Virus Research 191 (2014) 101–107

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Characterization of an Enterovirus species E isolated from naturally infected bovine in China

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ARTICLE INFO

Article history:
Received 14 March 2014
Received in revised form 28 July 2014
Accepted 28 July 2014
Available online 5 August 2014

Keywords:
Bovine enteroviruses
Enterovirus species E
Random priming PCR
VP1
Phylogenetic trees
Suckling mouse

ABSTRACT

Bovine enteroviruses, which belong to the Picornaviridae family, can cause clinical symptoms in cattle and are excreted in feces. In this study, a cytolytic virus was isolated from Madin-Darby bovine kidney (MDBK) cells from fecal samples of bovine with severe diarrhea and hemorrhagic intestinal mucosa that had been originally diagnosed with bovine viral diarrhea (BVD) by a bovine viral diarrhea virus Ag point-of-care test (IDEXX, American). Random priming PCR was used to amplify underlying viral sequences and identify the isolated virus. Phylogenetic analysis indicated that the isolated virus closely matches the EV-E2 species, which is different from other Chinese strains previously isolated. The newly identified virus was named HLJ-3531/2013. We infected the sulking mice with the isolated virus. Reverse-transcription PCR, hematoxylin and eosin (HE) staining, serum neutralization (SN) test, and virus isolation from various tissues revealed that HLJ-3531/2013 can infect the intestine, liver, and lung of suckling mice. The present work is the first to report the reproduction of clinical symptoms by an isolated virus in an experimental infection model of animals and lays a solid foundation for the development of the pathogenesis of bovine enteroviruses.

1. Introduction

Bovine enteroviruses are members of family *Picornaviridae*, genus *Enterovirus*, which are consist of Enterovirus species E (EV-E) and Enterovirus species F (EV-F) according to the Ninth Report of the International Committee on Taxonomy of Viruses states (ICTV). Bovine enteroviruses are small (27–30 nm) non-enveloped RNA viruses possessing icosahedral capsids constructed from 60 copies of each of four structural proteins, VP1–4 (Smyth et al., 1995). The first bovine enteroviruses were collected in the late 1950s (Kunin and Minuse, 1958; McFerran, 1958; Moll and Davis, 1959); since then, many other bovine enteroviruses have been isolated and studied. The viruses are isolated from the feces of cattle with symptoms of pneumonia, respiratory disease, enteritis, dysentery, and fertility disorders; fetal fluids from an aborted calf; feces of apparently healthy animals; and sewage, treated effluents, and materials from a farm environment (Dunne et al., 1973, 1974; Weldon et al., 1979). Two isolates of EV-F (BHM26 and BJ50) have also been obtained from rectal swabs from diarrheic cattle of dairy herds in China (Li et al., 2012). Despite the large amount of information available on bovine enteroviruses, difficulties in reproducing clinical symptoms following experimental infection of animals had led to the conclusion that bovine enteroviruses are of only minor veterinary medical importance.

In the present study, several serum samples and fecal samples were obtained from different bovine diagnosed with BVD by BVDV Ag point-of-care test (IDEXX, American). BVDV Antigen Test Kit/Serum Plus (IDEXX) was failed to identify BVDV antigens in serum and enzyme-linked immunosorbent assay (I-ELISA) for the detection of BVDV antibodies in serum was also failed. A cytolytic virus was isolated from fecal samples, nested RT-PCR for BVDV failed to detect viral genomes. Random priming PCR strategy was used to amplify an unknown fragment of the virus, which was proven to be EV-E by sequence analysis. The VP1 gene has been cloned by specific primers and phylogenetic analyses support the belief that the isolated virus closely matches the EV-E2 species. Suckling mice were infected by the isolated virus. Preliminary identification by RT-PCR, HE staining, SN testing, and virus isolation from infected mouse tissues indicated that the isolated virus

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http://dx.doi.org/10.1016/j.virusres.2014.07.032
0168-1702/

can infect certain tissues of mice and cause pathological lesions of the respiratory and digestive tract. The findings in this study establish a solid theoretical foundation for studying the pathogenesis of bovine enteroviruses. Further studies on the pathogenesis of bovine enteroviruses in cattle are recommended.

2. Materials and methods

2.1. Samples, cell culture, and virus isolation

Ten fecal samples and nine bovine serum samples were obtained from different bovines with severe diarrhea and hemorrhagic intestinal mucosa. Bovines were provided by Animal Husbandry and Veterinary Institute, Shanxi Academy of Agricultural Sciences. Fecal samples were diluted (1:10) in 10 mM PBS with 1% antibiotics, frozen and thawed three times, clarified by low-speed centrifugation, filtered through 0.22 μm filters, and kept at −70 °C until use. Madin-Darby bovine kidney (MDBK) cells and baby hamster kidney (BHK-21) cells (both preserved by Veterinary Immunology and Biological Laboratory) were used to isolate the virus. These cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS and 1% antibiotics and kept at 37 °C with 5% CO_2. Exactly 0.025 mL of the filtrate was diluted (1:10) and inoculated with cells that had been grown in 6-well tissue-culture plates and washed three times with PBS. DMEM was added to the last well of the plate as a negative control. Exactly 2 mL of maintenance medium (with 2% FBS and 1% antibiotics) was added to the wells after adsorption for 1.5 h at 37 °C. The cultures were incubated at 37 °C with 5% CO_2. The virus was collected and passaged after 72 h of inoculation or development of 80% cytopathic effects (CPEs).

2.2. Virus purification

The virus was purified on BHK-21 monolayers following the method of Mowat and Chapman (1962). BHK-21 cells were grown on 6-well tissue-culture plates at 37 °C with 5% CO_2, washed with PBS, and then inoculated with the virus suspension diluted to 10^{-4}–10^{-8} by DMEM without FBS. The supernatant was discarded after 1–2 h, and cells were recovered using a mixture of sterilized low-melting point agarose (2.2 μg/mL) and 2× DMEM at a ratio of 1:1. The cultures were incubated in an inverted position with a solid overlay. Plaques were observed after 48 h and stained with neutral red for counting. A single plaque was chosen and placed in culture medium without FBS. This mixture was then inoculated in the BHK-21 monolayer cultures in 6-well tissue-culture plates after three cycles of freezing and thawing. The purified virus was obtained after three passages as described above.

2.3. Identification of viral nucleic acid type

The nucleic acid type of the virus was detected using a DNA inhibitor (5-bromo-2′-deoxyuridine, BRDU, SIGMA) after CPEs had become visible and stable (Rovozzo and Burke, 1973). Briefly, MDBK cells were seeded on 96-well tissue-culture plates at 37 °C with 5% CO_2, and then inoculated with the virus suspension (diluted to 100 TCID50) 100 μL per well after washed with PBS. The supernatant was discarded after 2 h, and cells were recovered with 100 μL maintenance medium contained 50 μg/mL BRDU. Infectious bovine rhinotracheitis virus (IBRV) and NADL strain of BVDV were respectively used as positive and negative controls, 50 μg/mL BRDU without infection of virus was used to detect cytotoxicity. The result was observed after 48–72 h.

2.4. Physical and chemical properties of the virus

Lipid solvent sensitivity of isolated virus was assayed a described previously (Andrewes and Horstmann, 1949) following the treatment of 1 mL virus suspensions with 0.5 mL ether or chloroform for overnight at 4 °C, then centrifuge at 3000 × g for 30 min. Sensitivity to 0.25% trypsin was assayed following the treatment of 1 mL virus suspensions with an equal volume of 0.5% trypsin for 1 h at 37 °C. The stability of the virus toward varying pH (pH 3.0, 5.0, 9.0, and 10.0) for 1 h at 37 °C (Tyrrell and Chanock, 1963). Viral heat sensitivity was determined by mixing 1 mL of each virus suspension with an equal volume of 2 M $MgSO_4$ and incubation of each suspension at 50 or 56 °C for 30 min (Moll and Davis, 1959). In the control group, 2 M $MgSO_4$ was replaced by minimal essential medium. Viral titer of the each treated suspension was determined by the inoculation of 10-fold serial dilutions of each treated viral suspensions on BHK21 cells. The size and morphology of the virus were determined using an electron microscope.

Table 1
Primer sequences used for amplification of EV-E/HLJ/3531/2013 strain.

Primer name	Sequence (5′–3′)
Random 1[a]	ATGCCGCCGCCGCTCCGTCT
Random 2[a]	GGGTTGAACGGGACAGAG
BEV-S	ATTTACCCAACCAATTGCG
BEV-A	CGATGGTTACGGTGATTGG
5′UTR-F	GGGGAGTAGTCCGACTCCGC
5′UTR-R	CRGAGCTACCACYGGGDWTGTGG
VP1-S	CCATGTGGTAYCARACIAAYATGGT
VP1-A	GATTGCCAIACTTCATTYTCCCA

[a] Random 1 and 2 were designed for an unknown fragment amplification; primer pairs of BEV-S and BEV-A were designed for other four samples; primer pairs of 5′UTR-F and 5′UTR-R were designed for amplification of 5′UTR and amplification of mice tissues; primer pairs VP1-S and VP1-A were designed for amplification of VP1 gene.

2.5. Random priming PCR and nucleotide sequence analyses

Viral RNA was extracted from the supernatants of virus-infected cells using a viral RNA extraction kit (OMEGA, American). RT-PCR was performed using M-MLV 1st strand cDNA Synthesis Kit (TaKaRa, Japan) with random primers 1 and 2 (Table 1) following the manufacturer's instructions. Exactly 2 μL of cDNA was used for random priming PCR. PCR products were analyzed by electrophoresis in 1% agarose gel and purified using a DNA gel extraction kit (TIANGEN, Beijing). The purified products were then ligated into pMD-18T simple vector (TaKaRa, Japan) and transformed into *Escherichia coli* TG1 via a standard procedure.

The extracted RNA samples were also amplified by RT-PCR as described above using primers that designed for the conserved regions of published EV-E and EV-F 5′UTR (Ley et al., 2002) and VP1 sequences (Knowles, 2005). The VP1 sequence was subjected to BLAST analysis and aligned with the help of Clustal X (Thompson et al., 1997). Phylogenetic trees were constructed by the neighbor-joining method using Molecular Evolutionary Genetics Analysis (MEGA, version 5.0) software. Bootstrap resampling analysis of 1000 replicates was also performed.

2.6. Animal experiments

Outbred, specific-pathogen-free ICR mice (Yisi Laboratory Animal Technology, Changchun City, China) were used to build an animal model. All institutional (National Institute for Food and Drug Control) guidelines for animal care and use were strictly followed. Six one-day old mice were inoculated intraperitoneally with 50 μL of virus; another six mice were injected intraperitoneally with 50 μL of DMEM without FBS to form the control group. The mice in the control group were kept separately caged from the infected mice. The mice were observed daily for clinical illness and death until 21 d after treatment. Exactly 14 d after intraperitoneal inoculation with the virus or uninfected culture medium, three