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Title:
Establishment of serological test to detect antibody against ferret coronavirus

Running head
DETECTION OF ANTIBODY AGAINST FRC_OV

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ABSTRACT. Since there is no available serological methods to detect antibodies to ferret coronavirus (FRCoV), an enzyme-linked immunosorbent assay (ELISA) using recombinant partial nucleocapsid (N) proteins of the ferret coronavirus (FRCoV) Yamaguchi-1 strain was developed to establish a serological method for detection of FRCoV infection. Many serum samples collected from ferrets recognized both a.a. 1-179 and a.a. 180-374 of the N protein, but two serum samples did not a.a. 180-374 of the N protein. This different reactivity was also confirmed by immunoblot analysis using the serum from the ferret No.22.Therefore, the a.a. 1-179 of the N protein was used as an ELISA antigen. Serological test was carried out using sera or plasma of ferrets in Japan. Surprisingly, 89% ferrets in Japan had infected with FRCoV. These results indicated that our established ELISA using a.a. 1-179 of the N protein is useful for detection of antibody to FRCoV for diagnosis and seroepidemiology of FRCoV infection.

KEY WORDS: enzyme-linked immunosorbent assay (ELISA), ferret coronavirus (FRCoV), nucleocapsid (N)

63 Epizootic catarrhal enteritis (ECE), a new enteric disease of domestic ferrets
64 (*Mustelo putorius furo*), was first described in the United States in the early 1990s [11].
65 A novel alphacoronavirus, ferret coronavirus (FRCoV), was detected as the causative
66 agent of ECE in 2000 and designated as ferret enteric coronavirus (FRECV) [11, 12].
67 Ferrets with ECE show general clinical signs including lethargy, anorexia and vomiting,
68 and characteristic signs with foul-smelling, green mucous-laden diarrhea [12]. FRCoV
69 was also reported as the causative agent of feline infectious peritonitis (FIP)-like disease
70 in 2006, and the virus was designated as ferret systemic coronavirus (FRSCV) [2-4].
71 Ferrets with FIP-like disease show characteristic clinical signs of large palpable
72 intra-abdominal masses like dry type of FIP [2-4]. FRCoVs were divided into two
73 genotypes, I and II, based on differences in the spike (S) gene, and it was suggested that
74 genotype I was associated with FIP-like disease and genotype II was with ECE [13.
75 However, we previously showed that there was no significant relationship between the
76 genotypes of FRCoV and disease in Japan [19]. In addition, genotype I FRCoV was
77 also detected from many asymptomatic ferrets in the Netherlands [6]. The relationship
78 between genotypes of FRCoV and clinical symptoms remains unclear.
79 Although FRCoV genes were detected in ferrets by reverse
80 transcription-polymerase chain reaction (RT-PCR), there is no method to detect

81 antibodies to FRCoV. We attempted to isolate FRCoV using feline cell lines and our
82 newly established ferret cell line (manuscript in preparation), but the virus has not yet
83 been isolated. Because the nucleocapsid (N) is conserved between coronaviruses and
84 used as an antigen to detect antibody [5, 8], the N protein of FRCoV was one of the
85 most likely antigen candidates to detect antibody to FRCoV. In this study, an
86 enzyme-linked immunosorbent assay (ELISA) using recombinant N proteins was
87 established and applied to investigate the seroprevalence of FRCoV infection in Japan.
88
89 MATERIALS AND METHODS
90
91 *Samples from domestic ferrets*: From animal hospitals in Japan, 9 serum and 26
92 plasma samples were collected from domestic ferrets between Aug 1st, 2012 and Feb 4th,
93 2014 and used for ELISA and immunoblot analysis. We analyzed and reported the
94 results for 79 of the feces samples in our previous study [9]. One fecal sample from a
95 ferret in our animal facility was used to amplify the N gene of the FRCoV Yamaguchi-1
96 strain.
97 *Amplification of N genes*: RNA of the Yamaguchi-1 strain was extracted from
98 feces using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to

99 the manufacturer's instructions. N genes of the Yamaguchi-1 strain was amplified by
100 RT-PCR using TaKaRa RNA LA PCR^{TM} Kit (AMV) Ver. 1.1 (TaKaRa, Otsu, Japan).
101 RT was performed using random 9-mer oligonucleotide primers, and PCR was
102 performed using primer pairs, NF2 (5'-*TTA CAT ATG GTA TAA GAA CTA AAC*-3')
103 and NR2 (5'-*CGA TGT AGG AAC CTT CAA AAT A*-3'). PCR products were
104 electrophoresed on a 0.8% gel and extracted using a QIAEX II Gel Extraction Kit
105 (QIAGEN).

106 *Construction of expression plasmids*: Yamaguchi-1 strain fragments were
107 amplified using primer pairs, N1F (5'-*TGG GAT CCA TGG CTG GAA ACG GAC*
108 *CAC*-3') and N179R (5'-*GAC TCG AGT TAG TTA TTG GAT CTA TTG TTG GAC*-3')
109 for nt 1-537 encoding a.a. 1-179, and N180F (5'-*TGG GAT CCA TTA ACA GTA ACA*
110 *GTG GTG ATA T*-3') and N374R (5'-*GAC TCG AGT TAG TTT AGT TCA TCA ATA*
111 *ATT TCA*-3') for nt 538-1125 encoding a.a. 180-374. These forward and reverse primers
112 contained *Bam*HI and *Xho*I sites at the 5′-end, respectively. Fragments were purified
113 using a MinElute PCR purification Kit (QIAGEN) and digested with restriction
114 enzymes, *Bam*HI and *Xho*I. Two fragments of the Yamaguchi-1 strain were
115 electrophoresed on a 0.8% gel and extracted using a QIAEX II Gel Extraction Kit
116 (QIAGEN). Fragments were then cloned into *Bam*HI and *Xho*I sites of the expression

117 plasmid pGEX-6P-1 vector (GE Healthcare, Piscataway, NJ, U.S.A.) using a DNA
118 Ligation Kit Ver. 2.1 (TaKaRa). Plasmids were transformed into *Escherichia (E.) coli*
119 strain DH5α (TOYOBO, Osaka, Japan).

120 *Expression and purification of glutathione-S transferase (GST)-fusion proteins*:
121 Two N protein fragments, N1-179 and N180-374, were expressed as fusion proteins
122 with GST, GST-N(1-179) and GST-N(180-374), respectively. *E. coli* containing
123 recombinant or control plasmid was cultured in 2 × yeast extract and tryptone (YT)
124 medium (1.6% tryptone, 1% yeast extract and 0.5% NaCl, pH 7.0) containing 50 μg
125 ampicillin ml^{-1}. Expression of recombinant proteins was induced by the addition of
126 1mM isopropyl β-D-1-thiogalactopyranoside (Wako, Osaka, Japan) for 4 hr. The
127 bacterial cells were suspended in sonication buffer (50mM Tris–HCl, pH 8.0, 50mM
128 NaCl, 1mM EDTA and 1mM dithiothreitol) and lysed using a Multi-beads shocker
129 (YASUI KIKAI, Osaka, Japan). After centrifugation, supernatants were mixed with
130 Triton X-100 at a final concentration of 1% for 30 min, and then centrifuged at 20,630 ×
131 g at 4 °C for 30 min. The supernatants were collected, mixed with glutathione sepharose
132 4B beads (GE Healthcare) and incubated at 4 °C for 30 min. After centrifugation, beads
133 were washed four times with phosphate-buffered saline (PBS) containing 0.5% Triton
134 X-100 and once with sonication buffer. The beads were mixed with 300 μl of 10 mM

135 glutathione and incubated at 4 °C for 1 hr. After incubation, supernatants were
136 harvested as purified recombinant proteins and used for ELISA and immunoblot
137 analysis. The purified proteins were confirmed to be single bands by coomassie-brilliant
138 blue (CBB) staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis
139 (SDS-PAGE) analysis.

140 *SDS-PAGE analysis of recombinant proteins*: Purified recombinant proteins
141 were mixed in equal volumes of 2 × sample buffer (125 mM Tris–HCl, pH 6.8, 40%
142 glycerol, 4% SDS, 0.002% bromophenol blue and 10% 2-mercaptoethanol) and boiled
143 for 3 min. Samples were electrophoresed by SDS-PAGE and stained with CBB.

144 *Quantification of recombinant proteins*: Concentration of purified proteins was
145 measured using Bio-Rad Protein Assay Dye Reagent Concentrate (BIO-RAD, Hercules,
146 CA, U.S.A.) according to the manufacturer's instructions. A standard curve was
147 constructed using bovine serum albumin (Sigma-Aldrich, St. Louis, MO, U.S.A.). The
148 absorbance was measured using a spectrophotometer (BIO-RAD.) at 595 nm.

149 *ELISA*: The concentration of purified recombinant proteins was adjusted to 5
150 μg ml^{-1} with adsorption buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6). GST was
151 used as a control at 5μg ml^{-1}. One hundred microliters of purified recombinant proteins
152 and GST were added to 96-well microplates (Maxisorp; Nunc, Roskilde, Denmark).

153 After incubation at 37 °C for 2 hr, plates were placed at 4 °C overnight. The wells were
154 washed three times with PBS containing 0.05% Tween 20 (PBS-T) and then incubated
155 with 200 µl of 1% Block Ace (Dainippon Pharmaceutical, Osaka, Japan) in PBS at
156 37 °C for 30 min. After washing three times with PBS-T, 100 µl of diluted sera or
157 plasma were added to duplicate wells and incubated at 37 °C for 30 min. Sera or plasma
158 was diluted to 1:100 or 1:500 with PBS-T containing 0.4% Block Ace. Subsequently,
159 wells were washed three times with PBS-T before 100 µl of peroxidase-conjugated
160 anti-ferret immunoglobulin (ROCKLAND, Limerick, PA, U.S.A.) diluted with PBS-T
161 containing 0.4% Block Ace was added and incubated at 37 °C for 30 min. Following
162 three washes with PBS-T, 100 µl of Horseradish Peroxidase Substrate (BIO-RAD) was
163 added to each well. After incubation at room temperature for 30 min, the enzymatic
164 reaction was stopped by adding 100 µl of 2% oxalic acid to each well. The absorbance
165 was measured using a spectrophotometer (BIO-RAD) at 415 nm. All results were
166 subtracted from the value for GST and the cut-off value was arbitrarily set at 0.5.

167 *Immunoblot analysis*: Recombinant proteins mixed with 2 × sample buffer
168 were electrophoretically separated by SDS-PAGE, and then transferred to
169 polyvinylidene difluoride membranes (Millipore, Bedford, MA, U.S.A.). After
170 transferring, the membranes were incubated with Tris-buffered saline (TBS) (20 mM

171 Tris-HCl and 150 mM NaCl, pH 7.5) containing 3% gelatin (BIO-RAD) at 37 °C for 45
172 min. After washing three times with TBS containing 0.05% Tween 20 (T-TBS),
173 membranes were incubated with 2 ml of ferret serum or plasma diluted to 1:1,000 in
174 T-TBS containing 1% gelatin (BIO-RAD) at 37 °C for 45 min. After three washes with
175 T-TBS, membranes were incubated with 2 ml of peroxidase-conjugated anti-ferret
176 immuneglobulins with T-TBS containing 1% gelatin at 37 °C for 45 min. The
177 membranes were washed three times with T-TBS and then three times with TBS. The
178 reaction was visualized using 3,3'-diaminobenzidine tetrahydrochloride (Wako, Osaka,
179 Japan).

180 *Sequence analysis*: Nucleotide sequences were determined using a BigDye
181 Terminator Ver. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA,
182 U.S.A.) according to the manufacturer's instructions. The deduced amino acid
183 sequences of the N protein were compared with FRECV strain MSU-2 (GenBank
184 accession no. GU338457), FRECV strain MSU-1 (DQ340562) and FRSCV strain
185 MSU-1 (GU338456). The nucleotide sequences of N gene of the Yamaguchi-1 strain
186 was deposited into DDBJ (accession no. LC029423).

187 *Phylogenetic analysis*: A phylogenetic tree was constructed using the program
188 MrBayes Ver. 3.2.2 [7] for MrModeltest analysis with a WAG substitution matrix [10].

We referred to the following sequences to construct the phylogenetic tree of N protein sequences; FRECV strain MSU-2 (GU338457), FRECV strain MSU-1 (DQ340562), FRSCV strain MSU-1 (GU338456), mink CoV strain WD1127 (HM245925), mink CoV strain WD1133 (HM245926), CCoV type II strain fc1 (AB781790), FCoV type II strain M91-267 (AB781788), FCoV type I strain C3663 (AB535528), SARS-CoV strain BJ182-12 (EU371564) and FRCoV strain Yamaguchi-1 (LC029423). The tree was represented graphically using FigTree Ver. 1.4.2 [1].

Statistical analysis: Significant differences were statistically analyzed using Chi-square and Fisher's exact probability tests. *P* values of <0.05 were considered to be statistically significant.

RESULTS

Antigenic comparison of GST fused recombinant proteins GST-N(1-179) and GST-N(180-374): Nucleotide sequence of the Yamaguchi-1 strain N gene (1,125 bp) was determined and the deduced amino acid sequence of N protein (374 amino acids) was phylogenetically analyzed (Fig. 1). Two recombinant N proteins, GST-N(1-179) and GST-N(180-374), based on the Yamaguchi-1 strain were expressed as GST fusion

proteins in *E. coli*, and used as ELISA antigens with 7 sera and 15 plasma samples from ferrets. Although most samples reacted to both recombinant proteins, the plasma of ferret No.10 and serum of ferret No.22 only reacted to GST-N(1-179) and did not recognize GST-N(180-374) (Fig. 2). These results indicated that GST-N(1-179) was suitable for detection of antibodies to FRCoVs. Therefore, we decided to use GST-N(1-179) in the subsequent investigation. In addition, a cut-off value was arbitrarily set at OD = 0.5.

Comparison of the antigenic differences between GST-N(1-179) and GST-N(180-374) by immunoblot analysis: The plasma of No.10 and serum of ferret No.22 showed different reactivities from the other samples in ELISA (Fig. 2). To confirm the different antigenicity, immunoblot analysis was carried out using serum of ferret No.22. Plasma of ferret No.48 was used to compare with serum of ferret No.22. The purified proteins were confirmed to be single bands by CBB staining after SDS-PAGE analysis and used (Fig. 3A). Plasma of ferret No.48 and serum of ferret No.22 reacted with recombinant protein GST-N(1-179), but only plasma of ferret No.48 also reacted with GST-N(180-374) (Fig. 3B and 3C). The results of the immunoblot analysis were consistent with those of the ELISA.

Seroprevalence of FRCoV infection in ferrets in Japan: ELISA using

225 GST-N(1-179) was carried out with 1:100 dilutions of nine sera and 26 plasma samples
226 from domestic ferrets in 12 animal hospitals in five prefectures in Japan. The results
227 showed that 31 of the 35 (89%) ferrets were seropositive for FRCoV infection. There
228 was no significant difference between seropositivity and age or sex (Table 1).
229
230 DISCUSSION
231
232 In this study, we attempted to clarify the seroprevalence of FRCoV in Japan
233 and developed an ELISA using two Yamaguchi-1 strain recombinant N proteins,
234 GST-N(1-179) and GST-N(180-374). More ferret serum samples recognized
235 GST-N(1-179) than GST-N(180-374) (Fig. 2). In addition, identities of N(1-179)
236 between Yamaguchi-1 and the other FRCoVs (96.6-98.3%) were higher than those of
237 N(180-374) (90.7-93.8%) (data not shown). Therefore, we selected GST-N(1-179) as
238 the ELISA antigen for our serosurvey. Surprisingly, we found that 89% (31/35) of
239 domestic ferrets were seropositive to this antigen by ELISA (Table 1). There are reports
240 of FRCoV gene detection in 56%-61% of ferrets in Japan and the Netherlands [6, 9].
241 These data indicate that FRCoV has already spread within the ferret population and that
242 many ferrets may be persistently infected with FRCoV. However, there was no

243 significant difference between seropositivity and symptoms, age or sex. Further studies
244 are required to clarify the pathogenesis of FRCoV in ferrets.

245 Plasma from ferret No.10 and serum from ferret No.22 showed different
246 reactivities from those of other ferret samples in ELISA, reacting only with
247 GST-N(1-179), but not with GST-N(180-374) (Fig. 2). The different reactivity of ferret
248 No. 22 serum was also confirmed by immunoblot analysis using GST-N(180-374) (Fig.
249 3C). These results indicated that GST-N(1-179) is a better choice of antigen for ELISA
250 than GST-N(180-374). ELISA using GST-N(1-179) will be useful for serological
251 surveys for FRCoV. In future studies, this FRCoV infected with ferret No.22 should be
252 analyzed closely.

253 In conclusion, a new ELISA system using the recombinant N protein of
254 FRCoV, GST-N(1-179), was established. This ELISA will be useful for diagnosis and
255 epidemiological studies on FRCoV infection in ferrets.

256

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REFERENCES

1. Andrew, R. 2009. FigTree v1.4 computer program and documentation distributed by the author. Available: 〈http://tree.bio.ed.ac.uk/software/figtree/〉.
2. Garner, M. M., Ramsell, K., Morera, N., Juan-Sallés, C., Jiménez, J., Ardiaca, M., Montesinos, A., Teifke, J. P., Löhr, C. V., Evermann, J. F., Baszler, T. V., Nordhausen, R. W., Wise, A. G., Maes, R. K. and Kiupel, M. 2008. Clinicopathologic features of a systemic coronavirus-associated disease resembling feline infectious peritonitis in the domestic ferret (*Mustela putorius*). *Vet. Pathol.* **45**: 236–246.
3. Graham, E., Lamm, C., Denk, D., Stidworthy, M. F., Carrasco, D. C. and Kubiak, M. 2012. Systemic coronavirus-associated disease resembling feline infectious peritonitis in ferrets in the UK. *Vet. Rec.* **171**: 200–201.
4. Martínez, J., Ramis, A. J., Reinacher, M. and Perpiñán, D. 2006. Detection of feline infectious peritonitis virus–like antigen in ferrets. *Vet. Rec.* **158**: 523.
5. Motokawa, K., Hohdatsu, T., Hashimoto, H. and Koyama, H. 1996. Comparison of the amino acid sequence and phylogenetic analysis of the peplomer, integral membrane and nucleocapsid proteins of feline, canine and porcine coronaviruses. *Microbiol. Immunol.* **40**: 425-433.
6. Provacia, L. B., Smits, S. L., Martina, B. E., Raj, V. S., Doel, P. V., Amerongen, G. V., Moorman-Roest, H., Osterhaus, A. D. and Haagmans, B. L. 2011. Enteric coronavirus in ferrets, the Netherlands. *Emerg. Infect. Dis.* **17**: 1570–1571.
7. Ronquist, F. and Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574
8. Saijo, M., Ogino, T., Taguchi, F., Fukushi, S., Mizutani, T., Notomi, T., Kanda, H., Minekawa, H., Matsuyama, S., Long, H. T., Hanh, N. T., Kurane, I., Tashiro, M. and Morikawa, S. 2005. Recombinant nucleocapsid protein-based IgG enzyme-linked immunosorbent assay for the serological diagnosis of SARS. *J. Virol. Methods* **125**: 181-286.
9. Terada, Y., Minami, S., Noguchi, K., Maumoud, Y. A. H., Shimoda, H., Mochizuki, M., Une, Y. and Maeda, K. 2014. Genetic Characterization of Coronaviruses from Domestic Ferrets, Japan. *Emerg. Infect. Dis.* **20**: 284–287.
10. Whelan, S. and Goldman, N. 2001. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol. Biol. Evol.* **18**: 691–699.
11. Williams, B. H., Kiupel, M., West, K. H., Raymond, J. T., Grant, C. K. and Glickman, L.T. 2000. Coronavirus-associated epizootic catarrhal enteritis in ferrets.

297 *J. Am. Vet. Med. Assoc.* **217**: 526–530.
298 12. Wise, A. G., Kiupel, M. and Maes, R. K. 2006. Molecular characterization of a
299 novel coronavirus associated with epizootic catarrhal enteritis (ECE) in ferrets.
300 *Virology* **349**: 164–174.
301 13. Wise, A. G., Kiupel, M., Garner, M. M., Clark, A. K. and Maes, R. K. 2010.
302 Comparative sequence analysis of the distal one-third of the genomes of a systemic
303 and an enteric ferret coronavirus. *Virus Res.* **149**: 42–50.

304

FIGURE LEGENDS

Fig. 1. Phylogenetic tree based on the N protein amino acid sequences. We referred to the following sequences to construct a phylogenetic tree of N proteins: FRECV strain MSU-2 (GU338457), FRECV strain MSU-1 (DQ340562), FRSCV strain MSU-1 (GU338456), mink CoV strain WD1127 (HM245925), mink CoV strain WD1133 (HM245926), CCoV type II strain fc1 (AB781790), FCoV type II strain M91-267 (AB781788), FCoV type I strain C3663 (AB535528), SARS-CoV strain BJ182-12 (EU371564) and FRCoV strain Yamaguchi-1 (LC029423). Posterior probabilities are indicated above the branches. The sequences analyzed in this study are listed in boldface.

Fig. 2. ELISA using two recombinant proteins, GST-N(1-179) and GST-N(180-374). Seven sera and 15 plasma samples collected from domestic ferrets in Japan were diluted to 1:500. Peroxidase-conjugated anti-ferret immunogloblin at 1:2,000 was used as the secondary antibody. The absorbance was measured using a spectrophotometer at 415 nm. Horizontal and vertical axes indicate the ELISA absorbances using GST-N(1-179) and GST-N(180-374), respectively. White circles (○) indicate ferrets No.10 and No.22 with low reactivities to GST-N(180-374).

323

324 **Fig. 3.** Immunoblot analysis using recombinant proteins. Three purified proteins, GST
325 (lane 1), GST-N(1-179) (lane 2) and GST-N(180-374) (lane 3), were used as antigens.
326 The result of CBB staining after SDS-PAGE analysis was shown in A. Immunoblot
327 analysis was performed using plasma of ferret No.48 (B) and serum of No.22 (C)
328 diluted to 1:1,000. Peroxidase-conjugated anti-ferret immunoglobulin was diluted to
329 1:1,000 and used as a secondary antibody. The reaction was visualized with
330 3,3'-diaminobenzidine tetrahydrochloride.

331

332 Table 1. Detection of antibody to FRCoV from ferrets in Japan

	Age						Sex		Total
	<1y	1y	2y	3y	3y<	Unknown	Male	Female	
Number of examined animals	2	3	7	6	16	1	19	16	35
Number of antibody-positive animals	2	2	6	6	14	1	16	15	31
Percentage of antibody-positive animals	100%	67%	86%	100%	88%	100%	84%	94%	89%

333

Fig. 1

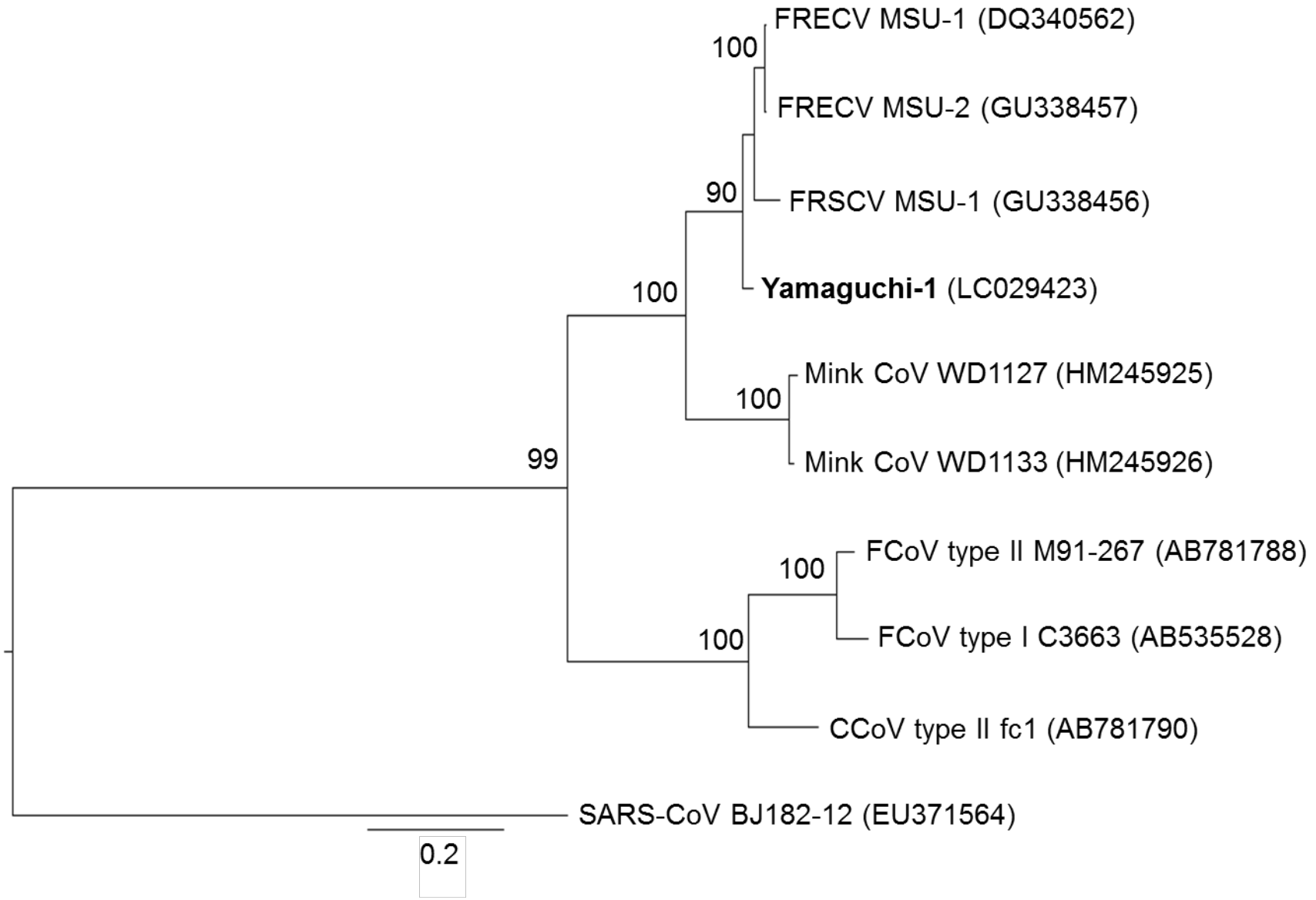

FRECV MSU-1 (DQ340562)
100
FRECV MSU-2 (GU338457)
90
FRSCV MSU-1 (GU338456)
100
Yamaguchi-1 (LC029423)
Mink CoV WD1127 (HM245925)
100
Mink CoV WD1133 (HM245926)
99
FCoV type II M91-267 (AB781788)
100
FCoV type I C3663 (AB535528)
100
CCoV type II fc1 (AB781790)
SARS-CoV BJ182-12 (EU371564)
0.2

Fig. 2

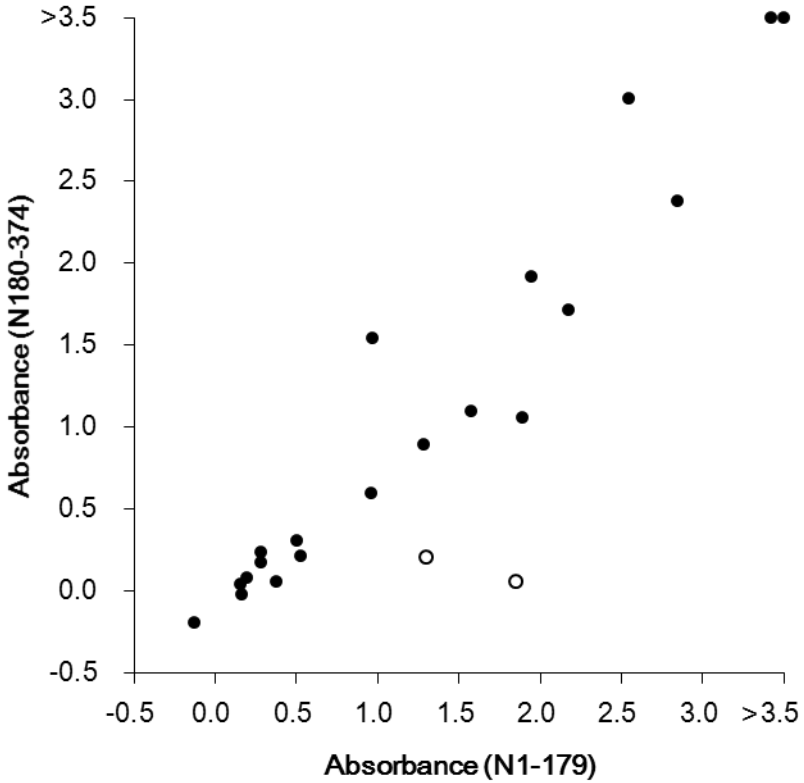
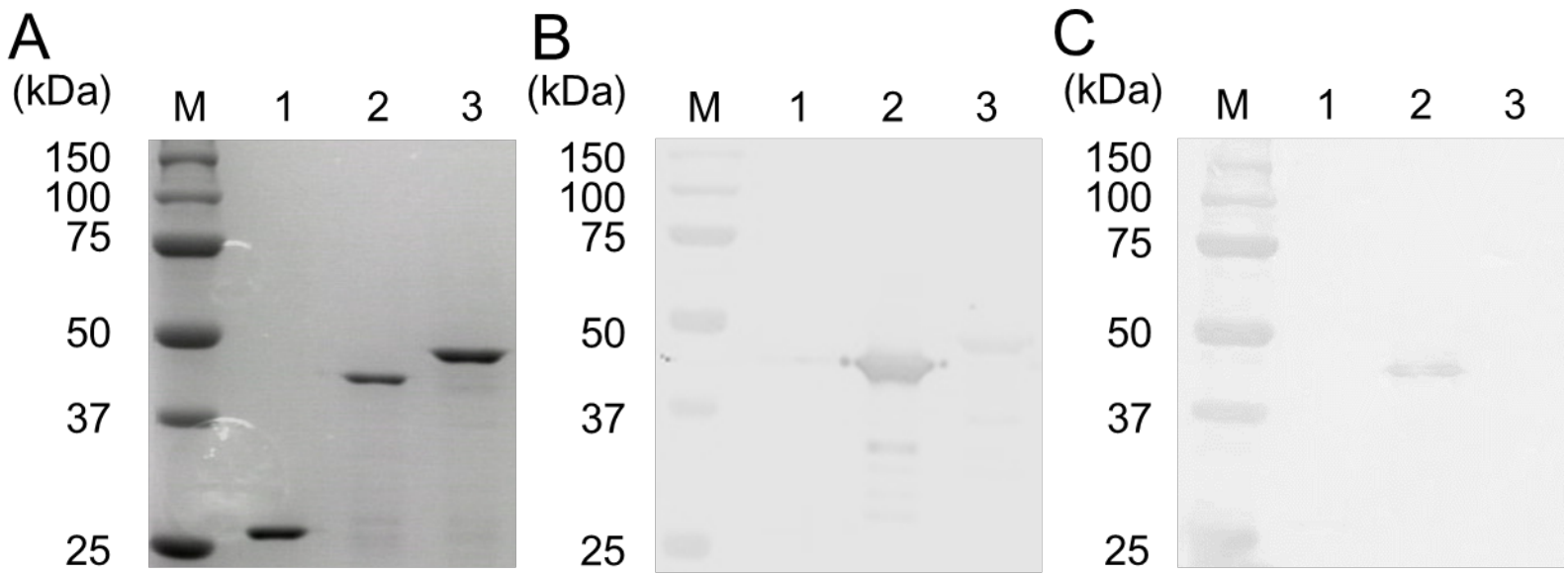

>3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
Absorbance (N180-374)
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
>3.5
Absorbance (N1-179)

Fig. 3


A
(kDa)
M
1
2
3
150
100
75
50
37
25
B
(kDa)
M
1
2
3
150
100
75
50
37
25
C
(kDa)
M
1
2
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25