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Letter

# Identification of bovine coronavirus in a Daurian ground squirrel expands the host range of *Betacoronavirus 1*

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*Dear Editor,*

Bovine coronavirus (BCoV) is a member of the species *Betacoronavirus 1* in the genus *Betacoronavirus*, subgenus *Embecovirus*. Other members of the species *Betacoronavirus 1* comprise porcine hemagglutinating encephalomyelitis virus (PHEV), human coronavirus OC43 (HCoV-OC43), equine coronavirus (ECoV), and rabbit coronavirus HKU14 (RbCoV HKU14) (Corman et al., 2018). BCoV is known to infect a wide range of mammals including domestic and wild ruminants, causing enteritis and pneumonia (Saif, 2010). BCoV is ubiquitous in cattle worldwide, even in the respiratory and intestinal samples of healthy individuals, causing major economic losses to the beef and dairy cattle industries (Vlasova and Saif, 2021). Importantly, it has been suggested that HCoV-OC43 originated in rodents, and that the major host of BCoV–cattle–acts as intermediate host (Kin et al., 2016; Forni et al., 2017; Corman et al., 2018; Cui et al., 2019).

Determining the host range of viruses is central to understanding their evolution and emergence (Woolhouse and Gowtage-Sequeria, 2005), and hence is of direct importance to zoonotic risk reassessments for future human epidemics and pandemics (Olival et al., 2017). Although ruminant animals such as cattle have been proposed to be the natural hosts of BCoV, this virus has been identified in other animals including dogs and camels which could similarly act as intermediate hosts for host jumping from animals to humans (Corman et al., 2018; Cui et al., 2019). However, because of its wide host range, the zoonotic source of BCoV remains obscure. Rodents (order Rodentia) are the most diverse and widely distributed mammalian group and important reservoirs and vectors of a number of viral pathogens (Meerburg et al., 2009). To our knowledge, however, BCoV has not been described in rodents.

In a regional surveillance effort for rodent-borne zoonosis in June 2021, a total of 54 Daurian ground squirrels (*Spermophilus dauricus*) were collected at Bashang grassland in Fengning Manchu Autonomous County, Hebei Province, China (Fig. 1A). Daurian ground squirrels, the dominant rodent species in this area, were captured by traps set both near the burrows and cattle farms. In a quadrat of 250 m by 250 m, a total of 300 traps were set in the morning and then checked and retrieved 6 h later. Sampling was performed at the same quadrat for two consecutive days. Animal species were morphologically identified by a trained expert, and subsequently confirmed by sequencing of a 457-bp amplicon from the mitochondrial cytochrome *b* gene (cyt *b*) (Li et al., 2021). Animal dissection was performed individually under ether anesthesia, and heart, liver, spleen, lung, kidney, rectum and brain tissues were collected and homogenized, respectively (Details were provided in Supplementary materials).

For each animal, equivalent supernatants of different tissues were pooled, and viral RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Germany). Complementary DNA (cDNA) was then obtained with the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Japan), and was used as the template for CoV screening with nested PCR primers targeting a 440-bp sequence of the RNA-dependent RNA polymerase (*RdRp*) gene (Falcon et al., 2011). One Daurian ground squirrel (DTA28) tested positive. Sanger sequencing (Tsingke, China) of the amplified *RdRp* gene (440-bp in length) revealed the 100.0% nucleotide sequence identity to BCoV. Real-time PCR targeting the *ORF1b* gene of BCoV DTA28S (Primers were shown in Supplementary Table S1) was then performed using the One Step PrimeScript RT-PCR Kit (Perfect Real Time) (TaKaRa, Japan) and showed a *Ct* value of 18.14 (Details see Supplementary materials). Transmission electron microscopy (TEM)

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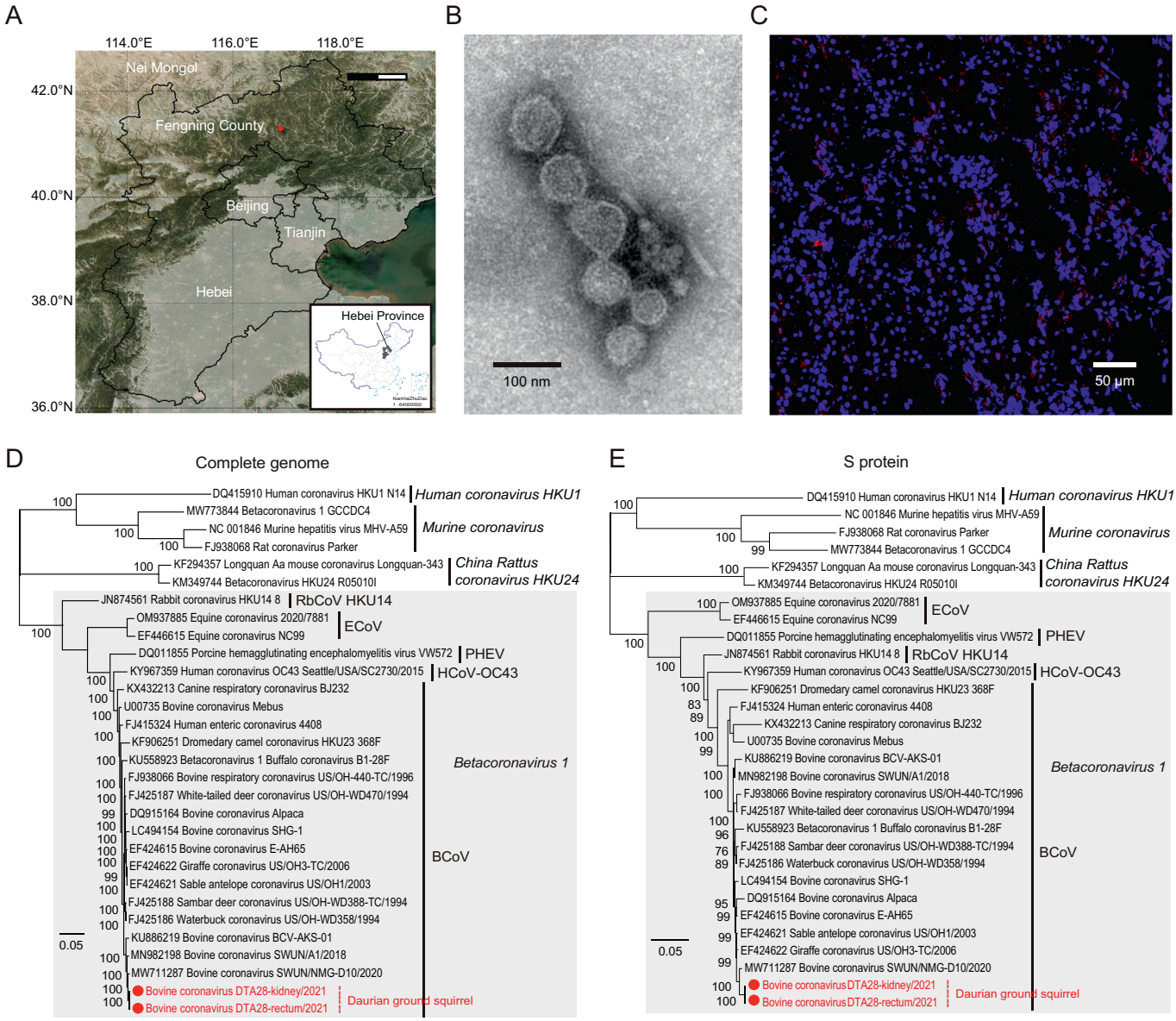


**Fig. 1.** Sample collection, viral characterization and phylogenetic analyses of BCoV DTA28 in a Daurian ground squirrel. **A** The ground squirrel was captured in Fengning County (marked with a red star) in Hebei Province, China. **B** Coronavirus-like particles were observed by transmission electron microscopy. **C** BCoV RNA (in red) in the kidney of the ground squirrel DTA28 was detected by FISH with cells being dyed by DAPI (in blue). Phylogenetic trees were estimated based on the (**D**) complete genomes and (**E**) spike proteins of DTA28 and other embecoviruses. Virus names in red indicate the strains identified in this study. Virus species names are shown in italics. The viruses belong to the species *Betacoronavirus 1* are shaded in grey. Virus abbreviations are as follows: BCoV, Bovine coronavirus (strain SWUN/NMG-D10/2020); HCoV-OC43, Human coronavirus OC43 (strain Seattle/USA/SC2730/2015); PHEV, porcine hemagglutinating encephalomyelitis virus (strain VW572); ECoV, Equine coronavirus (strain 2020/7881); RbCoV HKU14, Rabbit coronavirus HKU14 (strain HKU14-8). Experimental details were provided in Supplementary Materials.

examination was then performed (Details see Supplementary materials) and spherical virus particles of approximately 60–100 nm in diameter were observed in the pooled tissue sample of the squirrel DTA28 (Fig. 1B).

To obtain the complete viral genome sequence of the Daurian ground squirrel virus, RNA of the CoV-positive animal was sent for transcriptome sequencing (Novogene, China). The library was constructed with the NEBNext Ultra RNA Library Prep Kit (NEB, USA) and paired-end (150-bp) sequencing was performed on the NovaSeq6000 platform (Illumina, USA). The data was analyzed as published previously (Xu et al., 2021), and a sequence contig of 31,048-bp in length was annotated as BCoV. Overall, 8,204 clean reads were mapped onto this contig using Bowtie2 v2.3.3.1 (Supplementary Fig. S1), with the mean coverage of 39.2× (ranging from 1 to 175) across the genome. A total of 1,521 intra-host single-nucleotide variants (iSNVs) were initially found (Supplementary Table S2) (Lauring, 2020; Valesano et al., 2021). However, after filtering with the cut-off of variant frequency >5% and a variant $P$ value $< 10^{-6}$, only four iSNVs were identified, all of which were synonymous and fell in the coding region of the membrane (*M*) gene (Supplementary Table S3). These results suggested that the assembled BCoV contig was reliable.

Nested and real-time PCR were performed to screen the original specimens of DTA28. Accordingly, the kidney and rectum tissues were positive for BCoV, with *Ct* values of 16.41 and 23.57, respectively. Overlapping PCR primers (Supplementary Table S1) were designed, and two complete genome sequences were recovered, designated as BCoV DTA28-kidney/2021 and DTA28-rectum/2021, respectively. Fluorescence *in situ* hybridization (FISH) was then performed with the signal amplification by exchange reaction (SABER) (Kishi et al., 2019) (Details

see Supplementary materials), revealing the wide distribution of BCoV-specific RNA in the kidney (Fig. 1C).

The sequences of BCoV DTA28-kidney/2021 and DTA28-rectum/2021 were identical to each other (hereafter DTA28), with a G + C content of 37.07%, and had nucleotide identities of 97.2%–99.4% with other BCoVs. These two sequences were most closely related to BCoV SWUN/NMG-D10/2020 (accession no.: MW711287) (Supplementary Table S4). The predicted genomic organization of DTA28 showed a similar gene order to other BCoVs: 5′-ORF1ab-NS2-HE-S-NS-E-*M*-N(N2)-3′, possessing the highest identities with SWUN/NMG-D10/2020 at 98.3%–100.0% (nucleotide) and 98.8%–100.0% (amino acid) (Supplementary Table S5 and Supplementary Fig. S1). The 16 non-structural proteins (nsps) in the *ORF1ab* gene encoding the replicase were also predicted along with their known cleavage peptides (Supplementary Table S6).

To determine the genetic relationships of BCoV DTA28 to known BCoVs and other members of the subgenus *Embecovirus*, genome sequences of representative strains were downloaded from GenBank and multiple sequence alignment was performed using MAFFT v7.450. The complete genome sequence of BCoV DTA28 had nucleotide identities of 97.2%–99.4% with other BCoVs and 85.2%–94.4% to other members of the species *Betacoronavirus 1* (Supplementary Table S4). The spike (S) and hemagglutinin-esterase (HE) proteins are involved in receptor binding, host range, and tissue tropism (Corman et al., 2018). Our further analysis revealed that DTA28 had amino acid identities of 93.9%–98.8% in the S protein (Supplementary Table S7) and 95.5%–99.3% in the HE protein (Supplementary Table S8) to those of other BCoVs (Supplementary Fig. S2 and S3). The S protein N-terminal domain (NTD) of BCoV recognizes the sugar receptor, containing four amino acid residues that are critical for receptor binding in BCoV Mebus (Peng et al., 2012). DTA28 exhibited 39 amino acid differences in the S protein with BCoV Mebus, 14 of which fell in the NTD, including four amino acid differences at the critical receptor-binding amino acids (Supplementary Fig. S2). At the amino acid level, the HE protein was 95.5%–99.3% identical to other BCoVs and 71.9%–96.2% to other viruses in *Betacoronavirus 1* (Supplementary Table S8), possessing five amino acid differences with BCoV SWUN/NMG-D10/2020 (Supplementary Fig. S3).

We assessed the possibility of recombination between BCoV and other members of the subgenus *Embecovirus* using Simplot. No evidence for recombination was obtained (Supplementary Fig. S4). Phylogenetic analyses were performed using the maximum likelihood method in PhyML v3.0 with 1,000 bootstrap replicates, and the best-fit model of nucleotide substitution chosen using the Akaike Information Criterion using Smart Model Selection. The four phylogenetic trees estimated based on the complete genome (Fig. 1D), S protein (Fig. 1E), ORF1ab (Supplementary Fig. S5A), and HE protein (Supplementary Fig. S5B) exhibited similar topologies, with both BCoV sequences obtained here clustering together and closest to SWUN/NMG-D10/2020.

Together, these data provide strong evidence for the presence of a BCoV in Daurian ground squirrel. To our knowledge, this is the first report of BCoV in wild rodent, highlighting the expansion of host spectrum of BCoV. We speculate that the BCoV was replication active in the ground squirrel, because: (i) BCoV was identified with high viral loads in both the kidney and rectal tissues; (ii) transcriptomic sequencing exhibited high coverage across the BCoV genome including four iSNVs with high frequency, indicating that the within-host diversity was likely generated during virus replication, and (iii) TEM and FISH revealed the existence of intact virus particles and viral RNA of BCoV in the ground squirrel.

Our data also suggest that the ground squirrel virus might have been acquired through a transient spillover event from cattle to wild rodents. In particular: (i) the infection rate of BCoV was low, with only one of 54 ground squirrels testing positive; (ii) the BCoV genome recovered from ground squirrel was almost identical to those from cattle in China, and the close relationships of its S and HE proteins to other BCoVs suggested a similar pattern of receptor usage; (iii) the ground squirrels were collected in a grassland where animal husbandry and agriculture were highly developed, with cow feces often found near the ground squirrel burrows; (iv) although no BCoV had been reported in cattle in the sampling area, it had been previously identified in a different region of Hebei Province (accession nos.: MK903505–MK903508) as well as in Nei Mongol (Inner Mongolia, accession no.: MW711287) that neighbors Hebei; and (v) rodents are believed to be natural (*Murine coronavirus* and *China Rattus coronavirus HKU24*) and/or ancestral (*Human coronavirus HKU1* and *Betacoronavirus 1*) hosts for other embecoviruses. Notably, the tissue tropism of BCoV in the ground squirrel (kidney and rectum) seems different to those in cattle and wild ruminants in which the virus mainly infects the upper and lower respiratory tract, as well as the intestine (Saif, 2010; Vlasova and Saif, 2021). However, the role, if any, that rodents play in BCoV evolution and transmission clearly warrants further study, including continuous surveillance at the livestock-rodent interface.

## Footnotes

All sequence reads have been deposited in the Short Read Archive under BioProject PRJNA838119. Sequences of viral genome and the mitochondrial cytochrome *b* have been submitted to GenBank under accession numbers ON544072, ON544073, and ON532727. The procedures for rodents sampling and dissection were approved by the Administrative Committee on Animal Welfare of Shandong First Medical University and Shandong Academy of Medical Sciences. This study was funded by the Academic Promotion Programme of Shandong First Medical University (2019QL006) and the State Natural Sciences Foundation Monumental Projects (32090024). E.C.H. is supported by an ARC Australian Laureate Fellowship (FL170100022). The authors declare that they have no conflict of interest.

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