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Tissue tropism of bovine coronavirus may not be determined by viral genes

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Abstract

Bovine coronavirus (BCoV) causes respiratory and enteric diseases in cattle; however, the determinants of its tissue tropism remain unclear. In this study, we identified identical BCoV whole-genome sequences in tracheal and colonic samples obtained from a single adult cow that died from winter dysentery. After five passages in HRT-18 cells, virus isolation was successfully achieved only from the tracheal sample. Several amino acid substitutions were detected exclusively after isolation. These findings suggest that BCoV tissue tropism is not determined solely by viral genome sequences. It is important to analyze viral genomes directly from clinical samples prior to virus isolation.

Introduction

Bovine coronavirus (BCoV) is an important pathogen of cattle that causes both respiratory and enteric diseases [1]. Infected animals shed virus in nasal secretions and feces, respectively. BCoV is associated with three major clinical syndromes: calf diarrhea; winter dysentery, characterized by hemorrhagic diarrhea, in adult cattle; and respiratory infections. BCoV is a viral pathogen that contributes to the bovine respiratory disease complex (BRDC). BRDC results in substantial economic losses to the cattle industry worldwide [2]; thus, it is important to understand the transmission routes and tissue tropism of its causative agents.

BCoV is an enveloped, positive-sense single-stranded RNA virus belonging to the genus *Betacoronavirus*, subgenus *Embecovirus* [3]. The viral genome encodes several structural proteins, including spike (S), hemagglutinin esterase (HE), membrane (M), envelope (E), and nucleocapsid (N) proteins, as well as multiple non-structural proteins involved in viral replication and transcription [1]. Numerous molecular epidemiological studies have attempted to identify viral genetic determinants associated with respiratory or enteric tropism of BCoV [3–7]. However, the results remain controversial, and no consistent genetic markers that distinguish respiratory and enteric strains have been established [8].

A major limitation of previous studies is that viral genomes were typically compared between different animals, making it difficult to exclude host-related confounding factors. In the present study, we directly compared BCoV genomes detected in respiratory and intestinal tissues obtained from the same animal and examined the effects of virus isolation on viral genome sequences to investigate the relationship between viral genetics, tissue tropism, and cell culture adaptation.

Materials and methods

Sample collection

In February 2022, a 2-year-old adult cow at a farm in Gifu Prefecture, Japan, died from winter dysentery. The animal was necropsied at the Gifu Prefectural Central Livestock Hygiene Service Center. At this time, a tracheal swab and diarrheic intestinal content (colonic sample) were collected. All samples were stored at − 80°C until further analysis.

Virus isolation

HRT-18 cells (human rectal adenocarcinoma cell line) were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Ten percent (w/v) homogenates of tracheal swab and diarrheic samples in phosphate-buffered saline were inoculated onto HRT-18 cells. After incubation for 4–5 days, culture supernatants were collected and passaged onto fresh cells. Blind passages were performed a total of five times.

Immunofluorescence assay

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After blocking, cells were incubated with a rabbit anti-BCoV N protein peptide antibody, followed by Alexa Fluor-conjugated secondary antibodies. Fluorescence signals were visualized using a KEYENCE BZ-X800 fluorescence microscope.

Western blot analysis

Western blot analysis
HRT-18 cells infected with BCoV at a multiplicity of infection of 1 were lysed at 24 h post-infection and subjected to SDS-PAGE. BCoV N protein was detected using the same anti-BCoV N protein peptide antibody and horseradish peroxidase-conjugated secondary antibodies.

Whole-genome sequencing and BLAST analysis

Viral RNA was extracted from clinical samples and virus isolates, and complementary DNA was synthesized using random primers. Whole-genome sequencing was performed using next-generation sequencing combined with 5'- and 3'-RACE methods, as described previously [9]. BLAST analysis was performed using the NCBI database to assess nucleotide sequence identity against previously reported BCoV strains.

Results

In a previous study, we determined the whole-genome sequence of BCoV detected in diarrheic samples obtained from an adult cow that died from winter dysentery [9]. In the present study, we determined the whole-genome sequence of BCoV from a tracheal swab collected from the same animal. Comparison of the sequences revealed that the BCoV genomes detected in tracheal and colonic samples were completely identical (Fig. 1a). BLAST analysis demonstrated that the whole-genome sequence of this virus shared more than 99% nucleotide identity with previously reported BCoV strains (accession number, LC494186.1) detected in Japan, indicating that the virus analyzed in this study does not represent a genetically unusual or highly divergent variant.

Virus isolation was subsequently attempted from both tracheal and colonic samples. Infectious BCoV was successfully isolated only from the tracheal sample after five passages in HRT-18 cells, whereas virus isolation from the colonic sample was unsuccessful. Productive infection of HRT-18 cells was confirmed by immunofluorescence staining and western blot analysis targeting the BCoV N protein (Fig. 1b).

Whole-genome sequencing of the isolated virus revealed one synonymous substitution in the S gene and several non-synonymous substitutions in the pp1b, HE, and S genes compared with the viral genomes detected directly from the clinical samples. Notably, all amino acid substitutions were detected only after virus isolation, suggesting that these mutations arose during cell culture adaptation (Fig. 1a).

Discussion

In this study, we demonstrated that identical bovine coronavirus (BCoV) genomes were present in both respiratory and intestinal tissues of a single cow. This direct comparison within the same host provides evidence that viral genetic differences are not required to explain BCoV tissue distribution, indicating that tissue tropism is not determined solely by viral genome sequences. Consistent with this conclusion, BLAST analysis confirmed that the virus examined in this study is genetically close to previously reported Japanese BCoV strains, sharing more than 99% nucleotide identity at the whole-genome level. This finding indicates that the virus analyzed here does not represent a genetically unusual or divergent lineage. Therefore, the observed tissue distribution of BCoV cannot be attributed to infection with a distinct or atypical viral strain.

We found that virus isolation resulted in the accumulation of amino acid substitutions in nsp12, HE, and S proteins, all of which were absent in viral genomes detected directly from clinical samples. These findings are consistent with a previous study by Zhang et al. [4], who demonstrated that during cell culture adaptation, an enteric BCoV strain accumulated mutations that rendered it genetically similar to a corresponding respiratory BCoV isolate obtained from the same animal. In the present study, several mutations identified after serial passages in HRT-18 cells closely corresponded to those reported by Zhang et al. as culture-adaptive changes. Notably, the two HE substitutions (V103I and N104D) were identical to the AH65-E → AH65-E-TC mutations described in their study. Similarly, the S protein substitutions (D28N, Q179K, and E965D) were located within regions where mutation accumulation was observed during in vitro adaptation. In addition, the ORF1ab substitutions (M4891I and N4894Y) were detected within the same genomic region (nt 14600–15000) previously reported to harbor clusters of adaptive mutations. Collectively, these observations strongly suggest that the mutations identified in our isolates represent cell culture-associated adaptation events rather than genetic determinants of tissue tropism in vivo.

The amino acid substitutions detected in nsp12, HE, and S proteins during BCoV isolation are therefore likely to reflect viral adaptation to replication in HRT-18 cells. The two nsp12 substitutions (M4891I and N4894Y) are located within the thumb domain of the RNA-dependent RNA polymerase [10] and may

influence local conformational flexibility or interactions with replication cofactors. The HE substitutions (V103I and N104D) occur near regions implicated in receptor binding and esterase activity [11] and may modify surface charge or receptor interactions in human-derived cells. Similarly, the S protein substitutions (D28N, Q179K, and E965D) affect residues within the S1 N-terminal domain and S2 region, which are involved in receptor recognition and membrane fusion [12]. Together, these changes may contribute to coordinated adaptation of viral entry, replication, and release processes, thereby facilitating more efficient propagation of BCoV in vitro.

Although this study is based on a single case, the direct comparison of viral genomes derived from different tissues within the same animal minimizes host-related confounding factors. However, our findings do not exclude the possibility that viral genes influence tissue-specific pathogenicity under certain conditions. The recent development of reverse genetics systems for BCoV will enable future studies using genetically homogeneous viruses to more definitively address the contribution of viral genetic factors to tissue tropism and pathogenicity [13]. In this context, continuous epidemiological surveillance combined with careful genomic analysis of clinical samples will be essential for interpreting and validating such experimental findings.

Declarations

Author contributions

Keisuke Nakagawa: Conceptualization,methodology, analysis, wrote the first draft, reviewed and edited final draft. **Aimi Yoshida:** Methodology, reviewed and edited final draft. **Keisuke Kuwata:** Resources, reviewed and edited final draft. **Yuko Kitamura:** Resources, reviewed and edited final draft.

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Data availability

The complete genome sequences of strain GF2020 obtained from tracheal swab sample and isolate have been deposited in GenBank under accession numbers LC918946 and LC918947, respectively. The corresponding raw sequence data are available in the Sequence Read Archive (SRA) under accession numbers DRX326681 (BioSample SAMD00414418)

Conflicts of Interest: The authors declare no conflict of interest.

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Figures

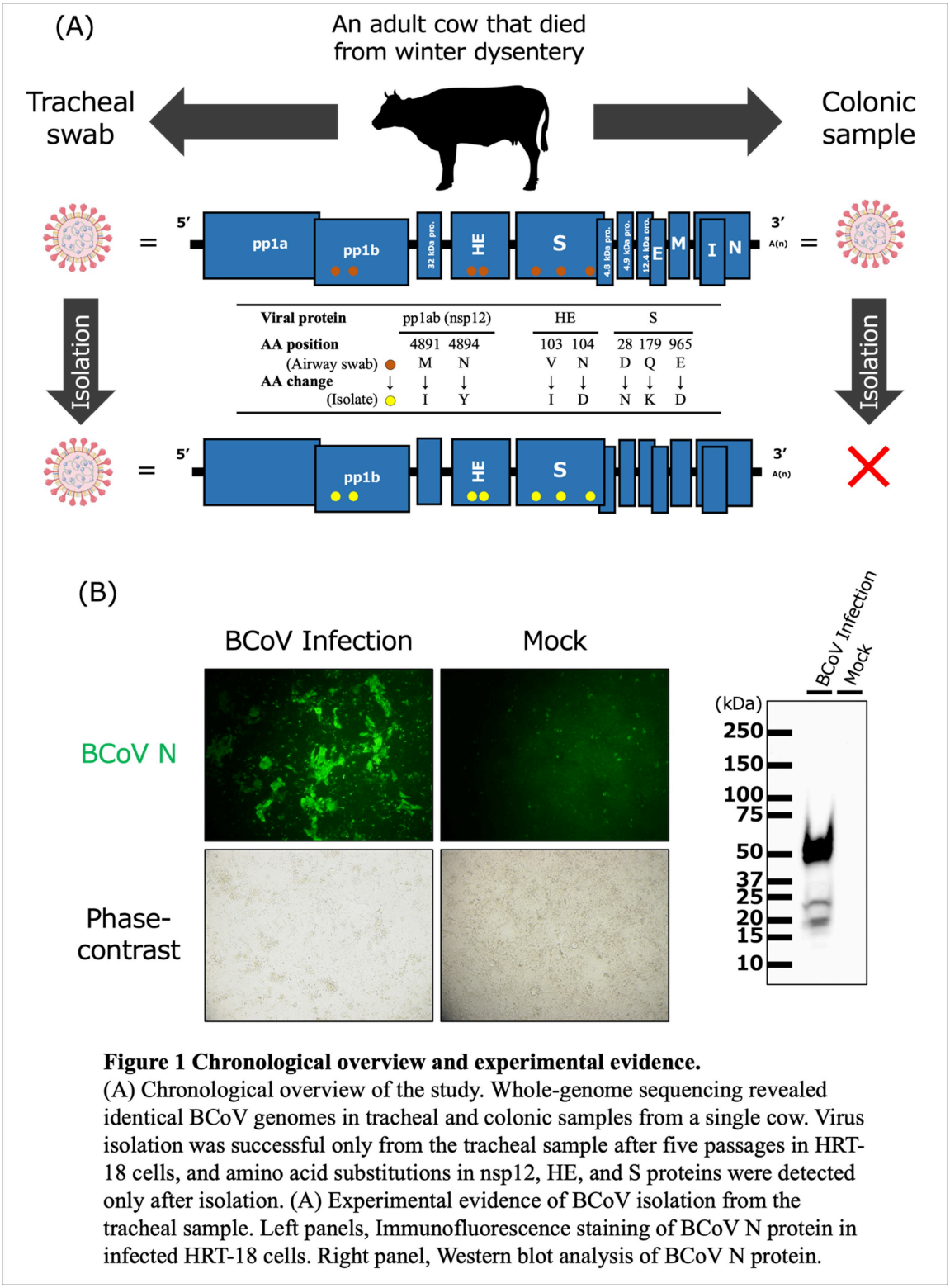


Figure 1 Chronological overview and experimental evidence.
(A) Chronological overview of the study. Whole-genome sequencing revealed identical BCoV genomes in tracheal and colonic samples from a single cow. Virus isolation was successful only from the tracheal sample after five passages in HRT-18 cells, and amino acid substitutions in nsp12, HE, and S proteins were detected only after isolation. (A) Experimental evidence of BCoV isolation from the tracheal sample. Left panels, Immunofluorescence staining of BCoV N protein in infected HRT-18 cells. Right panel, Western blot analysis of BCoV N protein.

Figure 1

See image above for figure legend