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1 *Virology*

2 **NOTE**

3 **A long-term animal experiment indicating persistent infection of bovine**

4 **coronavirus in cattle**

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18 Running head: PERSISTENT INFECTION OF BCoV IN CATTLE

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Abstract

A long-term animal experiment involving inoculation with bovine coronavirus (BCoV) was conducted to verify its persistent infection in cattle. Three colostrum-deprived Holstein calves were housed separately in individual rooms of a high-containment facility and inoculated with the BCoV strain Kumamoto/1/07. Until the end of the experiment (1,085, 700, and 280 days, respectively), viral RNAs were detected sporadically by RT-PCR and nested PCR from plasma, nasal discharge, and feces. Seroconversion and titer changes were validated by hemagglutination inhibition tests and neutralization tests. Among the samples, nasal discharge showed a higher viral positivity than feces, which seemed to be associated with positive detection in the plasma. These data demonstrate the existence of persistent infection of BCoV in the respiratory tissues of cattle.

Keywords: bovine coronavirus, long-term animal experiment, persistent infection

37 Bovine coronavirus (BCoV) is a member of the order *Nidovirales*, family
38 *Coronaviridae*, subfamily *Coronavirinae*, genus *Betacoronavirus*, species
39 *Betacoronavirus-1*. This species also includes *Human coronavirus OC43*, *Porcine*
40 *hemagglutinating encephalomyelitis virus*, *Equine coronavirus*, and *Canine respiratory*
41 *coronavirus* [1].
42 BCoV infects the respiratory and digestive organs of cattle and causes neonatal calf
43 diarrhea, bloody diarrhea in adult cattle (winter dysentery), and respiratory symptoms,
44 including shipping fever in feedlot cattle [6, 12, 16]. BCoVs have spread widely across
45 cattle farms all over the world, and thus, nearly all adult cattle have antibodies against
46 the virus. Outbreaks typically occur during autumn and winter [2] and are associated
47 with the housing period; however, several cases have also been reported during warmer
48 seasons [5, 10, 13]. Although the mortality of this disease is low, it causes substantial
49 economic losses owing to a reduction in milk production in dairy farms [14, 19] and
50 meat production in beef farms [17, 18].
51 The main transmission route of BCoVs is horizontal infection, i.e., ingestion or
52 inhalation of virus from feces or nasal discharges into the mouth or nasal cavity [7, 15].
53 There has been no report of vertical infection so far. BCoV antigens are detected in the
54 feces of clinically healthy cattle [4], and studies on BCoV shedding in feces showed the
55 virus to be detected over a long period on farms, even though the number of cattle
56 shedding the virus decreased [3]. This suggests the existence of persistently infected
57 cattle, which might also be the origin of transmission. To verify this hypothesis, we
58 conducted a longitudinal animal experiment.
59 The BCoV used in this experimental study was obtained from a clarified
60 suspension of feces from a cow on a farm where a BCoV case had been confirmed in
61 2007 by using HRT-18G cells (isolate Kumamoto/1/07) [11]. The culture supernatant of

62 the isolate was inoculated into cattle by the oral route. Feces were collected when the
63 cattle showed diarrhea. The isolate was passaged in colostrum-deprived Holstein calves
64 twice, and feces of the infected calves were harvested as inoculum for this study (data
65 not shown).

66 Three colostrum-deprived Holstein calves were used in this study. Each animal
67 was maintained in a separate room of a high-containment facility at our institute. In this
68 facility, the animal room area is separated from the preparation room by a shower room
69 area. To enter the animal room area, the investigator undresses and puts on work clothes
70 and boots in this area. The animal room area has 5 independent animal rooms and a
71 corridor. Each animal room has a ventilator with HEPA filers to prevent the
72 reintroduction of the virus from outside to the experimental cattle. During the animal
73 experiment, we adhered to the strict high hygiene and biosecurity measures to prevent
74 reinfection from other cattle. To enter the animal room, boots were removed at the
75 corridor, and the "inside" boots and waterproof clothes kept in the animal room were
76 worn. To exit the room after husbandry and sampling, put off the boots and waterproof
77 clothes were taken off after disinfection with sodium hypochlorite using a spraying
78 device.

79 The Animal Care and Use Committee of the National Institute of Animal Health
80 approved all animal procedures prior to the initiation of this study (authorization
81 number: 11-083).

82 One calf (11 days old, referred to as "calf 1") was inoculated orally using a catheter
83 that delivered a sample of the centrifuged supernatant at 1,500 × g for 10 min from a
84 mixture of 10 g feces as mentioned above and 50 m*l* phosphate-buffered saline (PBS)
85 containing 100 μg/m*l* gentamicin into the esophagus. Samples of nasal discharge, feces,
86 plasma, and sera were collected daily until 10 days post inoculation (dpi), followed by

87 weekly collection until 141 dpi and then twice-weekly collection until the end of the
88 experiment (1,085 dpi). The nasal discharge samples were collected by inserting cotton
89 into the nasal cavity for a few minutes and extracting the discharge using sterilized
90 disposable syringes. Feces were collected directly from the rectum and prepared as a
91 10% suspension in PBS containing 100 μg/m*l* gentamicin and 1 μg/m*l* trypsin for virus
92 isolation and RNA extraction. The same experiment was carried out on another calf (8
93 days old, "calf 2") using the centrifuged supernatant from a mixture of 50 m*l* PBS and
94 10 g feces of calf 1 collected at 3 dpi (after it showed diarrhea). Samples of nasal
95 discharge, feces, plasma, and sera were collected daily until 11 dpi, followed by weekly
96 collection until 78 dpi and twice-weekly collection until the end of the experiment (700
97 dpi). The same experiment was carried out on yet another calf (4 days old, "calf 3")
98 using centrifuged supernatant from a mixture of 50 m*l* PBS and 20 g feces of calf 2
99 collected at 5 dpi, when it showed diarrhea. Nasal discharge, feces, plasma, and serum
100 samples were collected daily until 8 dpi, followed by weekly collection until 33 dpi and
101 twice-weekly collection until the end of the experiment (280 dpi).

102 Viral RNA was extracted from plasma, nasal discharge, and 10% fecal suspensions
103 in PBS using the High Pure Viral RNA Kit (Roche Diagnostics, Tokyo, Japan)
104 according to the manufacturer's instructions. BCoV-specific genes were detected from
105 the extracted RNA by the RT-PCR assay using a Titan One Tube RT-PCR Kit (Roche
106 Diagnostics), with the following thermal cycling profile: 30 min at 50°C; 2 min at 94°C;
107 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and
108 extension at 68°C for 45 sec; and completion of amplification with a 7-min extension
109 step at 68°C [20]. The oligonucleotide primers used for nested PCR were designed from
110 the nucleotide sequence of the Kakegawa strain (GenBank accession no. AB354579) in
111 this study. The primers were as follows: BCoV-nu,

112 5′ -TGCTACTTCTCAGCAACCATCAG-3′ (nt 29,540-29,562, sense primer), and
113 BCoV-nd, 5′ -TTGGCATGCGGTCCTGTTCCAAG-3′ (nt 29,778-29,800, antisense
114 primer). The size of the amplification fragment is 261 bp. Nested PCR was performed
115 by using TaKaRa Ex Taq (Takara-Bio., Shiga, Japan), and the thermal cycling profile
116 was as follows: 1 min at 98°C; 30 cycles of denaturation at 98°C for 10 sec, annealing
117 at 55°C for 30 sec, and extension at 72°C for 45 sec; and completion of amplification
118 with a 7-min extension step at 72°C. To prevent laboratory contamination in RT-PCR
119 and nested PCR, we set up mixtures for these reactions in a laminar flow cabinet
120 equipped with an UV lamp, wore fresh gloves at each step, used pipette tips with
121 aerosol filters. Negative controls were also included in every PCR experiment to
122 detection of contamination. The RT-PCR and nested PCR products were visualized on
123 1.8% agarose gels stained with ethidium bromide.

124 The hemagglutination inhibition (HI) test was conducted by the microtiter method
125 [9]. Sera were treated with kaolin and chicken erythrocytes and heat inactivated. As
126 antigen for the HI test, the Kakegawa strain was used in this assay because the
127 hemagglutinating (HA) activity of the Kumamoto/1/07 strain was low and unstable with
128 chicken and mouse erythrocytes, similar to recent Japanese isolates (Kanno et al.,
129 unpublished data), whereas the HA activity of the Kakegawa strain was sufficient.
130 Briefly, twofold dilutions of serum were prepared in duplicate and reacted with the virus.
131 The HI titers were expressed as the reciprocal of the highest serum dilution that
132 completely inhibited the HA activity of 4 HA units of the virus.

133 Serum samples were also tested in a virus neutralization test by preparing serial
134 twofold dilutions of serum were reacted in duplicate with 100 of 50% tissue culture
135 infective doses ($TCID_{50}$) of Kumamoto/1/07, followed by incubation for 1 h at 37°C.
136 After incubation, the serum–virus mixture was transferred onto monolayered human

137 rectal tumor cells (HRT-18G) cultured in microplates and incubated for 5 days at 37°C.
138 The neutralization antibody titers were expressed as the reciprocal of the highest serum
139 dilution that completely inhibited the cytopathic effect (CPE).

140 All 3 calves showed clinical signs, such as loose or diarrheal stool, up to 8–12 dpi.
141 Viral RNA was detected from the plasma during the period of onset (Fig. 1A-C). After
142 recovery from the disease, viral RNA was sporadically detected by RT-PCR and nested
143 PCR from plasma. Final detection days (last day when a sample showed a positive
144 result) by RT-PCR were 792, 632, and 201 dpi, and by nested PCR, 1,085, 632, and 257
145 dpi, respectively, in the 3 calves. As with the plasma, viral RNA was detected from
146 nasal discharge on the basis of viral titers, and was detected sporadically by PCR-based
147 experiments. Final detection days by RT-PCR were 792, 589, and 173 dpi, and by
148 nested PCR, 932, 589, and 173 dpi, respectively, in the 3 calves. Viral RNA was not
149 detected from the feces after the disease onset in calves 2 and 3. However, nested PCR
150 was positive for the virus at 42 dpi in calf 2. In calf 1, viral RNA from feces was
151 detected sporadically by the PCR-based experiments. In this calf, the final detection day
152 was 28 dpi by RT-PCR and 1,058 dpi by nested PCR.

153 Virus isolation was conducted from the samples showing positive results by
154 RT-PCR and nested PCR. The samples were inoculated into HRT-18G monolayer cells
155 in 12-well plates and incubated for 5 days at 37°C and 5% CO_2. If a CPE was not
156 observed in the incubation period, passaging into fresh cells was conducted twice using
157 the inoculum after freeze-thawing the cultures thrice. However, the virus was not
158 isolated, except in the samples from the period of onset.

159 The HI and neutralization titers in the 3 calves increased several days after disease
160 onset and peaked at 19, 4, and 4 weeks post inoculation, and at 6, 10, and 20 weeks post
161 inoculation, respectively, in the 3 calves. Thereafter, the titers gradually decreased.

162 However, both titers showed a rapid rise at 421 dpi in calf 1 (640 and 724, respectively)
163 (Fig. 1A). Ten days before the sampling, calf 1 showed diarrhea at 411 and 412 dpi
164 (data not shown). Therefore, we surmised that the virus was reactivated in the cattle,
165 probably in the respiratory tissues, causing diarrhea followed by this booster effect. The
166 virus in the digestive tract might have been quickly inactivated and excreted; therefore,
167 viral RNAs were not detected from nasal discharge and feces at 421 dpi, 10 days after
168 the onset. Unfortunately, feces samples had not been collected at the onset, so no virus
169 was isolated. A rapid rise in these titers was also found in calf 2 at 632 dpi, just after the
170 detection of viral RNA from the plasma at 617 dpi by both RT-PCR and nested PCR
171 (Fig. 1B). This finding might also be attributed to virus reactivation in respiratory
172 tissues and quick inactivation by host immunity without clinical signs.

173 This study showed that the BCoV RNA was long-lasting, having been detected
174 from the nasal discharge of cattle that had been maintained in an isolated room of a
175 high-containment facility to prevent virus intrusion from outside. This indicated the
176 existence of persistent infection of BCoV in the respiratory tissues of cattle, although
177 further investigations are warranted because the virus was not isolated. Other
178 coronaviruses, such as *Transmissible gastroenteritis virus*, *Canine coronavirus*, and
179 *Feline coronavirus*, have also been reported to cause long-term infection [8, 21, 22].
180 Thus, coronavirus tends to cause persistent infection in host animals. Often, calf
181 diarrhea caused by BCoV repeatedly occurs at the same farm every year. This might be
182 explained by the presence of persistently infected cattle.

183

184 **Declaration of conflicting interests**

185 The authors have no potential conflicts of interest to declare with respect to the research,
186 authorship, and/or publication of this article.

187

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261

262 **Figure legends**

263 **Fig.1.** Detection of viral genes from clinical samples by RT-PCR and nested PCR assays
264 and of antibodies by the hemagglutination inhibition (HI) test and neutralization test
265 (NT) in calves 1 (A), 2 (B), and 3 (C). The days when the viral RNA was detected from
266 the plasma, nasal discharge, and feces are indicated by green, violet, and orange bars,
267 respectively. The numbers in parentheses indicate weeks post inoculation. The half-size
268 bar showed that the viral RNA was detected only by nested PCR. The antibody titers
269 measured in the HI and VN tests are on the left y-axes. The period of disease onset is
270 indicated by the black bar.

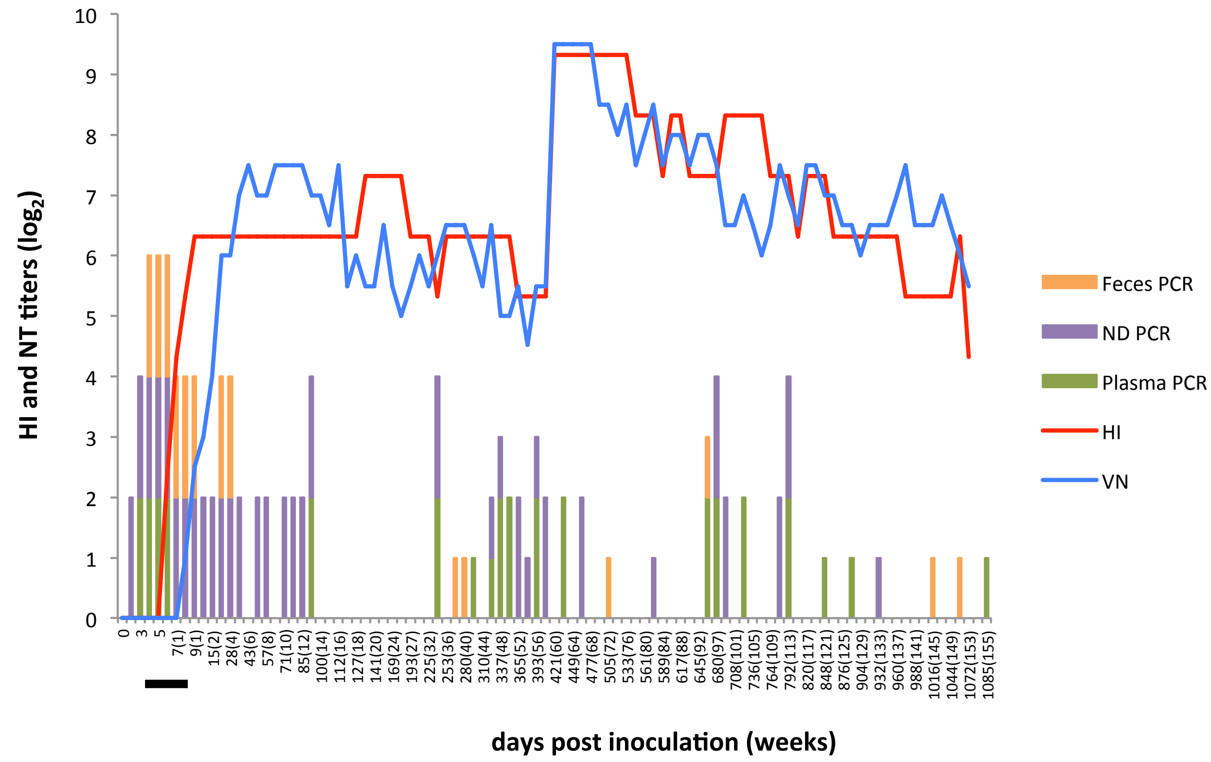

Fig. 1A

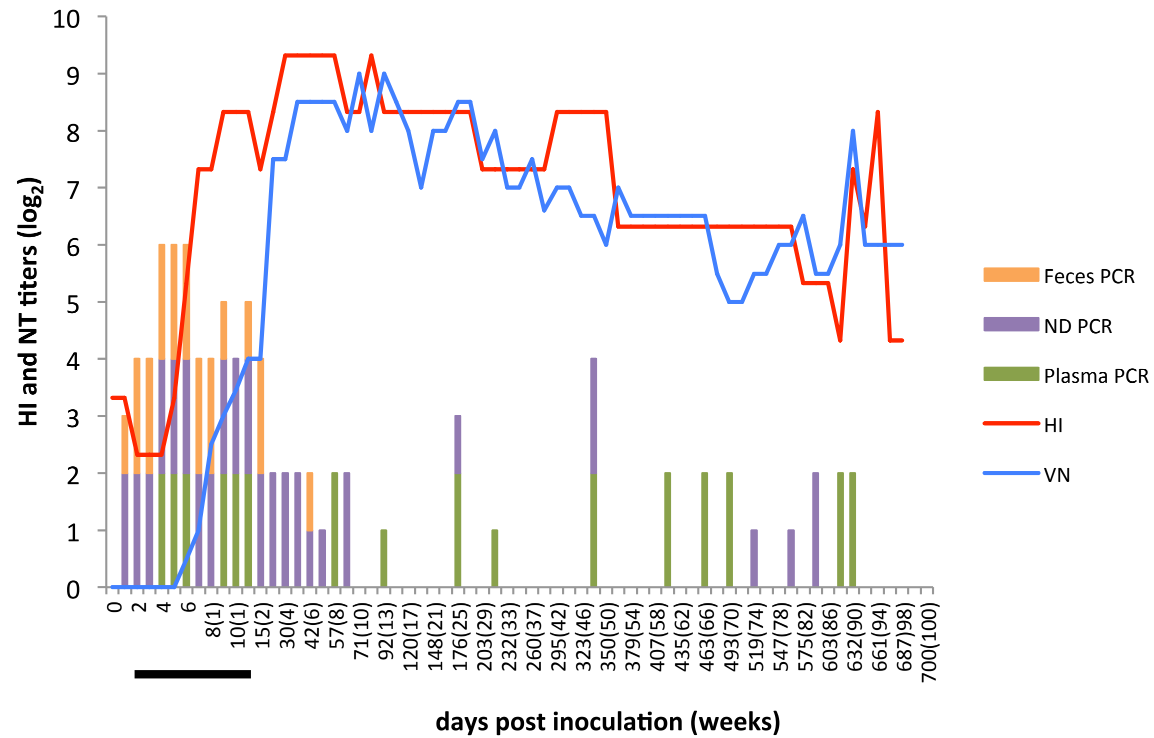

Fig. 1B

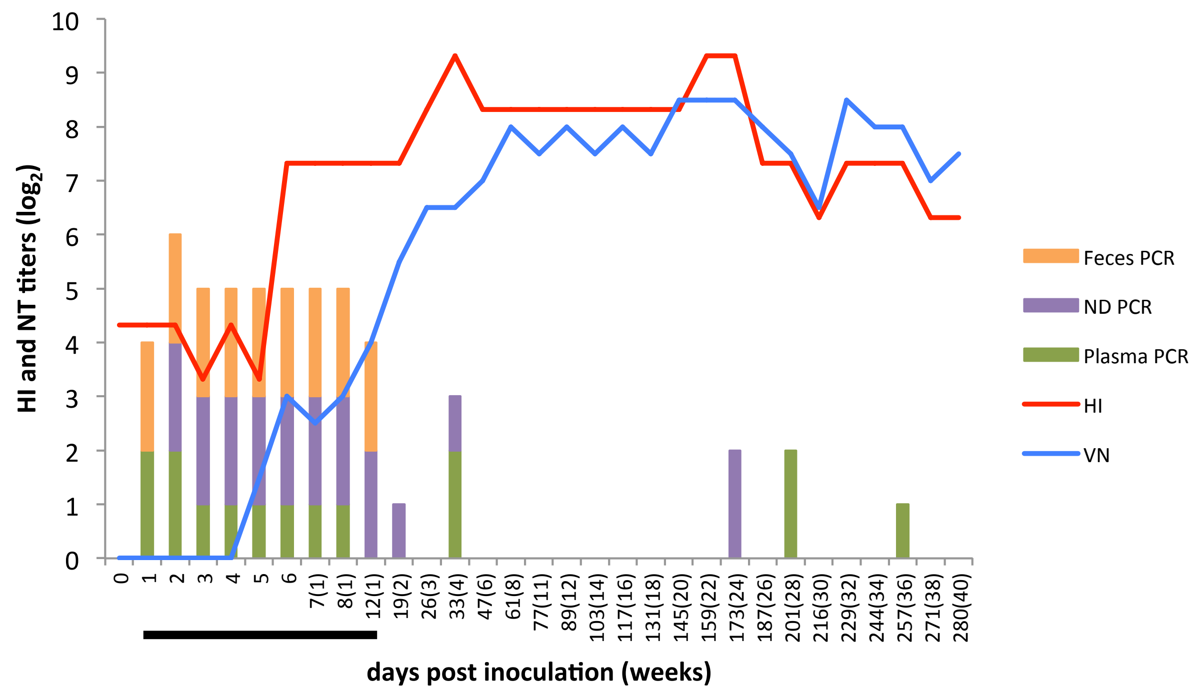

Fig. 1C