Archives of Virology (2020) 165:3011–3015
https://doi.org/10.1007/s00705-020-04840-y

ANNOTATED SEQUENCE RECORD

An emerging novel bovine coronavirus with a 4-amino-acid insertion in the receptor-binding domain of the hemagglutinin-esterase gene

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Received: 11 July 2020 / Accepted: 8 September 2020 / Published online: 6 October 2020

Abstract

The hemagglutinin-esterase (HE) protein of betacoronavirus lineage A is a secondary receptor in the infection process and is involved in the emergence of new betacoronavirus genotypes with altered host specificity and tissue tropism. We previously reported a novel recombinant bovine coronavirus (BCoV) strain that was circulating in dairy cattle in China, but this virus was not successfully isolated, and the genetic characteristics of BCoV are still largely unknown. In this study, 20 diarrheic faecal samples were collected from a farm in Liaoning province that had an outbreak of calf diarrhea (≤ 3 months of age) in November 2018, and all of the samples tested positive for BCoV by RT-PCR. In addition, a BCoV strain with a recombinant HE (designated as SWUN/A1/2018) and another BCoV strain with a recombinant HE containing an insertion (designated as SWUN/A10/2018) were successfully isolated in cell culture (TCID50: $10^{4.25}$/mL and $10^{4.73}$/mL, respectively). Unexpectedly, we identified the emergence of a novel BCoV variant characterized by a 12-nt bovine gene insertion in the receptor-binding domain in a natural recombinant HE gene, suggesting a novel evolutionary pattern in BCoV.

Betacoronavirus lineage A includes human coronavirus HKU1, human coronavirus OC43 (HCoV-OC43), equine coronavirus, mouse hepatitis virus (MHV), canine respiratory coronavirus (CRCoV), porcine hemagglutinating encephalomyelitis virus, and bovine coronavirus (BCoV). CRCoV and HCoV-OC43 are closely genetically related to BCoV [1, 2]. Members of this lineage have a unique gene encoding a surface hemagglutinin-esterase (HE) protein that is not found in other coronaviruses The HE protein serves as a secondary receptor in the infection process [3] and is involved in the emergence of novel genotypes and in shifts in host specificity and tissue tropism [4]. In addition, HE protein can induce the production of neutralizing antibodies against the BCoV [5].

BCoV causes gastrointestinal and respiratory diseases in cattle, leading to serious economic losses all over the word [6–8]. No consistent genetic or antigenic markers have been identified for discriminating BCoV in different clinical syndromes [9, 10], and the virus is thought to only has one genotype [10, 11]. BCoV can infect wild ruminant animals, including alpacas, sambar deer, and giraffes [8, 12, 13] and probably can be transmitted to dogs [1, 14, 15]. Interestingly, a coronavirus isolated from human diarrheic samples has been shown to be related to BCoV [16], indicating its public health significance.

Recently, we reported the wide circulation of a novel BCoV strain with a recombinant HE in dairy cattle in China [17]. In this study, in order to monitor the epidemic of the recombinant strain, a total of 20 diarrheic faecal samples were collected from a farm that had an outbreak of diarrhea in calves three months of age or younger in Liaoning province in November, 2018, and all of the samples tested positive for BCoV by RT-PCR as described previously [17]. Full-length HE genes (1275 nt) were successfully cloned from 18 of the 20 BCoV-positive samples as described previously [18–20], and PCR products were purified and cloned into the pMD19-T simple vector prior to sequencing. A

Handling Editor: T. K. Frey.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00705-020-04840-y) contains supplementary material, which is available to authorized users.

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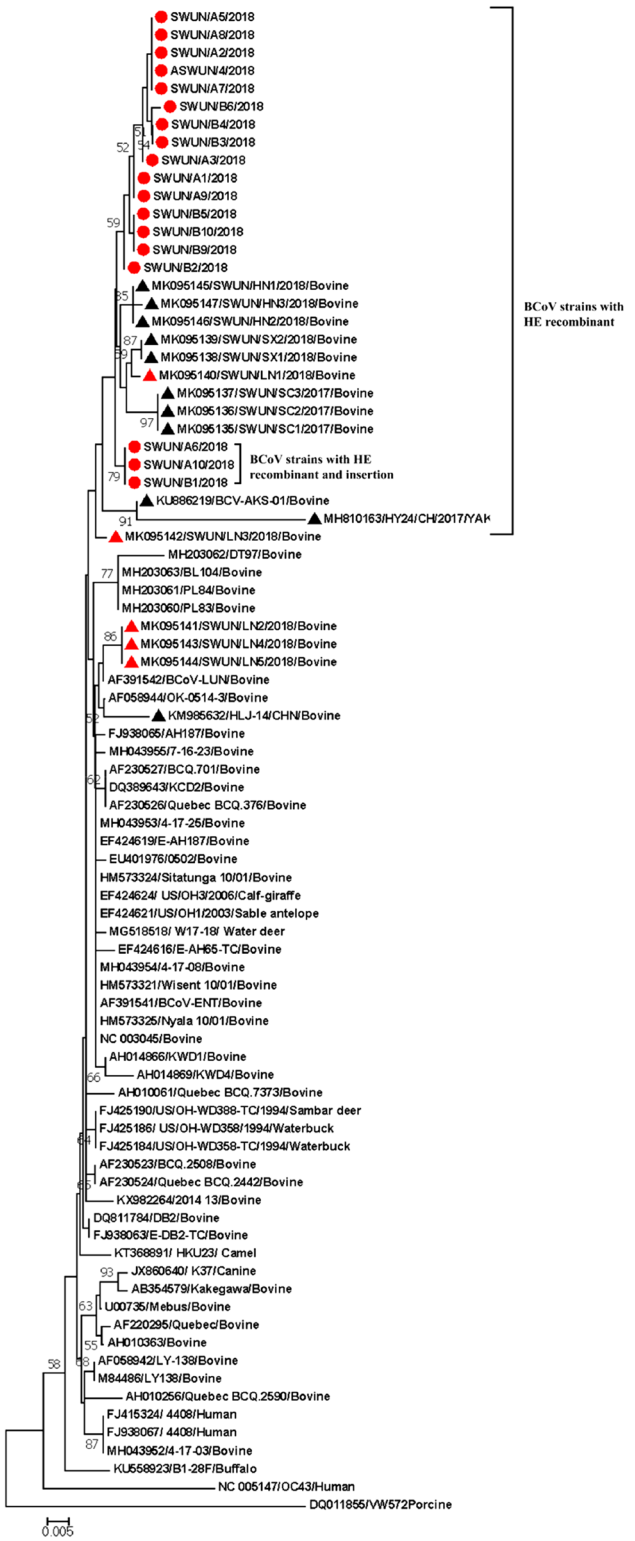

Fig. 1 Phylogenetic tree based on the deduced 424-aa sequence of the complete HE protein of BCoV. Sequence alignments and clustering were performed using ClustalW in MEGA 7.0 software. The tree was constructed by the neighbor-joining method with bootstrap values calculated for 1,000 replicates. The strains in this study are indicated by circles, and the other Chinese BCoV strains are indicated by triangles. The Liaoning strains are indicated in red (red circles and red triangles). The BCoV strain with recombinant HE and the insertion strain with recombinant HE with an insertion are indicated in the figure

phylogenetic tree based on the complete HE gene sequences of BCoVs using the neighbor-joining method showed that all 18 HE gene sequences (GenBank accession numbers: MN982182-MN982197, MN982198, and MN982199) together with 12 Chinese BCoV HE genes [17] clustered in a large independent branch (Fig. 1), and three of the 18 HE gene sequences (GenBank accession numbers: MN982186, MN982190, and MN982199) clustered in an independent small branch. Further analysis showed that all 18 HE genes identified in this study had undergone the same recombination event identified in our previous report [17]. This suggests an increase in the prevalence of this recombinant, since it was found in only two out of five strains detected in the earlier study (samples collected in January 2018 [17]) but was present in 18 of the 18 isolates from the same farm characterized in this study (samples collected in November 2018). This indicates that the HE recombinant BCoV strain is still present in Liaoning, China, and may be increasing in prevalence.

The BCoV HE receptor-binding domain is composed of six surface loops, five of which are grafted onto the beta-sandwich core of the lectin domain, the R1-loop, R2-loop, R3-loop, R4-loop, and the RBS-hairpin, and the other is present on the esterase domain, the E-loop (Fig. 2A). The 18 isolates with a recombinant HE were sequenced and found to contain the same amino acid (aa) variation (F181V) in the R2-loop that was described in our previous report [17]. The R2-loop in the lectin domain acts in ligand binding, and aa substitutions in this domain may affect receptor binding. For example, HCoV-OC43 may have evolved during adaptation of BCoV to a human host by gradually losing its lectin activity due to progressive accumulation of aa mutations in the R2-loop of the lectin-binding domain in the HE protein [2]. Unexpectedly, three isolates that clustered in an independent small branch all contained an identical insertion of 12 nucleotides (AAGGCTACTGTT), resulting in four additional amino acids in the R3-loop (Fig. 2B). This sequence was not present in any of the available BCoV HE sequences in the GenBank database. Interestingly, the inserted nucleotides were identified as originating from the bovine host, matching the sequence of a transcript variant of ORF159 on *Bos taurus* chromosome 16 C1 (GenBank accession no. XM027565276). It is worth noting that the R3-loop is composed of 13 aa (aa 207 -219) in BCoV HE, and residues F 211, L 212, S 213, and N 214 are essential for receptor-ligand interaction [3]. A 3D model constructed based on the crystal structure of bovine coronavirus

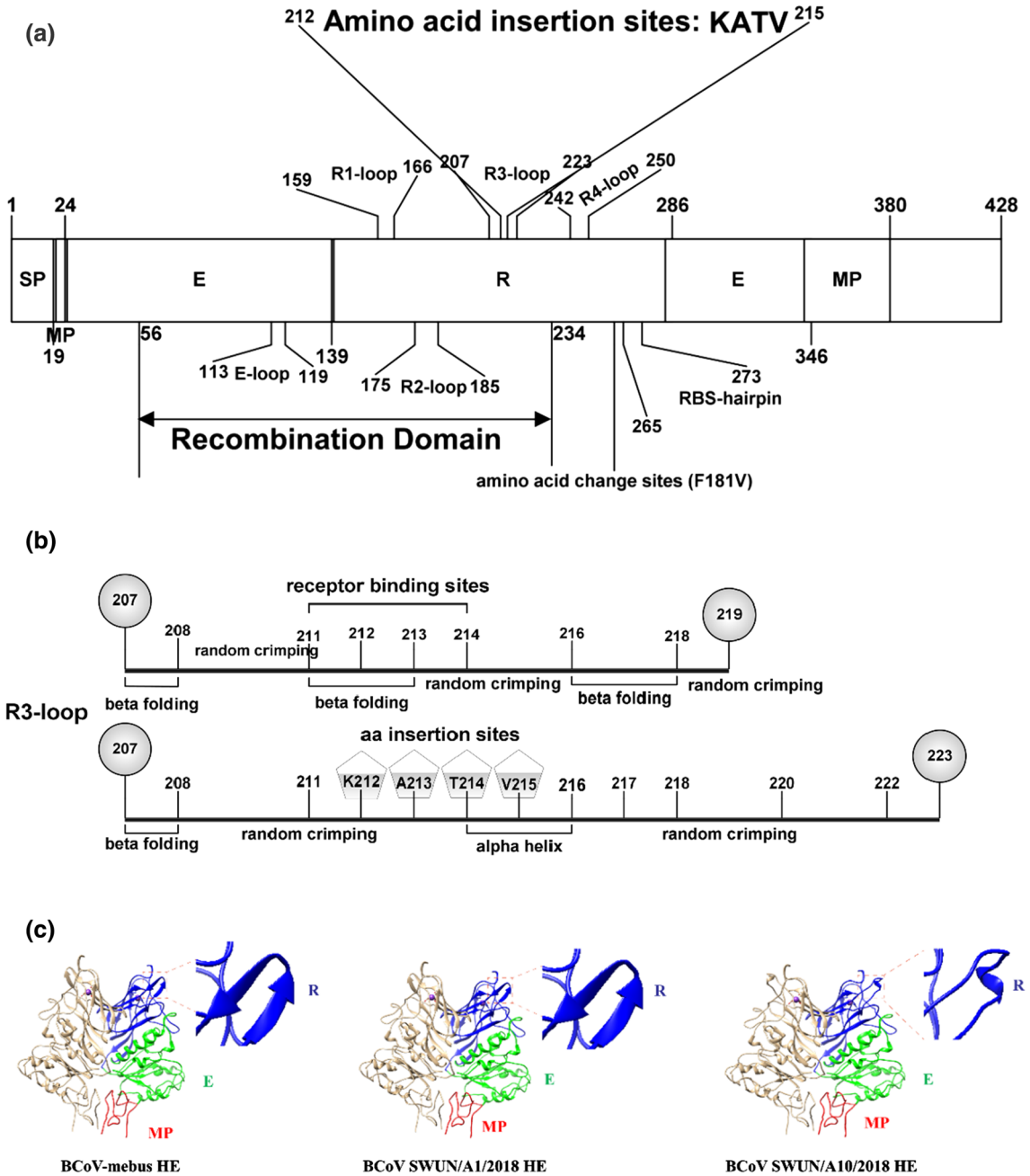

Fig. 2 **A.** Predicted structure of the recombinant HE protein containing the insertion. The insertion is located in the R3-loop of HE. E, esterase domain; MP, membrane-proximal domain; R, lectin domain; SP, signal peptide. **B.** Predicted structure of the R3-loop of the recombinant HE containing the insertion. The receptor binding site is at aa 211–214, and the 4-aa (KATV) insertion between F 211 and F 212, would cause a change in the structure of he R3-loop from FLS (beta folding)-NT (random crimping)-KYY (beta folding) for aa positions 211–218 to FKA (random crimping)-TVL (alpha helix)-SNTKYY (random crimping) for positions aa 211–222. **C.** Predicted crystal structures of the BCoV HE proteins (aa residues 19–380) from three strains: BCoV mebus prototype strain (GenBank accession number: U00735.2), SWUN/A1/2019 with recombinant HE, and SWUN/A10/2019 with recombinant HE with the insertion. The 3D models were constructed based on the crystal structure of bovine coronavirus hemagglutinin-esterase (SMTL ID: 3cl4.1) using the biological online software SWISSMODEL (https://www.swissmodel.expasy.org/interactive). These proteins have identical structures in the esterase domain and the membrane-proximal domain. SWUN/A10/2019 differs from the other two strains in the lectin domain. The boxed portions of the structures indicate the different conformations of the same R domain of BCoV strains. The domains are color-coded: lectin domain (R, blue); esterase domain (E, green); membrane-proximal domain (MP, red)

hemagglutinin-esterase (SMTL ID: 3cl4.1) using the online software SWISSMODEL (https://www.swissmodel.expasy.org/interactive) showed that the position of the 4-aa insertion in the R3-loop between F 211 and F 212 in HE in the recombinant BCoV strains would alter the spatial conformation of the receptor-binding site relative to that in the HE recombinant strains and prototype strains lacking this insertion (Fig. 2B and C). MHV is also a member of betacoronavirus lineage A, and a variant has been reported with an aa insertion in the R3-loop that causes a change in its receptor specificity to 4-O-Ac-Sia from 9-O-Ac-Sia [4]. It is reasonable to speculate that the 4-aa insertion in the R3-loop in HE may affect HE receptor binding in the BCoV strain. The betacoronavirus HE protein is involved in receptor binding, host spectrum and tissue tropism [2, 4, 21], and it would be valuable to further investigate the biological significance of this HE variant.

Betacoronavirus HE may have arisen from an influenza-C-like HE fusion protein (HEF), with transformation of the betacoronavirus HE from a trimer into a dimer for increased plasticity of the receptor-binding site (RBS) compared to that of HEF [3]. The integration of a bovine gene into other viruses has been reported previously. For example, insertion of the bovine SMT3B gene into the genome of bovine viral diarrhea virus correlates with the cytopathogenicity of the virus [22]. Interestingly, the inserted sequence in HE in this study may have originated from bovine chromosomal DNA, which may provide an evolutionary advantage for BCoV, and its identification should enhance our current understanding of the genetic evolution of BCoV.

The BCoV strain with a recombinant HE (designated as SWUN/A1/2018) and BCoV strain with a recombinant HE containing an insertion (designated as SWUN/A10/2018) successfully isolated in Vero cell culture. A cytopathic effect (CPE) was observed after three blind passages, and typical CPE characterized by cell rounding, cell lysis, and detachment from the culture flask was observed after 48 h at passage 6. After plaque purification, the $TCID_{50}$ values of strains SWUN/A1/2018 and SWUN/A10/2018 were calculated as $10^{4.25}$/mL and $10^{4.73}$/mL, respectively, by the Reed-Muench method [23]. To better understand the genetic evolution of SWUN/A10/2018, genomic RNA was successfully obtained from strains SWUN/A1/2018 and SWUN/A10/2018 (GenBank accession number: MN982198 and MN982199). The two complete genomes shared 99.4% nt sequence identity with each other, and 97.0%–98.7% nt sequence identity with all 47 other BCoV genomes with sequences in the GenBank database. Phylogenetic trees constructed by the neighbor-joining method based on the full-length genome sequence and the ORF1a, ORF1b and S genes showed that the two strains have a close genetic relationship (see appendix). Thus, the BCoV SWUN/A10/2018 strain might have evolved from the SWUN/A1/2018 strain.

In summary, we report a novel bovine coronavirus variant with an insertion of 12 nucleotides in the HE gene that results in the addition of four amino acids in the receptor-binding portion of the HE protein. This disruption could alter the receptor-binding site by changing its spatial conformation, and it may reflect an emerging pattern in the evolution of BCoV. The betacoronavirus HE gene is involved in the emergence of new *betacoronavirus* genotypes, host specificity, and tissue tropism [2, 4, 21]. Thus, further investigation of the biological and epidemiological characteristics of this variant BCoV strain is warranted.

Acknowledgements This work was funded by the 13th Five-Year Plan National Science and Technology Support Program (Grant number 2016YFD0500907), the Innovation Team for Emerging Animal Diseases on the Qinghai-Tibet Plateau, Southwest Minzu University, and Fundamental Research Funds for the Central Universities, Southwest Minzu University (Grant number 3300220239).

Data availability statement The data set supporting the conclusions of this article is available in the GenBank database.

Compliance with ethical standards

Ethical approval This study did not involve animal experiments other than collecting faecal samples from diarrhoeic dairy calves during visits to farms for clinical treatment.

Conflict of interest All authors report no conflict of interest related to the submitted work.

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