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Research Article

Detection and Molecular Characterization of BCoVs Circulating in Central China Based on the Full-length Spike Gene

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Abstract

Bovine coronavirus (BCoV) is prevalent throughout the world and is an important aetiological agent of diarrhoea in new-born calves. However, little is known about the genetic diversity and molecular epidemiology of BCoV in China. In this study, a total of 127 faecal samples from diarrhoeic dairy calves from nine cities in Henan Province, Central China, were collected between 2017 and 2018 and evaluated by RT-PCR for the N gene. BCoV was detected in 15% (19/127) of calves. In addition, the full-length sequence of the S gene from 13 representative BCoV strains was obtained and analysed. Sequencing results for the full-length S gene showed 97.3-97.5% nucleotide (nt) identity and 96.3-96.8% amino acid (aa) identity with the classical Mebus strain. Phylogenetic analysis based on the full-length S gene showed that the BCoV strains in this study clustered with Vietnamese and Cuban BCoV strains on a large branch of the tree. These results enrich the molecular characterization of the Chinese BCoV strains and provide the first large-scale epidemiological examination of the prevalence of BCoV in diarrhoeic calves in Henan Province, Central China.

Keywords: *BCoV, Spike gene, Phylogenetic analysis, China*

Orta Çin'de Sığır Koronavirusun Tam Uzunlukta Spike Genine Dayalı Tespiti ve Moleküler Karakterizasyonu

Öz

Sığır koronavirüs (BCoV) tüm dünyada yaygındır ve yeni doğmuş buzağılarda ishal olgularının etiyolojisinde önemli rol oynar. Bununla birlikte, Çin'de BCoV'nin genetik çeşitliliği ve moleküler epidemiyolojisi hakkında çok az şey bilinmektedir. Bu çalışmada, 2017 ile 2018 yılları arasında Orta Çin'in Henan eyaletindeki dokuz ilden ishalli süt buzağılarından toplam 127 adet dışkı örneği toplandı ve N geni için RT-PCR ile değerlendirildi. Buzağıların %15'inde (19/127) BCoV tespit edildi. Ek olarak, 13 temsilci BCoV suşundan S geninin tam uzunluktaki dizisi elde edildi ve analiz edildi. Tam uzunluktaki S geni için sıralama sonuçları, klasik Mebus suşu ile %97.3-97.5 nükleotit (nt) özdeşliği ve %96.3-96.8 amino asit (aa) özdeşliği gösterdi. Tam uzunluktaki S genine dayanan filogenetik analiz, bu çalışmada BCoV suşlarının, ağacın büyük bir dalında Vietnam ve Küba BCoV suşları ile kümelendiğini gösterdi. Bu sonuçlar, Orta Çin'in Henan Eyaletindeki ishalli buzağılarda BCoV suşlarının moleküler karakterizasyonunu zenginleştirerek BCoV prevalansının ilk büyük ölçekli epidemiyolojik incelenmesini sağlamış oldu.

Anahtar sözcükler: *BCoV, Spike geni, Filogenetik analiz, Çin*

INTRODUCTION

Bovine coronavirus (BCoV) is a lineage A member of the species *Betacoronavirus*, belonging to the family *Coronaviridae* in the order *Nidovirales*, as assigned by the International Committee on Taxonomy of Viruses (ICTV) [1]. *Betacoronaviruses* have a broad range of hosts, such as humans (Middle East respiratory syndrome coronavirus,

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MERS-CoV [2], and human coronavirus OC43, HCoV-OC43), mice (mouse hepatitis coronavirus, MHV), and horses (equine coronavirus [3], ECoV). BCoV infection can cause mild to severe diarrhoea in new-born calves, winter dysentery in adult cattle and respiratory disorders in cattle of all ages [4-6], which causes dramatic a reduction in milk production in dairy herds and loss of body condition in both calves and adult cattle, accompanied by depression and anorexia, leading to serious economic losses [7]. BCoV has been identified in many countries worldwide.

Bovine coronavirus has a single-stranded, positive-sense RNA genome that is 32 kb in length, and is the largest virus among known RNA viruses. BCoV is an enveloped and pleomorphic virus with a diameter ranging from 100 to 120 nm, consisting of five major structural proteins designated the nucleocapsid (N) protein, membrane (M) protein, small envelope (E) protein, haemagglutinin-esterase (HE) protein and spike (S) protein [8]. The S glycoprotein of BCoV forms a club-shaped structure on the viral surface and has an important role in the process of virus invasion and fusion. It cleaves two subunits, termed S1 and S2, at amino acid position 768 [9]. During virus entry, S1 is responsible for binding sugar on the host cell surface for viral attachment and inducting of neutralizing antibody expression and haemagglutinin activity in the host species [9]. S2 is a transmembrane protein that fuses the host and viral membranes, allowing viral genomes to enter host cells. The variation in host range and tissue tropism of coronaviruses is largely related to variations in the S protein [10].

Bovine coronavirus was first reported in the USA in 1972 from enteritic neonatal calves and termed the Mebus strain. Subsequently, the presence of BCoV has extended to other areas of the world. In the 1980s, BCoV infections occurred in the cattle population in China [11]. However, limited information is available about BCoV circulating in Henan Province, Central China. Therefore, we sought to identify the frequency of BCoV infection among calves in Henan Province and investigate the genetic evolution of Henan BCoVs.

MATERIAL and METHODS

Between 2017 and 2018, diarrhoeic faecal samples (n=127) were collected from calves at 14 different farms from nine cities in Henan Province [An Yang (AY), He Bi (HB), Qin Yang (QY), Yuan Yang (YY), Lan Kao (LK), Song Xian (SX), Lu Shi (LS), Shang Cai (SC) and Bi Yang (BY)]. All calves were less than 4 months old. BCoV-positive faeces and the full-length S gene were diagnosed and amplified using gene-specific primers *(Table 1*). The resulting sequence data were edited using Lasergene 7.0 Alignment Editor, and the sequence identities of the nucleotide and deduced amino acid sequences of the field strains in this study were performed using Clustal W software. A phylogenetic tree was constructed based on the deduced amino acid sequences encoded by the full-length S gene using the neighbour-joining (NJ) method with 1.000 bootstrap replicates in MEGA software, version 7.0. The NJ tree was visualized using Figtree 1.4.3 [12]. The positively selected sites were evaluated using maximum-likelihood (ML) method implemented in MEGA. A site model was selected for the positively selected sites, and the parameters under seven different codon substitution models and their performances were evaluated using likelihood ratio tests (LRTs) implemented in EasyCodeML 1.2 [13]. The animal experiments were carried out according to the Animal Experiment Committee of Henan Academy of Agricultural Sciences (Approval number SYXK 2014-0007). All animals

Table 1. *Primer pairs used to amplify the partial N and full-length S genes*

Primer Name	Sequence (5′ → 3′)	Size (bp)	Genome Position	Reference
NF	CCGATCAGTCCGACCAATC	460	N gene 80-539	[14]
NR	TAGTCGGAATAGCCTCATCGC			
S1F	ATGTTTTTGATACTTTTAATTTCC	920	S gene 1-920	[15]
S1R	ACACCAGTAGATGGTGCTAT			
S2F	GGGTTACACCTCTCACTTCT	769	S gene 782-1550	[15]
S2R	GCAGGACAAGTGCCTATACC			
S3F	CTGTCCGTGTAAATTGGATG	828	S gene 1459-2286	[15]
S3R	TGTAGAGTAATCCACACGT			
S4F	TTCACGACAGCTGCAACCTA	872	S gene 2151-3022	[15]
S4R	CCATGGTAACACCAATCCCA			
S5F	CCCTGTATTAGGTTGTTTAG	916	S gene 2691-3606	[15]
S5R	ACCACTACCAGTGAACATCC			
S6F	GTGCAGAATGCTCCATATGGT	653	S gene 3439-4092	[15]
S6R	TTAGTCGTCATGTGATGTTT			

received humane care in compliance with good animal practices according to the animal ethics procedures and guidelines of China.

RESULTS

Of the 127 faecal samples from the calves with diarrhoea, 19 (14.96%) were positive for BCoV. In terms of the geographical distribution, the percentages of BCoV samples by state were as follows: LK [4.72% (6/127)]; YY [3.94% (5/127)]; SC [4.72% (6/127)]; BY [0.79% (1/127)]; and LS [0.79% (1/127)] *(Fig. 1)*. These results indicate that BCoV is widely disseminated in cattle herds in Henan Province.

The full-length S gene was successfully obtained in 13 of the 19 positive samples, and they were designated SC-1, SC-2, SC-3, SC-4, SC-5, SC-6, LK-1, LK-2, YY-1, YY-2, YY-3, YY-4 and LS-1. Sequence comparisons of all 13 S genes in this study revealed that they share 99.5-100% nt identity and 98.9-100% aa identity with each other and 97.3-97.5% nt identity and 96.3-96.8% aa identity with the Mebus strain. They also share 98.6-98.9% nt identity and 98.4-99.3% aa identity with the other 7 Vietnamese BCoV S genes reported previously and 98.6% nt identity and 98.3% aa identity with the other two Chinese strains, AKS-01 from Xinjiang Province and HLJ-14 from Heilongjiang Province, respectively.

A phylogenetic tree based on the full-length S gene sequences using the NJ method showed that all 13 S genes from this study clustered on an independent small branch with each other, and clustered with 7 Vietnamese and 4 Cuban BCoV strains on a large branch of the tree *(Fig. 2)*.

The analysis of the predicted S proteins revealed that all 13 S genes from this study had 4092 nucleotides, encoded 1363 aa residues, and had a molecular weight of approximately 150 kDa. The S1 and S2 subunits were formed at the cleavage site aa 768, and their molecular weights were approximately 86 and 65 kDa, respectively. A total of 83 and 47 polymorphic nucleotides corresponding to 40 and 22 aa changes at 43 and 24 distinct sites were identified in the S1 and S2 subunits of the strains identified in this study compared with the Mebus strain, respectively. Of these amino acid changes, 19 were unique, while 43 were shared with other reference strains reported previously *(Fig. 3)*. In addition, the S1 N-terminal domain (S1-NTD, aa 15-298), which was shown to function as a receptor-binding domain (RBD) in BCoV, had a total of 20 aa changes in the strains of this study. In addition, the S1A and the S1B immunoreactive domains identified within the amino acids 351-403 and the amino acids 517-621 had 1 and 4 aa changes, respectively, in the strains in this study compared with the Mebus strain. Additionally, compared with the sequences of other BCoV S genes, six sequences of S genes from Shangcai city BCoV isolates in this study had unique N509H, T352I, I640I, S977A, and C1331F aa changes *(Fig. 3)*. No frameshifts, deletions or insertions were observed in the S gene sequences of strains in this study.

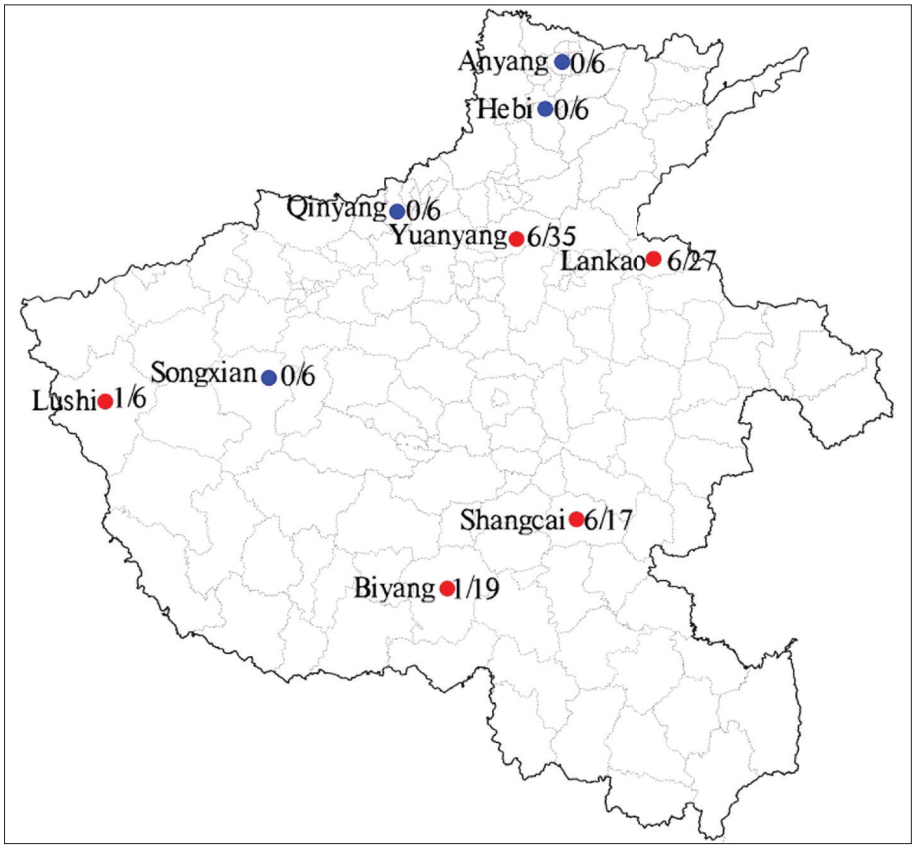

Fig 1. Map of Henan showing the number of samples and the geographic distribution of the sample collection sites. The red solid dot indicates an area where positive samples were located, and the blue solid dot indicates an area where no positive samples were located

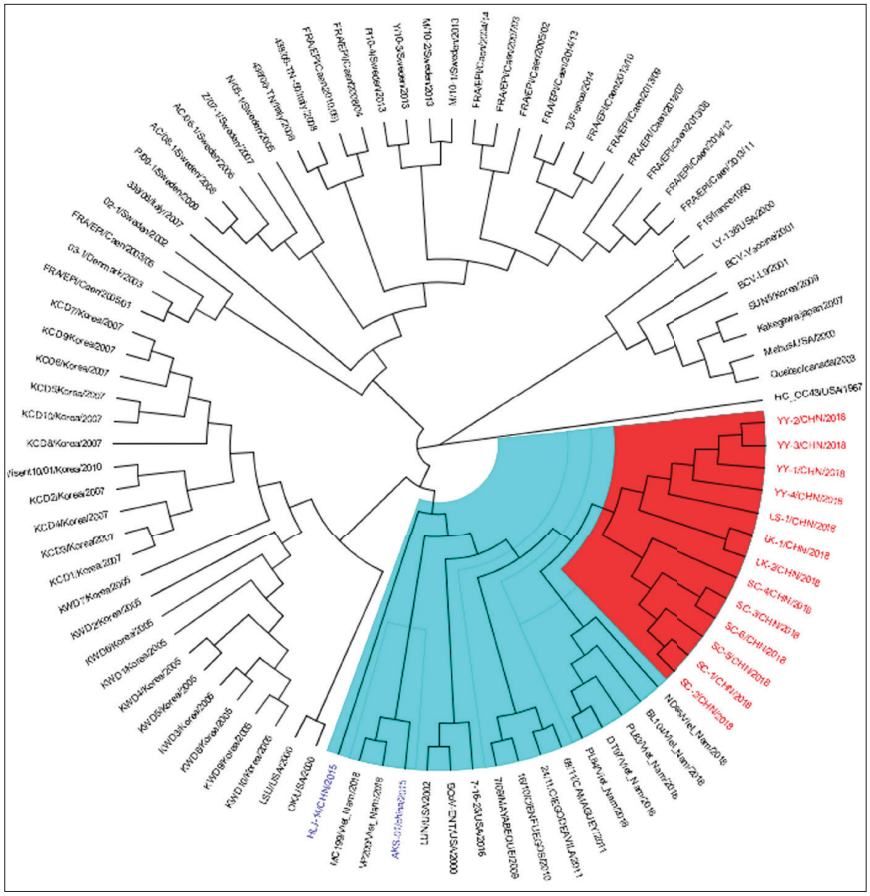

Fig 2. Phylogenetic tree generated by neighbour-joining analysis of the genetic distance in the deduced amino acid sequences of the full-length S gene of BCoV strains. The strains in this study are marked in red, and other Chinese BCoV strains are marked in blue

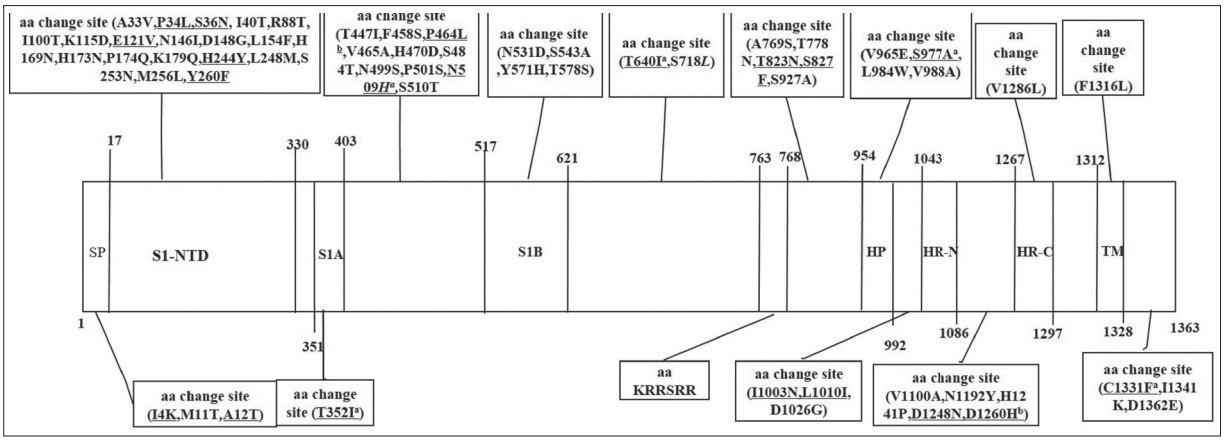

Fig 3. Amino acid variants of the 13 full-length S genes in this study. The unique aa changes in this study are underlined; [a] aa changes in six strains from Shangcai city; [b] aa changes in two strains from Lankao city

Likelihood ratio test analysis at the posterior probability p>95% level identified 11 positively selected sites. Of these 8 are in S1 and 3 are in S2. The following sites were under positive selection: 11, 115, 179, 499, 501, 524, 539, 716, 1235, 1350, and 1360 *(Table 2)*.

DISCUSSION

Bovine coronavirus, an important causative agent of diarrhoea in calves, is responsible for severe economic losses in the global farming industry. One recent study reported that BCoV infections are endemic in China and that the prevalence of BCoV in Henan Province is 46.7% [16], but the percentage in our study was 14.96%, which is lower than that in the previous study. The reasons for the difference in the detection rates of BCoV in calves in the same province could be explained by differences in sample number and sampling area. Fifteen samples from 3 farms were collected for detection in that study, while in our study, we screened 127 diarrhoeic faecal samples from calves, 19 of which were positive for BCoV. These positive samples were distributed across 10 of 14 farms across five different cities in the major

Table 2. *Parameter estimates and log-likelihood values under models of variable o-ratios among sites*

Model	Parameter Estimates	Likelihood Scores	Positively Selected Sites*
M0: one-ratio	ω=0.111175	-13568.203652	Not allowed
M1a: nearly neutral	ω0=0.04, ω1=1, (p0=0.91, p1=0.092)	-13336.145397	Not allowed
M2a: positive selection	ω0=0.04, ω1=1, ω2=1, (p0=0.91, p1=0.055, p2=0.038)	-13336.000953	None
M3: discrete	ω0=0.02, ω1=0.45, ω2=1.88, (p0=0.844, p1=0.13, p2=0.026)	-13325.821205	None
M7: β	p=0.041, q=0.217	-13385.024380	Not allowed
M8: $\beta+\omega_s>1$	p0=0.96, p1=0.042, p=0.047, q=0.335, ω=1.504	-13328.064083	11, 115, **179,** 499, 501, 524, 539, 716, 1235, 1350, 1360
M8a: $\beta+\omega_s=1$	p0=0.931, p1=0.069, p=0.302, q=5.403, ω=1	-13331.696625	Not allowed

** Sites with a posterior probability >95% having ω>1 and p>99% are in boldface*

dairy cattle production areas of Henan Province. This study is the first large-scale epidemiological examination to determine the prevalence of BCoV in diarrhoeic calves in Henan Province. The results showed that BCoV is widely disseminated in cattle herds in Henan Province.

The 13 Henan BCoV strains sequenced in this study show a higher similarity with the other seven Vietnamese BCoV strains, causing them to cluster as a unique clade in the phylogenetic tree *(Fig. 2)*. We also identified a high similarity between our strains and 2 other Chinese BCoV strains in distant regions of China, such as Heilongjiang and Xinjiang Provinces, implying that certain strains may have the potential to spread directly or indirectly to distant regions. These results also suggest that these BCoV strains were part of the main transmission chains in dairy herds in Asia. Interestingly, we found six strains from Shangcai city clustered in a smaller branch in the phylogenetic tree with strains from Lankao and Yuanyang cities *(Fig. 2)*. Compared with the BCoV S genes of other strains, the S genes of strains from these different cities have their own unique aa substitution changes, such as T352I, N509H, I640I, S977A, and C1331F aa changes in the Shangcai strains and P464L and D1260H aa changes in the Lankao strains *(Fig. 3)*. These results indicated that viral lineages can form natural groups based on geographical location [17]. These results may be linked to factors such as geographical distribution, different breeds and breeding systems and animal marketing [18].

Six BCoV strains from Shangcai city had the unique aa substitution S977A in the fusion peptide (FP) of the S2 subunit, and four BCoV strains from Yuanyang city in this study were observed to have the substitution V1285L located in the heptad repeat region C (HR-C). FP and HR-C are crucial for the fusion on viral and host cell membranes [19]. Membrane fusion is mediated by a major conformational rearrangement that exposes the fusion peptide and results in the formation of a six-helix bundle (6HB) [20], bringing the viral and host membranes together to fuse. The core of the 6HB is a triple-stranded coiled coil, and the HR-C elements pack within the grooves of the coiled coil in an antiparallel direction. Thus, aa substitutions in these regions may affect the interaction between the coiled-coil structure and the host cell receptor. Moreover, several reports have suggested that variation in the S2 subunit [21], particularly in FP and HR-C, determine host range expansion. Therefore, more sequence data and experimental studies are required to clarify the important role of these amino acid changes in the S2 subunit of BCoV.

The S protein is a major neutralizing antigen of BCoV. Researchers have previously confirmed that a single aa change within the S1B domain of the S protein of BCoV can confer resistance to virus neutralization [9]. In this study, the polymorphism T352I was identified in the S1A domain in only six strains from Shangcai city *(Fig. 3)*. However, whether this amino acid change affects the antigenicity of the virus is not known. Thus, the role of the T352I mutation in immunological properties requires further study.

In this study, two and five strong positive selection sites were detected within the receptor-binding domain (RBD) and an epitopic fragment of the S1 subunit of BCoV, spanning aa residues 15-298 and 324-720, respectively. Viral RBDs can recognize different receptors through structural variations and are a critical determinant of viral host ranges [19]. The epitopic fragments have been previously recognized using monoclonal antibodies (mAbs) [22]. Taken together, the strong positively selected sites within the S protein may be associated with the immune response and receptor binding and would thus be important in future BCoV vaccine development.

In conclusion, the results of our study have shown that BCoVs are circulating widely in dairy calves in Henan Province, Central China, and that most of these strains have unique evolutionary patterns based on our sequence analysis of the full-length S genes. Our findings will enrich the current understanding of the molecular characterization of BCoVs in China.

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