Penicillin or gentamycin yielded no effects on relieving the clinical signs. Blood samples obtained from cattle with diarrhea in Xinjiang Province, China in 2016 all tested with the IDEXX BVDV Ag/Serum Plus Test (Idexx Labs Inc, USA). Fecal samples were collected directly from the rectums of cattle to prevent contamination from the environment or between samples.

Virus Isolation

Fecal samples were diluted 1:9 (w/v) in sterile phosphate-buffered saline (PBS) with 1% antibiotics, and centrifuged at 10,000 × g for 10 min followed by filtration through a 0.22-µm filter. The filtrates were stored at -80°C until use for genomic analysis and virus isolation.

Virus isolation was conducted as described previously^[19]. Briefly, MDBK cells were infected with the filtrates in a 12-well culture plate. The cell cultures were frozen and thawed three times and passaged two to three times at 5-day intervals. Every passage of MDBK culture was observed for 5 days, with the presence or absence of cytopathic effects being recorded.

Immunofluorescence Assay

To detect BVDV in the infected MDBK cultures, an immunofluorescent assay was conducted as follows^[20]. Briefly, cell lysate was added to each of four wells of a 24-well MDBK tissue culture plate. Positive virus (NADL strain) and DMEM media (negative control) were also added to the four wells. After 24 h incubation at 37°C in a 5% CO₂ atmosphere, the plates were fixed in 4% cold polyoxymethylene and then washed with PBS. The fixed plates were incubated with fluorescein isothiocyanate (FITC)-conjugated polyclonal anti-BVDV (VMRD, Washington DC, USA) antibody, followed by a 1 h incubation in a 37°C humid box. The fluorescence signal was observed using a fluorescence inverted microscope (Zeiss Axioskop-40, Carl Zeiss, Jena, Germany).

RT-PCR Detection and Complete Genomic Sequence Analysis

Total RNA was extracted from the filtrate of ten samples and cell culture fluids of the virus isolates using a Mini BEST Viral

RNA/DNA Extraction Kit according to the manufacturer's instructions (TaKaRa Bio Inc, Beijing, China). The RNA was resuspended in 30 μ L of DEPC-treated water. The extracted RNA was reverse-transcribed using the M-MLV Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA) as specified by the manufacturer. Eleven primer sets were designed to amplify overlapping regions of the complete BVDV genome (Table 1). The amplified fragments were harvested and cloned into pMD19-T vector (TaKaRa Bio Inc, China). The three recombinant clones were submitted for DNA sequencing (Sangon Biotech (Shanghai) Co. Ltd, Shanghai, China). The retrieved sequences were edited and trimmed with the Edit Seq program in the DNASTar software (6.0). Clustal W was used to align the nucleotide sequences. Phylogenetic analysis was performed by the distance-based ClustalW method using Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software.

Experimental Infection of Animals

Six to nine-month-old, healthy calves were obtained from a calf farm in Xinjiang, China. All animals were confirmed to be free of BVDV, infectious bovine rhinotracheitis virus, bovine parainfluenza virus, and *Mycoplasma bovis* infection using enzyme linked immunosorbent assay (ELISA) kits (Idexx Labs Inc., Maine, USA) and by reverse transcription PCR (RT-PCR) or PCR for nucleic acid detection [21,22]. As we expected, all animals were BVDV mRNA-free. All animal

experiments were approved by The Shihezi State University Institutional Animal Care and Use Committee (NO.A2018-173-01).

Animal experiments are conducted in the veterinary biosafety level 3 laboratory (BL3) of Xinjiang Tiankang Biological Technology Co. Ltd. Infected animals are raised in the biosafety level III equipment (negative pressure isolator), and all operations are conducted in the biosafety level III cabinet. The six calves were divided randomly into treatment and control groups, with three animals in each group. Each calf in the treatment group was inoculated intranasally (IN) with $\sim 6 \times 10^{7.0}$ tissue culture infective dose (TCID₅₀) of the isolate BVDV LC virus. The animals in the control group were inoculated IN with DMEM. All animals were monitored daily for clinical signs as described previously [23]. Clinical assessments were made at the same time each morning by investigators who were blinded to the treatment groups. Clinical signs included depression, nasal discharge, diarrhea, coughing, and high rectal temperature. EDTA-blood samples from calves were collected at days -2 to 0 prior to inoculation and 2, 4, 6, 8, 10, 12, 14 and 16 days' post-inoculation (dpi) and were used to count white blood cells. Additionally, at -2, 0, 2, 4, 6, 8, 10, 12, 14 and 16 dpi, heparin blood was sampled for buffy-coat preparations to test for viremia in the infected calves. Deep nasal swab specimens were obtained from 1 day prior to challenge through to 14 dpi. The procedure to isolate BVDV from samples was conducted as described previously [19,24]. Two calves from each group were necropsied at 16 dpi, and tissue samples of liver, spleen, lung, heart, kidney, intestine and mesenteric lymph node were collected and fixed in 10% neutral buffered formalin and processed for histopathological examination following hematoxylin and eosin (H&E) staining and immunohistochemistry. After the animal experiment is finished, animal carcasses and all animal indoor wastes should be autoclaved before incineration or other final treatment.

Data Analysis

The two groups (treatment and control) were analyzed and compared with respect to the primary clinical signs including rectal temperature, nasal and ocular discharge, diarrhea, leukopenia, and virus shedding, using GraphPad Prism (version 4.0) software. The level of statistical significance was set at $P < 0.05$.

RESULTS

BVDV is the Causative Agents for the Outbreak

No obvious effect was observed the majority of sick cattle after they were treated with Penicillin or gentamycin, suggesting the outbreak is likely associated with viral agents. All samples were tested by the IDEXX BVDV Ag/Serum Plus Test (Idexx Labs Inc, USA), 420 antibody positive samples were detected, the positive rate of BVDV antibody

Table 1. Primers used for amplification of the complete genome sequence

| Primer Name | Sequence | Length |
|-------------|----------------------------------|--------|
| BVDV-A1F | GATCAATCTCTCGTATACAC | 375bp |
| BVDV-A1R | GCCATGTACAGCAGAGAT3' | |
| BVDV-AF | ATGCCCTTAGTAGGACTAGC | 1351bp |
| BVDV-AR | TCAATATTGTACCAGTTGCACCAACCATG | |
| BVDV-BF | GAAGGGATACAACGGCAATGTT | 1613bp |
| BVDV-BR | ACCATCTGGAAGGCCGGTCCATTGAGCA | |
| BVDV-CF | ATGGATGACAACTTGAATTTGG | 1167bp |
| BVDV-CR | ACCAGTGGCACTATAGTCGGGTC | |
| BVDV-DF | GTGATGATGGCAACTTGCTAACACATGAT | 1286bp |
| BVDV-DR | CATTTTGGGCAGGTGCCACCTTTCCACTT | |
| BVDV-EF | AACCTAATAATAAAACATAAGGT | 1431bp |
| BVDV-ER | AGTTCTGTGGTTTTCTGCCCTGTGTC | |
| BVDV-FF | AGGGTGGTTGGCAGAGTTAAGGTAGGGAAG | 1288bp |
| BVDV-FR | TATGCAAGTTGGATTGGCTCTGGGTG | |
| BVDV-GF | TATTATAGGAGCCAGGAAACAGCAAC | 1499bp |
| BVDV-GR | TGATCCAAGAAGTTCTTTACAAACACCTTCAT | |
| BVDV-HF | ATGGAAATCTGTACAAAACCCAGT | 1451bp |
| BVDV-HR | TTTGCTCTATTGACATGGAACCTC | |
| BVDV-IF | ACATCAGAGTTGGGATCACAATAATTGG | 1466bp |
| BVDV-IR | TCATTTTTTGGTATTGCTGTTTCATAATA | |
| BVDV-JF | TAAAACACACCTATGGTGAGGTGACGTGGGA | 1746bp |
| BVDV-JR | CCTCATACAGCTAAAGTGCTGTGTGCATT | |

was 100% (420/420). Collected blood samples were tested by RT-PCR, 98 positive samples were detected, approximately 23% (98/420) of BVDV antigen positive rates. The sequencing analysis of 5'-UTR fragment amplified by RT-PCR showed that the sick cattle were infected with BVDV virus.

BVDV-LC is a Noncytopathic Biotype BVDV Isolate

Fecal samples were cultured and passaged in MDBK cells, and no cytopathic effects were observed in MDBK cells after 15 passages. However, specific fluorescence signals were detected in the cells inoculated with 15 passage supernatants using FITC-labeled BVDV antibody, whereas no fluorescence signals were detected in the mock infected cells (Fig. 1). To confirm these results, PCR was performed and fragments were amplified from the infection group, and sequencing revealed that the sequences were BVDV-specific (data not shown). No fragments were amplified from the control group. One viral isolate was obtained from the fecal samples and this strain was designated as BVDV-LC.

Sequence and Phylogenetic Analysis of the Complete Genome

To investigate the evolutionary relationship between BVDV isolates, phylogenetic analysis was performed using MEGA software version 6.0. A phylogenetic tree constructed based on full-length genome sequences revealed that isolate BVDV-LC clustered with previous BVDV-1 isolates (Fig. 2-A). The genome sequence of BVDV-LC shared nucleotide sequence identities of 63.0% to 95.1% with other *Pestivirus* strains, 70.8% identity with BVDV-2 strains, 69.1% identity with BVDV-3, 67.9% with CSFV strains, and 63% identity

with the pronghorn strain (AY781152 antelope). The complete genome sequence of isolate LC was compared with those of nine BVDV-1 strains, three BVDV-2 strains, one strain of Pronghorn antelope pestivirus, two strains of BDV, two strains of CFSV, and one strain of BVDV-3 (Table 2). The sequences of BVDV-1 viruses showed a higher degree of divergence from that of isolate LC than from those of the BVDV-2 strains. In the coding sequences, the highest degree of shared identity was observed between the LC and SD0803 strains. The full-length genome sequence of SD0803 was 95.1% identical to that of the LC isolate.

To confirm the subtype assignment based on the full-length genome sequence and to compare our isolates to other reference strains, phylogenetic trees based on the 5'-UTR and N^{pro} genes were constructed (Fig. 2-B,C). For this analysis, sequences from members of 21 genetic subgroups of BVDV-1 from different regions of the world were used. The two phylogenetic trees showed that isolate BVDV-LC clustered in the same phylogenetic branch as in the phylogenetic tree based on the full-length genome (Fig. 2-B,C). The 5'-UTRs of the Shihezi148-1, SD0803, Changji, and Kuerle virus strains shared 92.5% and 98.5% sequence identity, respectively, to the isolate in the present study. The N^{pro} sequence of the SD0803 isolates shared 92.7% sequence identity with the BVDV-LC isolate. Furthermore, the BVDV-LC isolate clustered with strain SD0803, which belongs to the BVDV-1q subgenotype [25].

The complete genome of the BVDV-LC strain of BVDV-1 (GenBank accession No. MK102095) was 12.271 kb in length and contained a single ORF that was 11.694 kb in length, encoding a 3898-amino-acid polypeptide. The 5' and 3'-UTRs of the BVDV-LC virus were 388 and 186 bp in length,

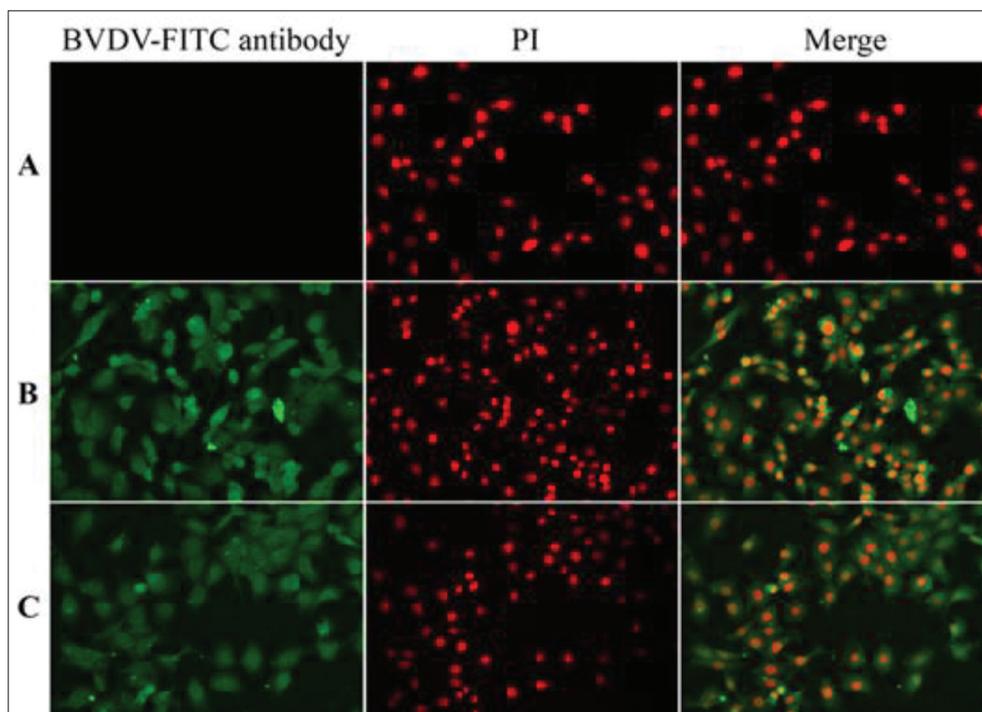
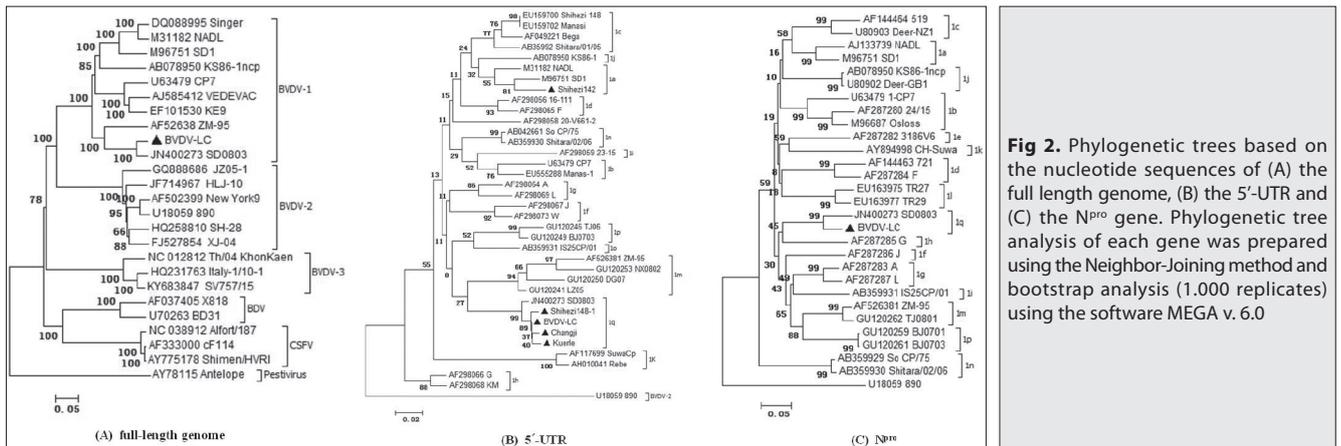


Fig 1. Immunofluorescent assay to detect BVDV-LC in cultured MDBK cells. A: Control cells; B: NADL-positive control; C: BVDV-LC

Table 2. Nucleotide sequence identity (%) of the BVDV-LC strain to the coding sequences of other whole genome sequences of Pestivirus isolates used in the trees

| Strain | Pestivirus | N ^{pro} | C | E ^{ms} | E1 | E2 | P7 | NS2 | NS3 | NS4A | NS4B | NS5A | NS5B |
|----------------|------------|------------------|------|-----------------|------|------|------|------|------|------|------|------|------|
| Singer-Arg | BVDV-1 | 81.3 | 76.1 | 80.9 | 77.6 | 71.7 | 74.8 | 78.0 | 83.0 | 81.8 | 81.8 | 76.9 | 79.6 |
| NADL | | 83.3 | 77.5 | 80.2 | 77.3 | 72.5 | 73.8 | 74.8 | 83.6 | 80.7 | 82.2 | 76.5 | 79.9 |
| SD1 | | 82.3 | 78.1 | 80.3 | 77.9 | 72.6 | 76.2 | 79.6 | 83.3 | 83.9 | 81.8 | 76.5 | 79.5 |
| KS86-1 | | 82.3 | 79.1 | 80.2 | 77.6 | 74.2 | 78.1 | 77.9 | 83.0 | 82.3 | 83.0 | 76.1 | 80.7 |
| CP7 | | 80.2 | 78.8 | 79.6 | 76.9 | 73.4 | 74.3 | 76.3 | 82.6 | 83.3 | 79.7 | 76.5 | 80.8 |
| VEDEVAC | | 79.6 | 80.1 | 79.1 | 75.6 | 72.9 | 71.4 | 75.9 | 82.3 | 82.3 | 79.9 | 76.6 | 79.9 |
| KE9 | | 79.2 | 77.5 | 81.4 | 75.9 | 73.4 | 78.6 | 76.8 | 81.6 | 84.4 | 81.4 | 76.9 | 79.7 |
| Zm95 | | 81.9 | 77.5 | 84.0 | 81.5 | 77.5 | 81.4 | 80.8 | 85.6 | 84.9 | 85.8 | 82.0 | 82.6 |
| SD0803 | | 92.7 | 95.1 | 94.7 | 94.4 | 92.0 | 94.3 | 94.8 | 96.5 | 95.3 | 95.9 | 96.4 | 96.0 |
| SH-28 | BVDV-2 | 70.2 | 66.3 | 71.1 | 71.1 | 64.4 | 59.0 | 57.6 | 78.2 | 75 | 75.8 | 66.4 | 70.2 |
| New York93 | | 71.8 | 66.7 | 70.8 | 69.7 | 57.9 | 60 | 62.0 | 78.5 | 74.0 | 74.9 | 65.1 | 70.9 |
| 890 | | 72.4 | 67.6 | 71.4 | 69.7 | 65.5 | 61.9 | 57.1 | 77.9 | 74.0 | 75.0 | 66.6 | 70.3 |
| Antelop | Pestivirus | 59.3 | 65.0 | 45.2 | 57.9 | 56.2 | 44.3 | 37.7 | 71.0 | 55.2 | 67.7 | 57.7 | 62.5 |
| X818 | BDV | 66.9 | 59.5 | 70.5 | 69.1 | 63.2 | 56.2 | 59.4 | 77.7 | 76.6 | 65.8 | 59.3 | 70.1 |
| BD31 | | 64.7 | 63.4 | 71.5 | 67.0 | 61.1 | 55.2 | 58.0 | 76.9 | 74.5 | 66.8 | 62.6 | 69.6 |
| cf114 | CFSV | 66.5 | 63.4 | 67.5 | 68.4 | 61.6 | 56.2 | 60.2 | 76.6 | 75.5 | 69.0 | 61.6 | 69.4 |
| Shimen-HVRI | | 65.9 | 63.1 | 67.5 | 68.2 | 59.9 | 56.7 | 60.4 | 76.6 | 75.5 | 69.0 | 61.7 | 69.4 |
| Th-04-KhonKaen | BVDV-3 | 67.3 | 66.7 | 73.7 | 69.7 | 61.9 | 52.4 | 54.8 | 77.1 | 77.1 | 73.6 | 62.0 | 70.8 |

**Fig 2.** Phylogenetic trees based on the nucleotide sequences of (A) the full length genome, (B) the 5'-UTR and (C) the N^{pro} gene. Phylogenetic tree analysis of each gene was prepared using the Neighbor-Joining method and bootstrap analysis (1,000 replicates) using the software MEGA v. 6.0

respectively. Based on the putative post-transcriptional processing sites of other BVDVs, the SD0803 genes and UTRs were mapped to the following nucleotide positions in the genome: 5'-UTR(1-388), N^{pro}(389-892), C(893-1197), E^{ms}(1198-1879), E1(1880-2464), E2(2465-3586), p7(3587-3795), NS2-3(3796-7204), NS4A(7205-7396), NS4B(7397-8437), NS5A(8438-9925), NS5B(9926-12082), and 3'-UTR (12083-12271).

Pathogenesis of Infection in Cattle Experimentally Infected with BVDV 1q

All three calves inoculated with BVDV-LC developed moderate clinical signs associated with BVDV infection, including depression, fever, leukopenia, and viremia. No clinical signs were observed in the control calves. The rectal temperatures of the calves were measured daily from day -2 (before inoculation) to day 16 (after inoculation). All calves

in the treatment group had elevated rectal temperatures after inoculation, and the highest temperature was over 40 degrees on day 8 to day 12. In the control group, there was no increase in the rectal temperature of the calves (Fig. 3-A). In the treatment group, the number of white blood cells began to decline on the second day after inoculation, and decreased the least on the sixth day after injection, to almost 40%. There was no change in the number of white blood cells in the control group (Fig. 3-B).

In the treatment group, the results of viral detection were positive from 4 to 12 dpi in nasal and blood samples. Viral shedding was detected by virus isolation in MDBK cells from nasal swabs and blood samples. Two of the infected calves showed viral shedding as early as 4 dpi and the longest shedding period reached 12 days after inoculation. Results showed that all infected calves had viremia and BVDV was isolated from blood from different calves on

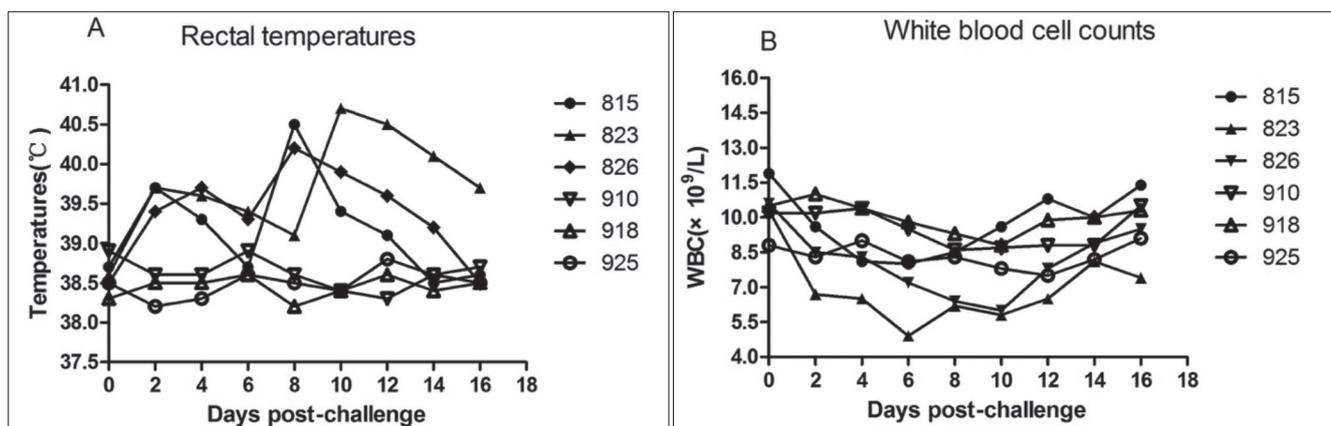
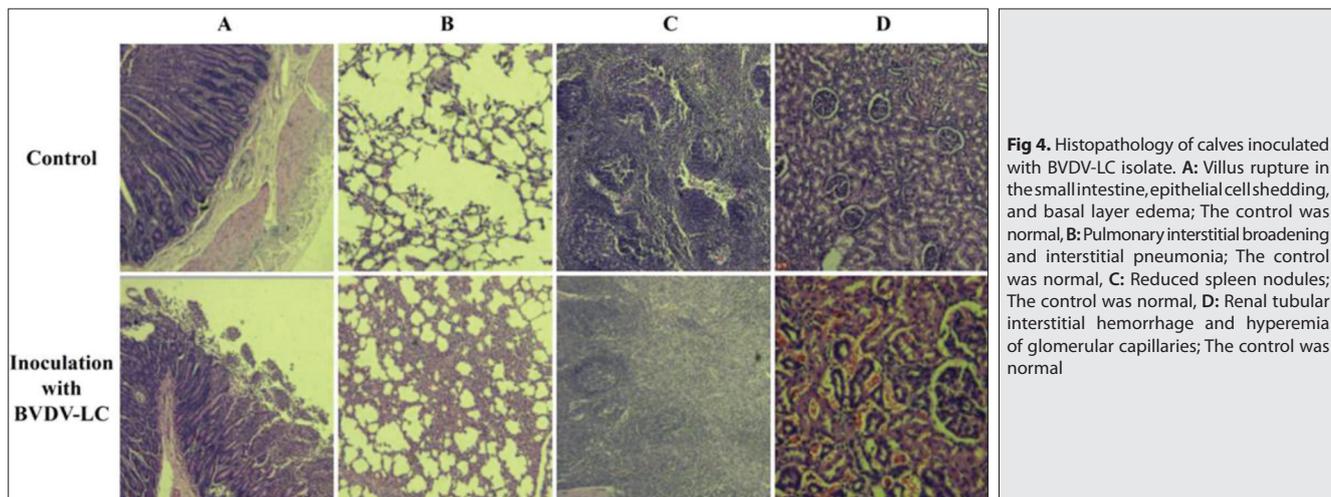


Fig 3. The clinical observation following inoculated with BVDV-LC virus. A: Elevated rectal temperatures were detected, B: Decreased white blood cell counts were also detected



days 4-12 days. Sera obtained before inoculation with BVDV-LC and on day 14 was subjected to neutralization testing. Results showed that all of the virus-infected calves developed virus-neutralizing antibodies by day 14. The results of viral detection were negative after inoculation in the control group.

All experiment calves were euthanized at 16 dpi, one mock-infected calf (918) and two randomly chosen infected calves (815 and 823) for histopathology analysis. Gross pathological findings included enlarged mesenteric lymph nodes, obvious renal hemorrhage, intestinal inflammation, and severe intestinal hemorrhage. Compared with the control group, all of the organs in the challenge group showed obvious pathological changes, including: villus rupture in the small intestine, epithelial cell shedding, and basal layer edema (Fig. 4-A); pulmonary interstitial broadening and interstitial pneumonia (Fig. 4-B); reduced spleen nodules (Fig. 4-C); renal tubular interstitial hemorrhage and hyperemia of glomerular capillaries (Fig. 4-D); and finally, no pathological changes in the heart. The central venous and interlobular veins of the bovine liver were filled with blood.

Immunohistochemical staining was performed on the

tissues of the heart, liver, spleen, lung, kidney and small intestine of calves in the challenge group. Virus infection was detected in the liver and lung, which had low viral loads, and the small intestine and heart tissues, which showed higher viral loads (Fig. 5). In the control group, there were no signs of virus infection in any of the tissues.

DISCUSSION

In this study, a new BVDV-LC strain was isolated from calves afflicted with bovine viral diarrhea/mucosal disease, which was similar to a BVDV isolated in Xinjiang Province, China in 2016. The pathogenesis of this isolate was tested by experimental infection. A virus of this genetic subtype has previously been detected in pigs in China^[25]. BVDV1-q was isolated from cattle and pigs, suggesting that this virus has evolved to replicate well in different species. At present, there are 22 subtypes of BVDV1, and the diversity of genotypes is one of the main features of BVDV. Genetic evolutionary analysis is an effective tool for tracing the origin of newly emerged viruses for epidemiological and vaccine research^[26-28].

A previous study indicated that the BVDV 1a subtype was

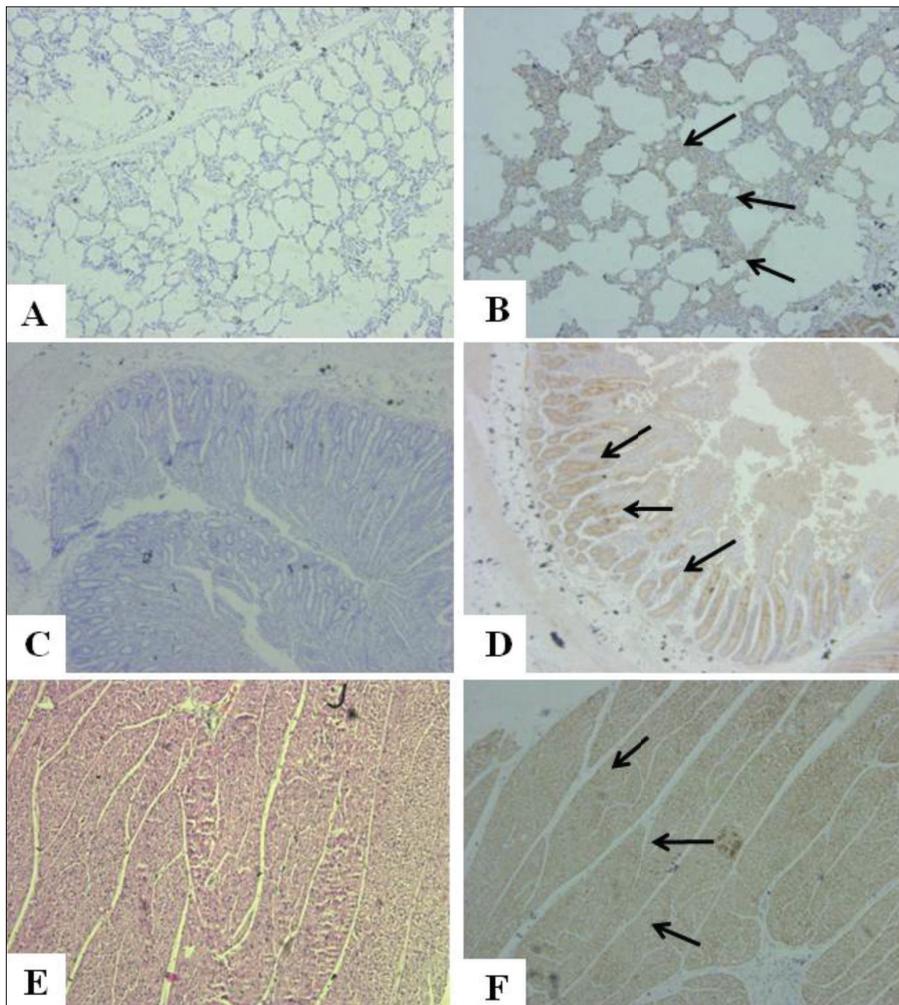


Fig 5. Immunohistochemical analysis of the tissues of calves inoculated with BVDV-LC isolate. A: Section of the lung of a calf experimentally infected with BVDV-LC. Hybridization signals are detected by anti-BVDV antibody (arrows). B: Section of the lung of a mock-infected calf. There is no signal. C: Section of the intestine tenue of a calf experimentally infected with BVDV-LC. Hybridization signals are detected by anti-BVDV antibody (arrows). D: Section of intestine tenue of a mock-infected calf. There is no signal. E: Section of the heart of a calf experimentally infected with BVDV-LC. Hybridization signals are detected by anti-BVDV antibody (arrows). F: Section of heart of a mock-infected calf. There is no signal

predominant and widespread in countries neighboring China, such as Korea and Japan^[29,30], whereas, in China and India, BVDV 1b was the predominant subgenotype^[23,31]. Four isolates of BVDV, the Shihezi148-1, Changji, Kuerle, and SD0803 viruses, were recently isolated from a dairy in northwestern China. All four isolates were classified as belonging to a potentially novel subgenotype, and a partial 5'-UTR sequence of each isolate was obtained. To determine the genetic relationship between the BVDV-LC virus and these novel subgenotypes, a phylogenetic tree was constructed based on the 5'-UTR sequences, which showed a single branch containing four isolates clustered with the BVDV-LC virus (Fig. 1B). The 5'-UTR of the SD0803 virus shared a high degree of sequence homology (98.5%) with the SD0803 viruses, which indicates that these viruses should thus be classified as strains of the BVDV-1q subgenotype. Similar to the phylogenetic tree based on the N^{pro} sequences, the BVDV-1q branch was most closely related to the BVDV-1m, BVDV-1o, BVDV-1p, and BVDV-1g

clusters in the 5'-UTR-based phylogenetic tree.

In this study, the pathogenesis of BVDV-LC was studied and only mild clinical symptoms associated with BVDV infection were observed. These included depression, fever, leukopenia, and viremia. Calves infected with the virus showed no symptoms of diarrhea. Furthermore, the experimental animals showed no secondary infections with other pathogenic microorganisms such as pasteurellosis or *Escherichia coli*. Histopathological observations showed lymphoid depletion and shedding of the villi of the small intestine. Many studies have reported that animals infected with non BVDV show leukocyte depletion, failure to induce the production of type I interferon α/β , immune suppression, and often secondary infections^[32-35].

In summary, a BVDV-LC strain was successfully isolated from cattle in northwest China. We determined the complete genome sequence of the cattle-derived LC strain of BVDV-1 and phylogenetic and sequence analyses based on the N^{pro} gene and the 5'-UTR showed that the BVDV-LC strain belongs to the BVDV-1q subgenotype. This is the first report of the genomic sequence of a BVDV-1q virus isolated from cattle. Calves

inoculated with this isolate showed mild clinical signs, a high rectal temperature, and lymphopenia, which suggested that the strain remains virulent and can be used as a BVDV challenge virus to evaluate the efficacy of BVDV vaccines. This report forms a solid basis for further studies on BVDV in China.

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