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Isolation and Characterization of a Bovine Viral Diarrhea Virus Subgenotype 1b Strain from Diarrheal Cattle in Inner Mongolia, China

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Abstract

Bovine viral diarrhea virus (BVDV) is a major pathogen threatening the global cattle industry, causing severe economic losses. In China, BVDV is widely prevalent, with BVDV-1 being the main circulating genotype. Herein, a cytopathic (CP) BVDV strain, designated BVDV-HH03, was isolated and identified from diarrheic calves on a dairy farm in Hohhot, Inner Mongolia, via RT-PCR, Madin–Darby Bovine Kidney (MDBK) cell isolation, and neutralization assays. Subsequent whole-genome sequencing and phylogenetic analysis identified the isolate as belonging to the BVDV-1b subgenotype, sharing the highest nucleotide identity (>99%) with the BVDV-1 Nebraska strain isolated in the USA in 1990. Preliminary calf infection experiments indicated that BVDV-HH03 exhibited low pathogenicity under the experimental conditions. Two groups of 2-month-old calves received BVDV-HH03 inoculation by the combined intranasal–oral route; each group received inoculum with a titre of $10^{6.5}$ or $10^{7.5}$ $TCID_{50}$/mL at an inoculation volume of 7 mL per calf. No obvious clinical signs or fever were observed in inoculated calves throughout the 14-day monitoring period. Nevertheless, all infected calves showed decreased peripheral white blood cell counts within the 8-dpi monitoring period, with a maximum reduction exceeding 40%, and viremia was detected in 3/5 and 4/5 animals from the low- and high-dose inoculation groups by RT-PCR, respectively. This study represents the first isolation of BVDV-1b in Inner Mongolia, providing valuable data for the prevention and control of BVDV, as well as for further research on the epidemiology and pathogenicity of BVDV-1b.

Keywords: bovine viral diarrhea virus; BVDV-1; virus isolation; molecular characterization; pathogenicity

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1. Introduction

Bovine viral diarrhea virus (BVDV) is the causative agent of bovine viral diarrhea (BVD), a contagious cattle disease with severe economic impacts on global livestock production [1]. Typical clinical signs of BVD include fever, enteritis (diarrhea), respiratory disorders (coughing), reproductive failure (abortion or fetal malformations) and leukopenia; however, infected cattle are often asymptomatic. BVDV infection induces immunosuppression in cattle, which increases susceptibility to other viral and bacterial pathogens, impairs production performance, and elevates mortality rates [2–4]. In addition, fetal intrauterine BVDV infection occurring during the first trimester may lead to the delivery of persistently infected (PI) calves with immunotolerance [5]. Such PI animals continuously excrete high

viral loads throughout their lifetimes and serve as the primary reservoir for transmitting BVDV to susceptible herds [6].

BVDV, a member of the genus Pestivirus in the family Flaviviridae, possesses a positive-sense single-stranded RNA genome of 11.3–13.0 kb [7,8]. The genome consists of a single large open reading frame (ORF) that encodes a polyprotein of approximately 3900 amino acids (aa). This ORF is flanked by 5′ and 3′ noncoding regions (NCRs; i.e., 5′UTR and 3′UTR) [9,10]. This polyprotein is proteolytically processed by host cellular and viral proteases to yield four structural proteins and eight non-structural proteins [11]. Historically, based on genetic and antigenic variations, BVDV was classified into three genotypes, namely BVDV-1, BVDV-2 and BVDV-3 (the latter is also termed the HoBi-like pestivirus). Under current ICTV taxonomy, these agents correspond to distinct species: Pestivirus bovis (BVDV-1), Pestivirus tauri (BVDV-2), and Pestivirus brazilense (HoBi-like pestivirus), respectively. For consistency with the established literature, we use the traditional common names (BVDV-1, BVDV-2, and BVDV-3) throughout this manuscript. BVDV-1 is further subdivided into at least 25 subtypes, including 1a–1x as well as the porcine-isolated strain ZM-95 [6,12–17], whereas BVDV-2 and BVDV-3 each contain five subtypes (2a–2e and 3a–3e, respectively) [18–22]. BVDV-1 is widespread worldwide, with particularly high circulation in Asia. In contrast, the distribution of BVDV-2 is more restricted, while BVDV-3 is endemic in a limited number of countries, such as Bangladesh, India, and Russia [1,6]. In China, BVDV-1 is the predominant circulating genotype, covering multiple subtypes including 1a, 1b, 1c, 1d, 1m, 1q, 1v, 1u, and 1w [8,12,15,23–25]; by contrast, BVDV-2 and BVDV-3 have also been reported but only sporadically [8,23,26–28]. The high genetic diversity inherent to BVDV leads to constant shifts in viral antigen profiles, which poses great challenges to reliable diagnostic assays and effective vaccine-based prevention and control of BVDV infection [29–31].

In vivo, BVDV exhibits broad tissue distribution and cellular tropism; its common target cells include lymphocytes, epithelial cells, neurons, oocytes and macrophages [11]. In vitro, BVDV can infect various bovine primary cells, such as bovine uterine endometrial cells, bovine testis cells, bovine kidney cells, bovine turbinate cells, bovine bronchial epithelial cells, bovine lung cells, and bovine skin fibroblast cells (review in [32]). Beyond primary cells, the immortalized Madin–Darby bovine kidney (MDBK) cell line also supports BVDV replication and is widely used for BVDV isolation and propagation [26,29,33–36]. Based on its ability to induce visible cytopathic effects (CPE) in cultured cells, BVDV can be classified into cytopathic (CP) and non-cytopathic (NCP) biotypes. Among field clinical isolates, NCP BVDV predominates, whereas CP BVDVs are infrequently detected [37,38]. In this study, a BVDV-1b strain designated BVDV-HH03 was isolated from diarrheic cattle in Hohhot, Inner Mongolia, China. Its biological characteristics were comprehensively characterized both in vitro and in vivo. In vitro assays confirmed that BVDV-HH03 belongs to the cytopathic biotype, while animal experiments revealed that this strain exhibits low pathogenicity to cattle under the experimental conditions tested.

2. Materials and Methods

2.1. Sample Collection and Cells

Serum samples were collected from eight diarrheic calves, aged approximately 2–3 months, at a dairy farm in Hohhot, Inner Mongolia, China.

The Madin–Darby bovine kidney (MDBK) cell line was purchased from the YSK BIOSCIENCES Co., Ltd. (Shenzhen, China). Cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Globe Kang, Qinhuangdao, China) and 1% penicillin–streptomycin (Gibco, Waltham, MA, USA).

2.2. Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total viral RNA was extracted from samples using an RNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China) following the manufacturer's instructions. BVDV detection was carried out by one-step RT-PCR with TransScript One-Step RT-PCR SuperMix (TransGen Biotech Co., Ltd., Beijing, China), according to a previously reported study [39]. The specific primer pairs targeting 5′UTR of BVDV were as follows: forward primer (Left primer) 5′-CATGCCCATAGTAGGAC-3′ and reverse primer (Right primer) 5′-CCATGTGCCATGTACAG-3′. The expected size of the amplified target fragment was 288 bp. Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The PCR products were analyzed by 1% agarose gel electrophoresis.

2.3. Virus Isolation

For BVDV isolation, BVDV PCR-positive serum samples were first filtered using a 0.22 μm filter. Then, 200 μL of the filtrate was inoculated onto MDBK cell monolayers and incubated for 2 h at 37 °C under 5% CO_2. Subsequently, the inoculum was removed, and the cell monolayers were washed twice with phosphate-buffered saline (PBS). Fresh DMEM containing 2% heat-inactivated FBS (Globe Kang, China) was added, and cell cultures were maintained at 37 °C under 5% CO_2 for 5 days with daily observation of cytopathic effects (CPE) under an inverted microscope. Uninoculated MDBK cells were maintained in parallel as negative controls throughout the experiment. Culture supernatants were harvested and inoculated onto fresh MDBK cell monolayers, and this procedure was repeated for a total of three consecutive passages. The cell supernatant from the third passage was subjected to RT-PCR for BVDV identification, as described in Section 2.2, and served as the virus stock, and this virus was designated BVDV-BHH03.

2.4. Viral Titration

The 50% tissue culture infectious dose ($TCID_{50}$) of the BVDV-BHH03 was determined using MDBK cells cultured in 96-well plates. The virus stock was serially 10-fold diluted in serum-free DMEM, ranging from 10^{-2} to 10^{-9}. Subsequently, eight replicate wells of MDBK cell monolayers were each inoculated with 100 μL of the respective viral dilution, the plates were incubated at 37 °C under 5% CO_2 for 1 h. Following incubation, 100 μL of maintenance medium supplemented with 2% FBS was added to each well. The plates were further incubated for an additional 5 days, and the occurrence of CPE was observed and recorded daily. The $TCID_{50}$ value was calculated by the Reed–Muench method.

2.5. Determination of Viral One-Step Growth Curve

MDBK cells were infected with BVDV-HH03 at a high multiplicity of infection (MOI = 10) in 25 cm^2 cell culture flasks. After 1 h of viral adsorption at 37 °C, the inoculum was removed, and the cell monolayers were washed twice with serum-free DMEM to eliminate residual unbound virus. Fresh maintenance medium was supplemented, and the cells were continuously cultured. Culture supernatants were collected at 12 h intervals from 0 h to 96 h post-infection (hpi). Viral titers were determined via the $TCID_{50}$ assay as described in Section 2.4.

2.6. Virus Neutralization Test (VNT)

BVDV-HH03 was diluted in serum-free DMEM to obtain a concentration of 100 $TCID_{50}$/0.1 mL. The BVDV-antibody-positive serum (purchased from the China Institute of Veterinary Drug Control, IVDC; internal reference No. Z57) was heat-inactivated at 56 °C for 30 min prior to use, and then two-fold serially diluted from 1:16 to 1:2048. An equal volume of diluted virus was mixed with each serum dilution, and the mixtures were

incubated at 37 °C for 1 h. Each virus–serum mixture was inoculated into six replicate wells of MDBK cell monolayers in 96-well plates at 200 μL per well. The plates were incubated at 37 °C under 5% CO_2 level for 5 days, and the number of wells showing CPE in each group was observed. In addition, three control groups were included: a BVDV-negative serum control (serum mixed with virus), a mock control (cells cultured with medium only), and a virus-only control (cells inoculated with virus without serum).

2.7. Whole-Genome Sequencing and Phylogenetic Analysis

Viral RNA from BVDV-HH03 was extracted using the TIANamp Virus DNA/RNA Kit (Tiangen Biotech Co., Ltd., Beijing, China; Cat. #DP315). RNA purity was verified with an OD_{260}/OD_{280} ratio of 1.8–2.1, and DNase I treatment was performed to eliminate residual genomic DNA. Random primers were used for reverse transcription. The resulting cDNA was amplified with random primers prior to WGS library preparation. Whole-genome sequencing of BVDV-HH03 was performed on the Illumina NovaSeq 6000 PE150 platform at Sangon Biotech Co., Ltd. (Shanghai, China). Raw reads were cleaned and assembled de novo using SPAdes (v3.14.1) and SOAPdenovo (v2.04). Depth statistics were performed on the assembled contigs to evaluate the assembly quality and coverage uniformity, considering only contigs ≥1500 bp in length. Finally, gene functional annotation of the assembly results was conducted using Prokka (v1.14.5). Subsequently, the homology of the obtained BVDV-HH03 sequence with reference sequences published in GenBank was analyzed. Specifically, the assembled nucleotide sequences were subjected to BLASTn, while the translated amino acid sequences from the predicted large ORF was analyzed via BLASTp to determine the genetic homology, identify the closest matching BVDV isolates. BVDV-HH03 recombination analysis was performed using the Recombination Detection Program (RDP) (versions 5.93). A maximum-likelihood phylogenetic tree was constructed in MEGA 11 using the Tamura–Nei model with 1000 bootstrap replicates. The analysis included 38 BVDV-1 full-genome sequences: BVDV-HH03 generated in this study, plus 37 reference sequences downloaded from GenBank. This dataset contained 20 BVDV-1b sequences, together with at least one representative full-genome sequence for each of the BVDV-1 subgenotypes 1a, 1c, 1d, 1e, 1f, 1h, 1i, 1j, 1k, 1m, 1n, 1o, 1q and 1u. Only bootstrap values >70% are shown on the tree.

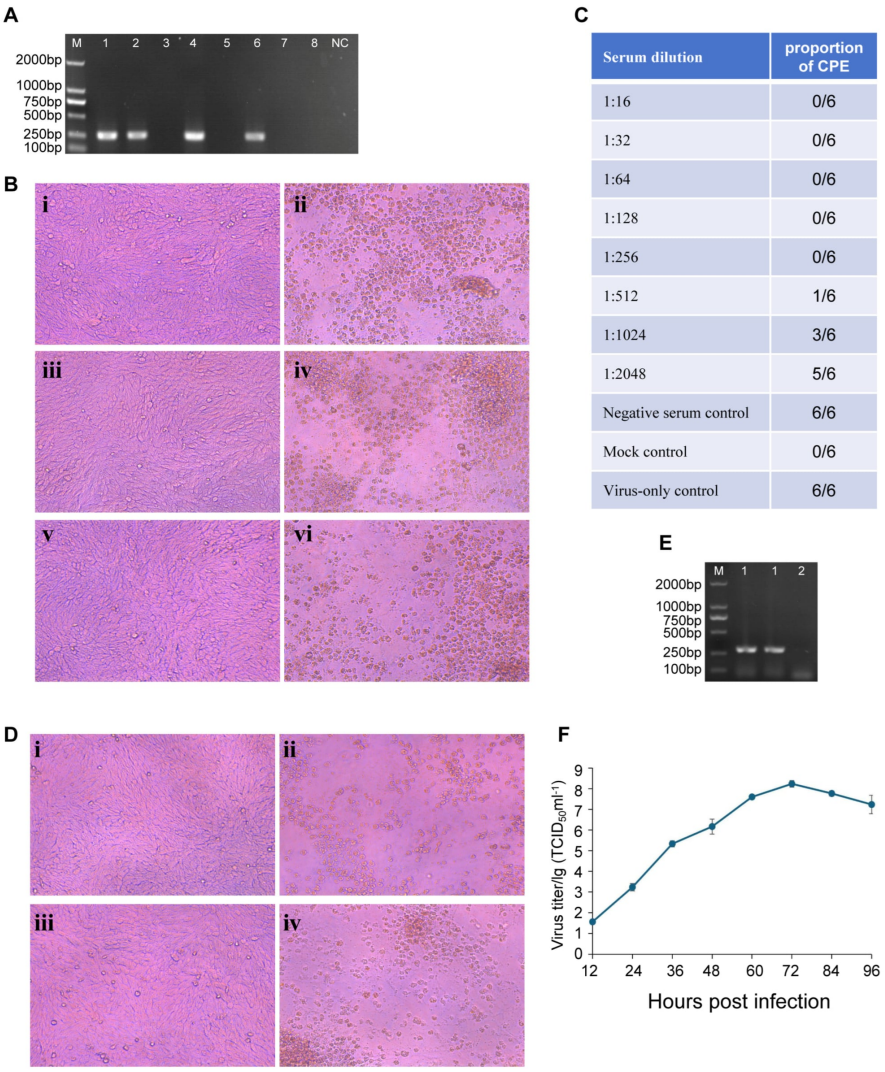
2.8. Calf Infection Experiment

Thirteen clinically healthy female Holstein calves, with an average age of 76 ± 4.5 days, were selected from a dairy farm in Hohhot, China. Prior to the experiment, all animals tested negative for BVDV antigens and antibodies in serum using ELISA kits (Idexx Laboratories, Inc., USA; Cat, No. 99-43830). The calves were divided into three groups: a high-dose infection group ($n = 5$), a low-dose infection group (n = 5), and a control group ($n = 3$). Calves in the two infection groups were inoculated with BVDV-HH03 via a combined intranasal and oral route, each calf received 3.0 mL of viral suspension intranasally and 4.0 mL of viral suspension via oral gavage. The viral titers of the stock for the high-dose and low-dose groups were $10^{7.5}$ and $10^{6.5}$ $TCID_{50}$/mL, respectively. The control group received an equivalent volume of sterile saline via the same combined intranasal and oral routes. To establish baseline health status and monitor disease progression, rectal temperatures and clinical signs were monitored continuously from 3 days prior to infection through 14 days post-infection (dpi). Additionally, peripheral blood samples were collected daily for the first 8 dpi to analyze white blood cell counts and detect serum viral RNA levels using RT-PCR.

3. Results

3.1. Isolation and Identification of BVDV

Serum samples were collected from eight calves presenting with severe diarrhea. RT-PCR was performed using primers capable of amplifying both BVDV-1 and BVDV-2, as described previously [39], and four samples tested positive for BVDV (Figure 1A).



Serum dilution	proportion of CPE
1:16	0/6
1:32	0/6
1:64	0/6
1:128	0/6
1:256	0/6
1:512	1/6
1:1024	3/6
1:2048	5/6
Negative serum control	6/6
Mock control	0/6
Virus-only control	6/6

Figure 1. Isolation and identification of BVDV in MDBK cells. (**A**) RT-PCR amplification of BVDV from eight bovine serum samples Lane M: DNA marker; Lanes 1–8: serum samples collected from eight cattle; NC: PCR negative control. (**B**) Virus isolation via three serial passages of serum sample 4 in panel A in MDBK cells. (**i**,**iii**,**v**): Passage 1, Passage 2, Passage 3; (**ii**,**iv**,**vi**): negative controls for Passage 1, Passage 2, Passage 3. (**C**) Proportion of wells displaying cytopathic effects (CPEs) in MDBK cells inoculated with the third-passage virus derived from serum sample 4 in panel A. Prior to inoculation, the virus was pre-incubated with BVDV antibody-positive serum at serial dilutions. Six replicate wells were tested for each serum dilution, and relevant control groups were included for comparison. (**D**) CPE observation in MDBK cells inoculated with the third-passage virus derived from serum sample 4 in panel A after pre-incubation with 1:256 diluted BVDV antibody-positive serum. (**i**), Mock control; (**ii**), virus pre-incubated with BVDV-negative serum; (**iii**), virus pre-incubated with 1:256 diluted BVDV-antibody-positive serum; (**iv**), virus-only group. (**E**) RT-PCR amplification of BVDV using supernatants from MDBK cells infected with the 3rd-passage virus derived from serum sample 4 in panel A. Lane M: DNA marker; Lane 1: supernatant from MDBK cells infected with the 3rd-passage virus; NC: supernatant from uninfected normal MDBK cells, as a negative control. (**F**) The one-step growth curve of BVDV-HH03 in MDBK cells.

One RT-PCR-positive serum sample (Sample 4) with the largest available volume was inoculated onto MDBK cell monolayers and blindly passaged three times, with visible CPEs observed at 4–5 dpi in every passage (Figure 1B). The third-passage virus was then used in a VNT to further confirm that the isolated pathogen was BVDV. Viral supernatant was pre-incubated with serially diluted BVDV-antibody-positive serum prior to inoculation onto MDBK cells. No CPEs were observed in this neutralization group at serum dilutions ≤ 1:256, indicating complete neutralization (Figure 1C,D). No CPEs were detected in the uninfected normal cell control (Mock control). In contrast, typical CPEs developed in the virus-only Control and negative serum control (viral supernatant incubated with BVDV-negative serum) (Figure 1D). These findings imply that the observed CPE was likely induced by BVDV-HH03 and may exclude contamination with unrelated adventitious pathogens.

RT-PCR was performed on the third-passage supernatant using the same primer set described above, and the target-specific band of expected size was clearly detected (Figure 1E), indicating successful isolation of BVDV. Hence, this isolate was designated BVDV-HH03. We also characterized the one-step growth curve of BVDV-HH03 in MDBK cells, with the viral titer reaching a maximum of 10^8 TCID50/mL at 72 h post infection (Figure 1F).

3.2. Genomic Characterization and Phylogenetic Analysis of BVDV-HH03

Whole-genome sequencing was performed on the Illumina NovaSeq 6000 PE150 platform to further characterize the genome of BVDV-HH03. The near-complete genome of this isolate, 12,297 nucleotides (nt) in length, was successfully assembled and uploaded to GenBank (GenBank accession number: PZ879143). Annotation of the genomic sequence identified a single major open reading frame (ORF) spanning 11,697 nt (381–12,077 nt), a partial 5′UTR of 380 nt (1–380 nt), and a partial 3′UTR of 220 nt (12,087–12,297 nt).

Web BLAST analyses of the full genome, ORF coding sequence, and 3′ UTR of BVDV-HH03 revealed that this isolate exhibited the highest nucleotide identity (>99% across all regions) to a non-cytopathic BVDV-1b strain Nebraska (MH231153.1) isolated in the USA in 1990 (Tables 1, 2 and S2). In contrast, the 5′ UTR of BVDV-HH03 shared the greatest nucleotide identity of 99.09% with a BVDV-1 strain CNIM-2202 (GenBank accession number: PP920639.1) isolated from China in 2022, for which neither the genetic subtype nor biotype has been annotated (Table S3). Accordingly, recombination analysis using RDP software was performed to examine whether BVDV-HH03 originated from a recombination event between BVDV-1 Nebraska and BVDV-1 CNIM-2202. BVDV-1 Draper (GenBank accession number: KC963967.1), another BVDV-1b strain sharing high full-genome nucleotide identity with BVDV-HH03 (Table 1), together with four Chinese BVDV-1b isolates (GenBank accession numbers: PP488512.2, PP770551.1, KF772785.1, and KF501393.1), were also included in this analysis. As shown in Figure 2, the majority of the BVDV-HH03 genome, including the major ORF, the 3′ UTR, and partial 5′UTR (1–63 nt and 313–339 nt) sequences, exhibited high sequence similarity to the BVDV-1b strain Nebraska, whereas the remaining 5′ UTR region (64–312 nt) displayed significantly higher similarity with the BVDV-1 strain CNIM-2202, suggesting a possible recombination event between these two parental strains.

We further performed BLAST analysis on the amino acid sequence encoded by the BVDV-HH03 ORF. The results showed that this sequence shared the highest amino acid identity of 99.05% with BVDV-1 Nebraska (Table 3). Sequence alignment revealed 36 amino acid substitutions in the polyprotein between BVDV-HH03 and BVDV-1 Nebraska (Figure 3A). Notably, among the top 20 isolates with the highest identity with BVDV-HH03, all were derived from the USA except one originating from Japan (Table 3).

Table 1. Nucleotide identities of the full genome of BVDV-HH03 with the top 20 matched reference strains based on BLAST analysis.

Accession	Identity (%)	Genotype/ Subgenotype	Collection Year	Collection Location
MH231153.1	99.35	BVDV-1/1b	1990	USA
PV626360.1	94.32	BVDV-1/1b	1993	USA
PV626358.1	93.95	BVDV-1/1b	1986	USA
KC963967.1	93.83	BVDV-1/1b	2012	South Korea
PV346712.1	93.2	BVDV-1/-	2022	Senegal
PV346698.1	93.06	BVDV-1/-	2022	Senegal
PV626373.1	93.6	BVDV-1/1b	1989	USA
PV626374.1	93.56	BVDV-1/1b	1992	USA
PV346708.1	92.98	BVDV-1/-	2022	Senegal
PV346713.1	92.88	BVDV-1/-	2022	Senegal
PP770551.1	93.82	BVDV-1/-	2023	China
PV626355.1	93.86	BVDV-1/1b	-	USA
PV346706.1	92.8	BVDV-1/-	2022	Senegal
PV346707.1	92.76	BVDV-1/-	2022	Senegal
PV346711.1	92.75	BVDV-1/-	2022	Senegal
PV346710.1	92.66	BVDV-1/-	2022	Senegal
PV346709.1	92.67	BVDV-1/-	2022	Senegal
PV346714.1	92.61	BVDV-1/-	2022	Senegal
PV346715.1	92.62	BVDV-1/-	2022	Senegal
KP941587.1	93.62	BVDV-1/-	2014	USA

Note: - indicates that relevant information is not annotated.

Table 2. Nucleotide identities of the ORF coding sequence of BVDV-HH03 with the top 20 matched reference strains based on BLAST analysis.

Accession	Identity (%)	Genotype/ Subgenotype	Collection Year	Collection Location
MH231153.1	99.57	BVDV-1/1b	1990	USA
PV626360.1	94.55	BVDV-1/1b	1993	USA
PV346712.1	93.37	BVDV-1/-	2022	Senegal
PV346698.1	93.25	BVDV-1/-	2022	Senegal
PV346708.1	93.14	BVDV-1/-	2022	Senegal
PV346713.1	93.06	BVDV-1/-	2022	Senegal
PV626358.1	94.23	BVDV-1/1b	1986	USA
PV346706.1	92.97	BVDV-1/-	2022	Senegal
PV346711.1	92.93	BVDV-1/-	2022	Senegal
PV346707.1	92.94	BVDV-1/-	2022	Senegal
PV346710.1	92.82	BVDV-1/-	2022	Senegal
PV346709.1	92.83	BVDV-1/-	2022	Senegal
PV346714.1	92.79	BVDV-1/-	2022	Senegal
PV346715.1	92.79	BVDV-1/-	2022	Senegal
PP770551.1	94.04	BVDV-1/-	2023	China
PV626355.1	94.02	BVDV-1/1b	-	USA
JX419398.1	93.97	BVDV-1/-	2008	Japan
JX419397.1	93.95	BVDV-1/-	2008	Japan
KT355592.1	93.83	BVDV-1/1b	-	USA
KC963967.1	93.83	BVDV-1/1b	2012	South Korea

Note: - indicates that relevant information is not annotated.

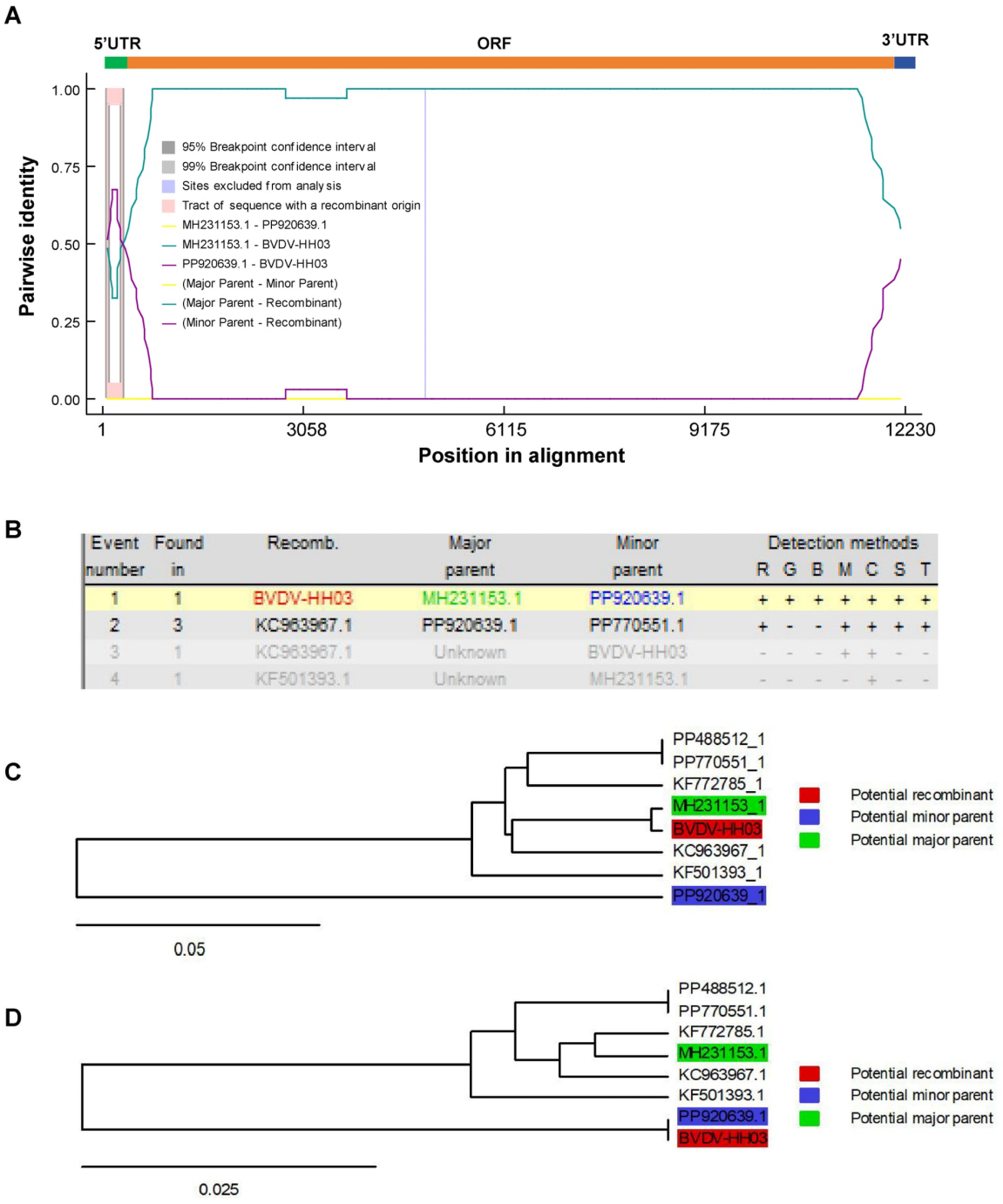


Figure 2. Recombination analysis of the BVDV-HH03 genome based on eight BVDV-1 genomic sequences. (**A**) Recombination detection plot based on pairwise distance identity, showing recombination signals within viral genomic sequences of the indicated BVDV-1 strains. (**B**) Summary of recombination events detected in BVDV-1 genomic sequences, as shown in panel (**A**). The abbreviations R, G, B, M, C, S, and T represent seven independent recombination detection algorithms implemented in RDP5, namely RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, and 3Seq, respectively. (**C**) Unweighted Pair-Group Method with Arithmetic Means (UPGMA) phylogenetic tree based on nucleotides 1–63 and 313–12,230 corresponding to the BVDV-HH03 genome. (**D**) UPGMA phylogenetic tree of nucleotides 64–312 corresponding to the BVDV-HH03 genome. Both trees (**B**,**C**) were constructed with the indicated BVDV-1 reference strains.

Table 3. Amino acid identities of the deduced amino acid sequence from the ORF of BVDV-HH03 with the top 20 matched reference strains based on BLAST analysis.

Accession *	Identity (%)	Genotype/ Subgenotype	Collection Year	Collection Location
AZQ00677.1/MH231153.1	99.05	BVDV-1/1b	1990	USA
XTP83278.1/PV626377.1	96.65	BVDV-1/1b	1989	USA
XTP83268.1/PV626360.1	96.1	BVDV-1/1b	1993	USA
XKQ99521.1/PQ613796.1	95.9	BVDV-1/-	-	USA
XKQ99523.1/PQ613798.1	95.85	BVDV-1/-	2021	USA
XTP83277.1/PV626374.1	95.8	BVDV-1/1b	1992	USA
XTP83274.1/PV626368.1	95.8	BVDV-1/1b	1986	USA
XKQ99519.1/PQ613794.1	95.8	BVDV-1/-	-	USA
XTP83266.1/PV626358.1	95.8	BVDV-1/1b	1986	USA
XTP83276.1/PV626373.1	95.72	BVDV-1/1b	1989	USA
ADX31693.1/HQ174294.1	95.72	BVDV-1/1b	-	USA
AWW87353.1/MG950351.1	95.69	BVDV-1/1b	2015	USA
QLB38953.1/MT265675.1	95.69	BVDV-1/1b	-	USA
BDR06656.1/LC727644.1	95.69	BVDV-1/-	2016	Japan
AFC37640.1/JN380088.1	95.68	BVDV-1/1b	-	USA
AWW87371.1/MH311876.1	95.67	BVDV-1/1b	2016	USA
AWW87372.1/MH311877.1	95.67	BVDV-1/1b	2016	USA
AWW87352.1/MG950350.1	95.67	BVDV-1/1b	2015	USA
AWW87369.1/MH311874.1	95.67	BVDV-1/1b	2016	USA
XTP83263.1/PV626355.1	95.67	BVDV-1/1b	-	USA

Note: * Accession lists the protein accession of the reference sequence and its corresponding BVDV nucleotide DBSOURCE accession; - indicates that relevant information is not annotated.

To determine the genetic subtype of BVDV-HH03, a phylogenetic tree was constructed based on the complete genome sequences of representative BVDV-1 strains. As illustrated in Figure 3B, the BVDV-HH03 isolate clustered tightly with multiple reference strains of the BVDV-1b subtype, among which 15 sequences were from Chinese field isolates, within an independent monophyletic clade, confirming that BVDV-HH03 belongs to the BVDV-1b subtype.

A

position	160	288	344	410	466	605	664	704	742	945	983	1022
Nebraska	N	R	H	K	G	A	A	I	D	G	E	L
BVDV-HH03	H	S	Y	E	E	P	P	L	V	D	A	I
position	**1063**	**1106**	**1162**	**1225**	**1363**	**1423**	**1482**	**1543**	**1583**	**1643**	**1783**	**2384**
Nebraska	M	K	V	V	P	E	K	S	H	T	S	Q
BVDV-HH03	V	R	G	G	A	A	N	R	Y	S	N	H
position	**2723**	**2824**	**2904**	**3020**	**3048**	**3143**	**3263**	**3408**	**3462**	**3522**	**3608**	**3683**
Nebraska	D	D	L	E	G	H	K	N	K	S	S	V
BVDV-HH03	G	G	R	D	R	P	T	T	N	I	I	D

Figure 3. *Cont.*

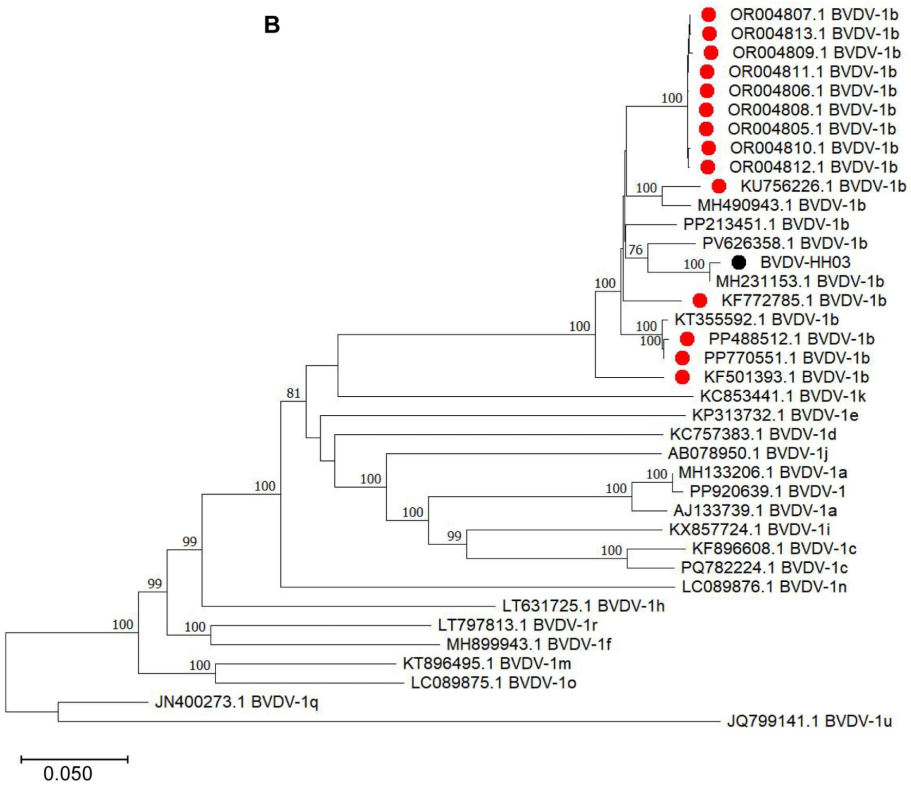
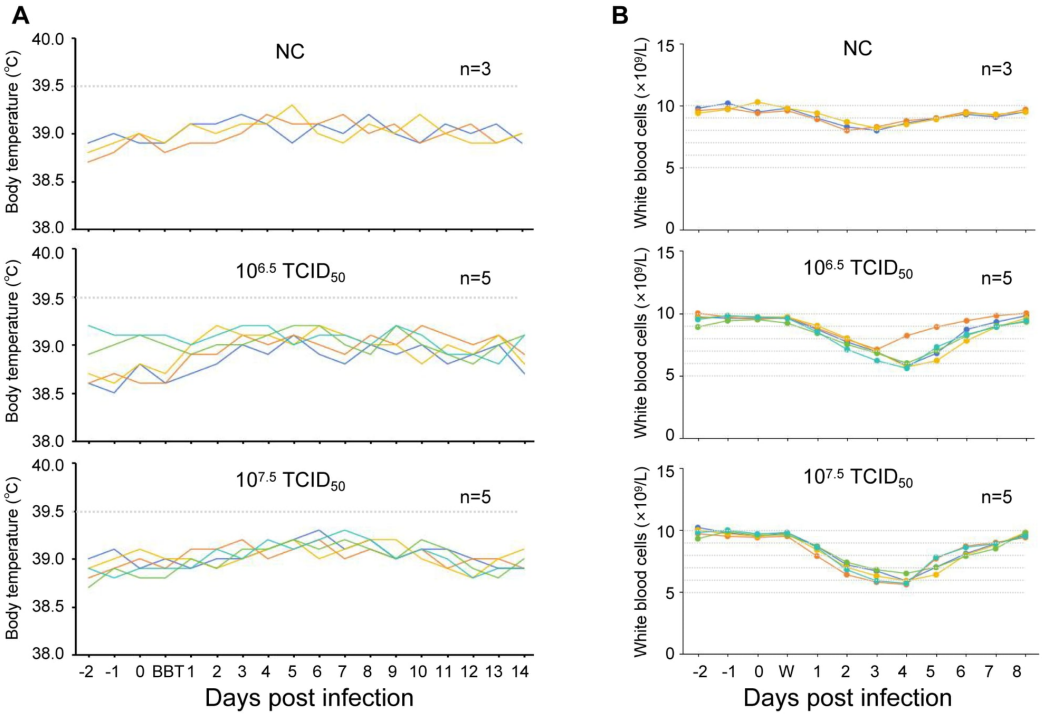


Figure 3. Genomic characterization of the BVDV-HH03. (**A**) Amino acid substitutions in the polyprotein between BVDV-HH03 and the BVDV-1 Nebraska strain. (**B**) A phylogenetic tree was constructed in MEGA 11 using the Maximum Likelihood method and Tamura–Nei model based on 38 complete BVDV-1 genome sequences. The black dot indicates the BVDV-HH03 sequence; the red dots indicate BVDV-1b sequences from Chinese isolates; accession numbers correspond to other BVDV-1 subgenotype sequences retrieved from GenBank. Bootstrap values above 70% are indicated.

3.3. Calf Infection Experiment

To evaluate the virulence of BVDV-HH03, two groups of calves (n = 5 per group) were inoculated with BVDV-HH03 via a combined intranasal and oral route. The two groups received virus stocks at titers of $10^{6.5}$ and $10^{7.5}$ $TCID_{50}$/mL, respectively. Briefly, each calf received 3.0 mL of viral suspension intranasally and 4.0 mL via oral gavage. An additional three calves were inoculated with sterile normal saline according to the identical inoculation protocol and served as mock controls. No obvious clinical signs were observed throughout the 14 dpi monitoring period, and measured rectal temperatures of all calves stayed within the normal physiological range for cattle (38.0–39.5 °C) (Figure 4A). In addition, hematological analysis of peripheral blood total leukocytes was performed within 8 dpi. As shown in Figure 4B, leukocyte counts gradually declined after virus inoculation in the BVDV-HH03-inoculated groups, reaching the lowest level at 3–4 dpi with an approximately 40% maximum reduction, followed by a gradual recovery. In contrast, only mild fluctuations in leukocyte counts were detected in the mock control group throughout sampling, with the maximum reduction below 20%.



C

days	$10^{7.5}$ TCID50					$10^{6.5}$ TCID50					NC		
	1	2	3	4	5	6	7	8	9	10	11	12	13
0	-	-	-	-	-	-	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	+	+	+	-	-	-	-	+	-	-	-
4	+	-	+	+	-	+	-	+	-	+	-	-	-
5	+	-	+	+	+	+	-	+	-	-	-	-	-
6	+	-	-	+	-	-	-	+	-	-	-	-	-
7	+	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 4. Characteristics of clinical signs and virological profiles in calves experimentally infected with BVDV-HH03. Panels show rectal temperatures (**A**), peripheral white blood cell counts (**B**), and BVDV RT-PCR detection in serum samples (**C**) from calves inoculated with BVDV-HH03 at the indicated time points. The numbers $10^{6.5}$ and $10^{7.5}$ indicate the viral doses used for BVDV-HH03-inoculated calf groups; NC denotes the control group injected with sterile saline. Symbols "+" and "−" indicate RT-PCR-positive and RT-PCR-negative results, respectively. In panel (**A**), DBT (day-before-treatment) represents the mean rectal temperature measured 3 days prior to inoculation.

Furthermore, RT-PCR amplification of BVDV from serum samples was performed throughout the 8 dpi monitoring period to verify the establishment of BVDV-HH03 infection. As shown in Figure 4C, in the $10^{7.5}$ TCID50/mL infection group, BVDV was initially detectable in four animals from 3 to 4 dpi, with one animal persistently negative during the 8-day monitoring window; all animals in this group were RT-PCR negative at 8 dpi; in the $10^{6.5}$ TCID50/mL infection group, three animals yielded positive BVDV RT-PCR results starting at 3–4 dpi, and two animals remained negative throughout the 8-day monitoring window; all animals of this group tested BVDV-negative from 7 dpi. All animals in the

negative control (NC) group remained BVDV-negative at all time points. These results indicated that BVDV-HH03 could induce viremia in the majority of infected animals.

Collectively, these findings would suggest that BVDV-HH03 may cause subclinical infection under the present experimental conditions.

4. Discussion

BVDV is recognized as a major pathogen contributing to bovine respiratory disease complex worldwide and leads to substantial economic losses in the cattle industry [1]. In China, BVDV is widespread, and the main circulating BVDV strains belong to BVDV-1 [8,12,24]. In this study, a BVDV-1 strain was isolated from diarrheic cattle on a farm in Hohhot, Inner Mongolia, and designated BVDV-HH03. In vitro cell culture and whole-genome sequencing verified that BVDV-HH03 belongs to the BVDV-1b genotype and represents a CP biotype. Further in vivo infection experiments in calves suggested that this strain exhibits low virulence.

Previously, Xiao et al. performed a BVDV epidemiological survey of dairy cows in 13 northern Chinese provinces from June 2022 to June 2024 and confirmed that all prevalent strains were BVDV-1, with BVDV-1a, 1m and 1c as the dominant subgenotypes [24]. Another epidemiological investigation of BVDV in dairy cattle herds from five provinces in East China revealed that the prevailing subgenotypes were BVDV-1a and BVDV-1c [40], Consistently, a 2019 survey covering 17 dairy cattle, beef cattle and yak herds across four western Chinese provinces detected circulating BVDV-1a and BVDV-1c [41]. In addition, Deng et al. surveyed 92 large-scale dairy farms with more than 500 cattle in 19 provinces of China in 2017, and identified BVDV-1a, 1c, and 1m as the dominant strains [12]. Nevertheless, other BVDV-1 subgenotypes, including BVDV-1b, can still be sporadically detected in certain regions and cattle herds within China [1,12,15,34,35]. According to published global epidemiological data, BVDV-1b is the predominant subgenotype across the Americas, Asia and Europe worldwide [6]. In the present study, we isolated and identified a BVDV isolate of the CP biotype from a cattle herd in Inner Mongolia, China. Whole-genome sequencing and phylogenetic analysis confirmed that this isolate belongs to the BVDV-1b subtype. To our knowledge, this represents the first isolation of the BVDV-1b subgenotype in Inner Mongolia. Earlier studies have successfully isolated BVDV-1d and BVDV-2b in the same area [26,33], indicating considerable genetic diversity of circulating BVDV in this region.

Recently, two independent studies have reported the isolation of BVDV-1b strains in Hebei and Tianjin, respectively. The NCP BVDV-1b strain HB2411 was isolated from a dairy farm in Hebei, where affected cattle exhibited clinical signs including diarrhoea and abortion [36]. Meanwhile, nine BVDV-1b strains (GenBank numbers: OR004805-OR004813) comprising both CP and NCP biotypes were isolated from PI cattle suffering from mucosal disease in Tianjin [42]. BVDV-HH03 shared 93.16–93.28% genomic identity with these nine Tianjin strains (Table S1); whereas the complete genome sequence and accession number of HB2411 could not be found in the corresponding reference, making pairwise genomic comparison impossible. Interestingly, BVDV-HH03 shared the highest nucleotide identity (99.35%) with the BVDV-1b strain Nebraska, which was isolated in the USA in 1990. Nevertheless, distinct differences were still observed between the two strains, with 36 amino acid substitutions distributed throughout the polyprotein. This finding highlights the high genetic diversity characteristic of BVDV. Notably, the previously reported BVDV-1b strains from Tianjin and Hebei also possessed their highest nucleotide identity with the BVDV CP7 strain (U63479.1), isolated in the USA roughly 40 years ago [43,44]. A recent study identified BVDV-1b in commercially imported foetal bovine serum of Israeli origin [35]. Nevertheless, the origin tracing of Chinese BVDV-1b strains is a complex systematic task, and additional comprehensive epidemiological and molecular evidence is still required to validate the above hypothesis. Even so, these findings highlight the urgent

necessity of sustained surveillance for BVDV in imported cattle, foreign-derived FBS, semen, and embryos.

BVDV-free, seronegative calves aged 2–6 months represent a reliable animal model for evaluating the pathogenicity of BVDV. In this study, 2-month-old calves received BVDV-HH03 through combined intranasal and oral gavage administration. During the 14-day observation period, no obvious clinical signs or elevated body temperatures were detected in animals from the two infection groups and the control group. Nevertheless, peripheral blood leukopenia was observed in both infection groups, with a maximum reduction of approximately 40%, indicating that the strain can induce hematological injury. Additionally, BVDV viral nucleic acid was detectable in serum samples from infected animals. Specifically, viral RNA was positive in four animals within the $10^{7.5}$ $TCID_{50}$/mL infection group, whereas three animals tested positive in the $10^{6.5}$ $TCID_{50}$/mL infection group. The presence of persistently RT-PCR-negative individuals within both infection groups may be attributable to inter-individual variation among animals, the relatively weak infectivity of BVDV-HH03, or limited analytical sensitivity of the RT-PCR assay. In addition, as noted by WOAH, acute BVDV infection and viraemia may be transient, which highlights the requirement for serial sampling and multiple appropriately timed diagnostic endpoints. Collectively, these results suggest that BVDV-HH03 exhibits low pathogenicity under the experimental conditions tested. Nevertheless, this study has key limitations, including the small sample size, short observation period, lack of standardized clinical and pathological scoring, absence of histopathological examination, and insufficient statistical analysis of leukopenia. Further comprehensive investigations involving extended observation periods, additional inoculation doses, histopathological analyses, and larger numbers of experimental calves are still required to fully characterize its pathogenic features. Notably, although BVDV-HH03 was isolated from clinically diseased cattle, experimental infection of calves with this strain did not trigger obvious clinical signs. This apparent discrepancy may be explained by the hypothesis that the source animal was co-infected with other unidentified pathogens or diarrhoea-causing agents. We also noted that several reported BVDV-1b strains were isolated from clinically diseased cattle herds. Unfortunately, most relevant studies lack in vivo infection experiments in calves to characterize the virulence of these field isolates [36,42]. Hence, the pathogenicity of BVDV-1b requires further systematic evaluation. In contrast, the pathogenicity of strains belonging to other BVDV-1 subtypes (e.g., 1a, 1m, 1v) and BVDV-2 subtypes (e.g., 2b) have been assessed in cattle in previous studies, and certain strains have been confirmed to possess distinct pathogenic potential [26,34].

5. Conclusions

In summary, a BVDV-1b strain of the CP biotype was successfully isolated and identified from a cattle farm in Inner Mongolia in the present study. Whole-genome analysis revealed that this isolate shared the highest nucleotide homology with a strain isolated in the USA in 1990. Preliminary calf infection experiments indicated that this isolate was of low virulence. These findings provide valuable data for the prevention and control of BVDV infection, as well as further research on the epidemiology and pathogenicity of BVDV-1b.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/microorganisms14112480/s1: Table S1: BLAST search results for the full genome of the BVDV-HH03; Table S2: Nucleotide identities of the 3′UTR of BVDV-HH03 with the top 20 matched reference strains based on BLAST analysis; Table S3: Nucleotide identities of the 5′UTR of BVDV-HH03 with the top 20 matched reference strains based on BLAST analysis.

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contributed to conceptualization, constructive discussions, manuscript drafting, review and editing, and project administration. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of Inner Mongolia Huaxi Biotechnology Co., Ltd. (HXSW-GCP-LL-01).

Informed Consent Statement: Before sample and data collection, all animal owners were fully informed of the study's objectives, procedures, and potential implications, and oral informed consent was obtained from all eligible participants. Because most participants in the rural survey areas had limited formal education and local field conditions constrained on-site procedures, formal written consent could not be obtained from every individual during the investigation. Subsequently, written informed consent for sample use and publication was obtained from some animal owners through personally signed documents.

Data Availability Statement: The data that support the findings of this study are available in the Supporting Information of this article. The dataset presented in this study is openly available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The accession number is PZ879143.

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Conflicts of Interest: Author Xiaojun Zhao was employed by the company Inner Mongolia Yili Industrial Group Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Author Yueqiang Duan was employed by the company Inner Mongolia Huaxi Biotechnology Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

BVDV	Bovine viral diarrhea virus
CP	cytopathic
NCP	non-cytopathic
PI	persistently infected
ORF	open reading frame
MDBK	Madin–Darby bovine kidney
CPE	cytopathic effect

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