Molecular Identification and Phylogenetic Analysis of Bovine Coronavirus Isolated from Diarrheal Yak (*Bos grunniens*) in Qinghai Tibetan Plateau Area, China

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ABSTRACT

Bovine coronavirus (BCV) is viral pathogen causing severe diarrhea, respiratory system infections, and winter dysentery in calves. In the present study, we detected BCV in diarrhea newborn yaks in Qinghai Tibetan Plateau Area (QTPA), China. The molecular identification and phylogenetic analysis of BCV were performed by using the hemagglutinin (HE) gene and nucleocapsid protein (N) gene. The two genes sequencing homology were 99.0% to the reference strains (China isolates). The phylogenetic trees were all composed of four major clusters, the QTPA strains always were located in the same cluster along with the China strains. The evolutionary rate of BCV HE gene and N gene was estimated to be 4.1805×10−4 and 1.3338×10−4 substitutions/site/year. This study is a timely investigation for BCV and provides the epidemiological information of this virus in QTPA. Therefore, further studies are needed to carry out to determine the prevalence of BCV infection in yaks and other calves of diarrheic disease, and the study highlights the urgency to develop effective measures to control BCV in QTPA of China.

INTRODUCTION

Bovine coronavirus (BCV) is one of the

genus coronaviruses that belongs to the Nidovirales order, Coronaviridae family, and contains a single-stranded, non-segmented RNA positive-sense genome of 31 kb in length.[1] The virion possesses five structural proteins: spike (S) protein, nucleocapsid (N) protein, hemagglutinin-esterase (HE), transmembrane (M) protein, and small envelope (E) protein.[2]

BCV is a major viral pathogen causing severe diarrhea in neonatal calves, respiratory system infections in cows, calves, and feedlot beef cattle, and winter dysentery in adult cattle.[3] Although the affected animals rarely die, BCV infection is characterized as a hemorrhagic enteritis with dehydration, anorexia, and emaciation, then it can cause significant loss of weight of both calves and adults and reduction in milk production of cows, resulting in serious economic losses of the beef and dairy industries worldwide.[3] Actually, this virus has been reported in many countries such as Uruguay, Korea, USA, China, Vietnam, and Australia in nearly 2 years.[4-9] As far as China is concerned, BCV is prevalent in the cattle population in Sichuan, Shandong, Shanxi, Henan, Liaoning, Jilin, Xizang, Yunan, and other provinces.[9, 10] Therefore, it is urgent and essential to investigate the molecular epidemiology and genetic characterization of BCV for establishing effective controls for this virus.

In the present study, we performed the immunochromatographic strip test and molecular identification on newborn yak diarrheal feces samples from 2 farms in Qinghai Province to investigate the prevalence and genetic diversity of BCV in infected yaks in the Qinghai Tibetan Plateau Area (QTPA) of China.

MATERIALS AND METHODS

Sample Collection

In the current study, we collected the newborn yak feces from Gangcha County (10) and Huzhu County (10) of QTPA in which not only diarrhea symptoms and hemorrhagic enteritis with dehydration, anorexia, and emaciation were observed in newborn yaks (Fig. 1). The diarrhea samples were detected firstly by the Speed V-Diar4TM rapid immunochromatographic strip test (Virbac Feance Lt., La Seyne-sur-Mer, France).

Figure 1 *The diarrhea newborn yaks (A and B) and the diarrhea fecal samples (C and D).*

Molecular Identification

Virus RNA was extracted from the positive samples by using the TIANamp Virus RNA Kit (DP315-R, TIANGEN Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. Then the 1st Strand cDNAs were synthesised by using the TaKaRa RNA PCR Kit (AMV) Ver.3.0 (RR019A, TaKaRa Biomedical Technology (Beijing) Co., Ltd., Beijing, China) following the manufacturer's instructions. The molecular PCR identification was performed using the N gene and HE gene by the primers designed from the published nucleotide sequences of the Mebus strain (GenBank accession NO. U00735). The primers were: BCV-NF: 5`-ATGTCTTTTACTCCTGGTAAGCAA-3` and BCV-NR: 5`- TTATATTTCTGAGGTGTCTTCAGTATAGG-3` (product=1347 bp), BCV-HEF: 5`- ATGTTTTTGCTTCTTAGATTTGTTC-3` and BCV-HER: 5`-CTAAGCATCATGCAGCCTAGTA-3`

(product=1275 bp). The PCR reactions were carried out using using the commercial PCR kit (RR019A, TaKaRa Biomedical Technology (Beijing) Co., Ltd., Beijing, China) in a standard mixtures of 50 µl containing 0.25 µl TaKaRa Ex Taq HS (5 U/µl), 5 µl 10×Ex Taq Buffer (Mg2+plus) (20 mM), 4 µl dNTP Mixture (2.5 mM each), 2 µl cDNA, 4 µl primer mixtures (10 µM of each primer) and 34.75 µl PCR-Grade water. PCR was performed in a BIO-RAD MyCycler Thermal Cycler programmed as follows: an initial denaturation step of 5 min at 95°C; 35 cycles of 98°C for 10 s, (N=54°C and HE=52°C) for 30 sec, and 72 °C for (N=1.5 min and HE=1.5 min); then at 72 °C for 10 min. The amplification PCR products were analyzed by electrophoresis loading 10 µl on a 1.5% gel agarose (Biowest Regular Agarose G-10, manufacturerd to specifications distributed by GENE COMPANY LTD, Hong Kong, China, origin: Spain) stained with 4S Red Plus Nucleic Acid Stain (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China), and observed and captured using a GeneSys Image Acquisition Software System (G:BOX F3, Biocompare, Cambridge, U.K.) (Fig. 2). Virus isolation was carried out on the yak fecal sample containing the BCV as determined by the molecular identification according to Decaro et al.[11]

Figure 2. *Distribution of the locations (●) of samples collection in this study. Qinghai Province located on the Qinghai Tibetan Plateau of China. The number represented sampling site. The black circle represented the positive sample in this study.*

Phylogenetic Analysis

Direct sequencing was performed for the PCR products by GENEWIZ Company (Tianjin, China). The obtained sequences were aligned with reference sequences available in GenBank database for sequence similarity by using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Clustal X version 2.0.[12] The best-fit phylogenetic model was estimated by the software jModelTest 2 based on Akaike and Bayesian information criteria.[13] All the BCV sequences were used to generate a BEAST input file using BEAUti within BEAST package v1.8.0. The rates of nucleotide substitution per site/per year, and the most recent common ancestor (tMRCA) were estimated using a Bayesian MCMC approach.[14] Each dataset was simulated using the following options: generation, 100 000 000; burn-in, 10%; and ESSs > 300, and analyzed by Tracer 1.7. Trees were presented as MCC trees using TreeAnnotator 1.8.0 and visualized using Figtree 1.4.2.

RESULTS

One from Gangcha and one from Huzhu sample tested positive for BCV by rapid immunochromatographic strip test. The samples were designated as QHGC1 and

QHHZ1, respectively. Then the amplification of N gene and HE gene were performed by the PCR method using specific primers, and the expected PCR products, a single band of about 1,300 bp for N gene and 1,200 bp bp for HE gene were observed on 1% w/v agarose gel electrophoresis. The sequencing results showed that the N gene and HE gene sequences of BCV (QHGC1 isolate N gene Genbank accession NO. MH475915 and HE gene MH475914; QHHZ1 isolate N gene Genbank accession NO. MK688458 and HE gene MK688459) contained 1,344 and 1,272 nucleotides, which encoded 448 and 424 amino acid (aa) residues, respectively.

The two isolates N gene and HE gene sequences showed 99.93% and 99.61% homology with each other, while The amino acid sequences showed 100% and 99.53% homology with each other, respectively. The sequences of the fragments of N gene (MH475915 and MK688458) and HE gene (MH475914 and MK688459), which provided 99.7% homology to N gene of China isolates (MK095167/8/9) with the highest score (2401) and 99% homology to HE gene of China isolates (MK095145/6) with the highest score (2,322), when blasted against the NCBI's database.

The best-fit model for the BCV N and HE genes was the GTR+I+G model. The MCC tree for the N and HE genes indicated that BCV strains were divided into three and four diverse clusters, respectively (Fig. 2 and 3). For the MCC tree of N gene:

- cluster 1 (C1) comprised 21 BCV isolates (identified between 2003 and 2014) derived from France (n = 20) and Italy (n = 1)
- cluster 2 (C2) contained 37 BCV isolates identified between 1983 and 2019 came from the USA (n = 18), China (n = 16) and Korea (n = 3)
- cluster 3 (C3) comprised 11 BCV strains identified between 1965 and 2015

Figure 3. *The phylogenetic tree of BCV based on sequences of the partial hemagglutinin-esterase (HE) gene. The scale bar represents the number of substitutions per site. The black circle represented the positive sample in QTPA of China. The phylogenetic tree is denominated by: Sequence name/Country/year.*

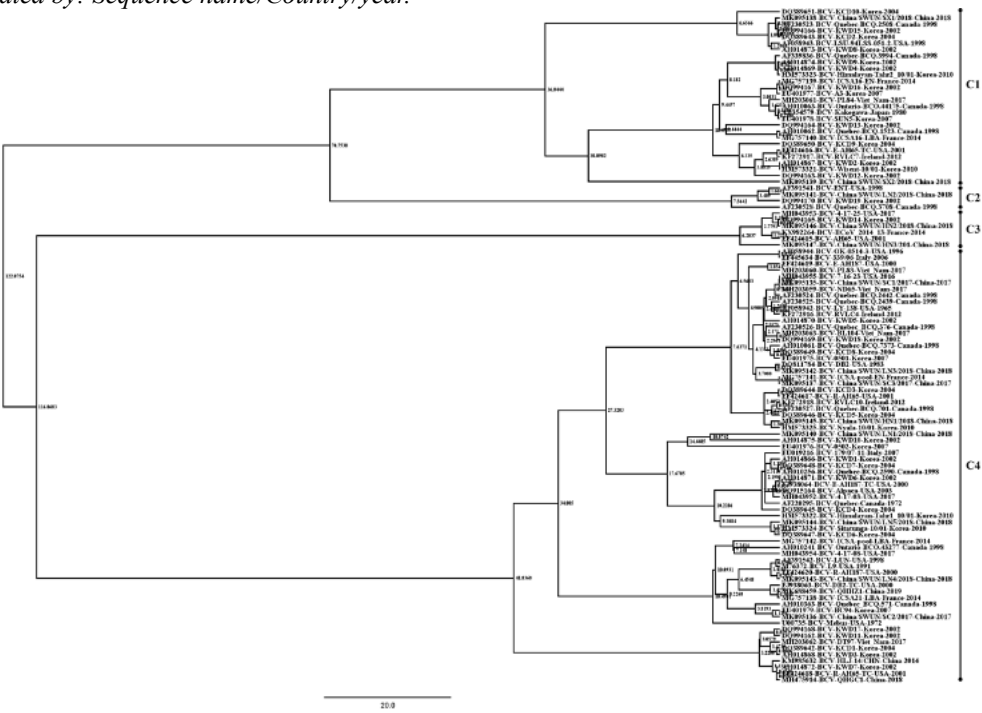
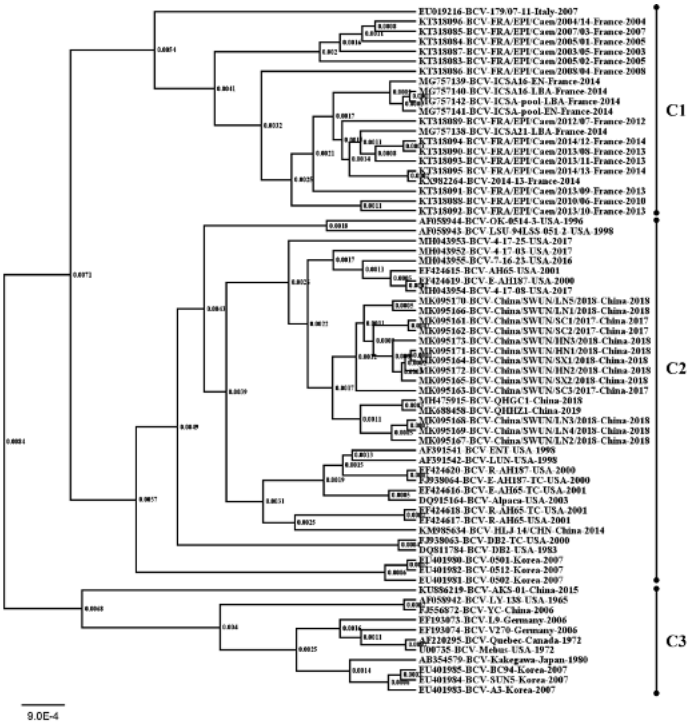


Figure 4. *The phylogenetic tree of BCV based on sequences of the partial nucleocapsid protein (N) gene. The scale bar represents the number of substitutions per site. The black circle represented the positive sample in QTPA of China, The phylogenetic tree is denominated by: Sequence name/Country/year.*



in Korea (n = 3), China (n = 2), USA (n = 2), Germany (n = 2), Canada (n = 1) and Japan (n = 1).

On the other hand, for the MCC tree of HE gene:

• cluster 1 (C1) comprised 28 BCV isolates (identified between 1998 and 2018) derived from the Korea (n = 15), China (n = 2), Canada (n = 4), USA (n = 2), France (n = 2), Vietnam(n = 1), Japan (n = 1), and Ireland (n = 1)

• cluster 2 (C2) contained 4 BCV isolates identified between 1998 and 2018 in four countries: USA (n = 4), China (n = 1), Canada (n = 1), and Korea (n = 1);

• cluster 3 (C3) contained 6 BCVs identified between 2001 and 2018 in four countries: USA (n = 2), Korea (n = 1), China (n = 2), and France (n = 1)

• cluster 4 (C4) comprised 69 BCV strains identified between 1965 and 2019 in USA (n = 16), Italy (n = 2), China (n = 11), Vietnam(n = 4), Canada (n = 9), Ireland (n = 2), Korea (n = 22), and France (n = 3). The evolutionary rate of BCV HE gene and N gene was estimated to be 4.1805×10−4 and 1.3338×10−4 substitutions/site/year (95% highest posterior density (HPD) 3.4727×10−4 – 4.9234×10−4) according to ESS (206.3572) and ((95% highest posterior density (HPD) 8.7067×10−5–1.8074×10−4) according to ESS (133.402), respectively.

This study was aimed at identifying BCV in QTPA of China, comparing and performing phylogenetic analysis of our three partial genes (N and HE gene) sequences with some other BCV strains from GenBank database around the world. During clinical examination, the diarrhea samples were detected firstly by the rapid immunochromatographic strip test, then molecular identified

by PCR amplification and sequencing, and high percent identity scores and expect (E) values from the BLAST software using the QTPA strain genes sequences were demonstrated.

DISCUSSION

Previous studies were conducted in China, especially in Xinjiang where Zhang et al.[15] detected by RT-PCR, BCV in 16.9% (12/71) of the analyzed diarrheic calves fecal samples in dairy farms. As is known, the QTPA is also located in northwestern China closely next to Xinjiang, which may be a possibility of transmission of BCV infections. BCV infections were mostly prevalent in young animals worldwide, such as with 64% prevalence in diarrheic calves in Canada,[16] 42.9 % prevalence of a diarrhea outbreak in pasture-feeding steers in the south of Brazil,[17] 1.71% prevalence in calves of diarrhea outbreaks in dairy and beef cattle herds from Argentina,[3] 78.8 % prevalence in Croatian cattle,[18] and 40.0% prevalence in Québec dairy calves.[19] The positive prevalence ranged from 76.8% to 85.3% for BCV among Swedish organically managed dairy herds and conventionally managed herds,[20] the sinusitis prevalence of BCV at the individual level was 40.1% in Australian live export cattle,[21] 68.6% prevalence in calves in Brazilian dairy herds,[22] and 14% prevalence in calves in New Zealand dairy farms.[23] As Abi keha et al, reported in China, with different prevalence in dairy calves in Sichuan (16%), Shandong (8.7%), Shanxi (5.0%), Henan (46.7%), Liaoning (77.8%), and Jilin (5.3%). Moreover, they found an HE gene recombination event occurring in BCV firstly.9 But the HE gene recombination event did not occur in our study.

The N gene is highly conserved and plays an important role in viral pathogenicity, and the gene recombination events in HE have been reported in BCV, so these two genes were used for phylogenetic analysis. The phylogenetic trees of two BCV genes were all composed of four major clusters by using the reference sequences from GenBank and showing the evolutionally distance, China QTPA strains always were located in the same cluster.

From the phylogenetic tree of BCV N gene showing, China QTPA (QHHZ1 and QHGC1) strain clustered together with China strains (China/SWUN/L2/3/4, SC1/2/3, SX1/2, HN1/2/3, LN1/5), which implied that it may form geographical isolation and adaptive mutation based on the evolutionary analysis of N gene (Fig. 1), and which may also provide an explanation for transmission of BCV in China. However, for the phylogenetic tree of BCV HE gene, the two strains (QHHZ1 and QHGC1) did not cluster together, QHHZ1 clustered together with USA strain DB2-TC and France strain ICSA21-LBA, for QHGC1 strain clustered together with USA strain AH65-TC, Korea strain KWD7, and China strain HLJ-14, which was associated with allelic variation. The evolutionary rate of the BCV HE gene (4.1805×10^{-4}) in this study, was similar to the data of Jihye study (4.5630×10^{-4}).[7]

Actually, how was the yaks infected with BCV, when there was no outbreak of large-scale epidemic diarrhea occurring. Recently, the yaks were free-range on the plateau grassland in QTPA with the moderate livestock density, where there were a lot of direct contact among wild animals carrying the virus with yaks. In addition, with the development of transportation of yaks and cattle trade, it may all introduce in virus disease. Moreover, the yaks were not vaccinated with BCV vaccine generally. Therefore, the yaks can be infected with BCV easily for the above reasons.

The molecular and phylogenetic analysis of BCV infections in yaks in QTPA of China was reported in this study. Importantly, as the data showing that there was BCV infections in QTPA, China, which may spread in this area. Moreover, the phylogenetic trees indicated close relation between the the isolate and enteric BCV strains. Therefore, further studies are needed to carry out to determine the prevalence of BCV infection

in yaks and other calves of diarrheic disease in QTPA, China.

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