

Establishment of serological test to detect antibody against ferret coronavirus

Shohei MINAMI¹⁾, Yutaka TERADA¹⁾, Hiroshi SHIMODA¹⁾, Masaki TAKIZAWA²⁾, Mamoru ONUMA³⁾, Akihiko OTA⁴⁾, Yuichi OTA⁴⁾, Yoshihito AKABANE⁵⁾, Kenichi TAMUKAI⁶⁾, Keiichiro WATANABE⁷⁾, Yumiko NAGANUMA⁷⁾, Eiichi KANAGAWA⁸⁾, Kaneichi NAKAMURA⁹⁾, Masanari OHASHI¹⁰⁾, Yoshinori TAKAMI¹¹⁾, Yasutsugu MIWA¹²⁾, Tomoaki TANOUE¹³⁾, Masao OHWAKI¹³⁾, Jouji OHTA¹³⁾, Yumi UNE¹⁴⁾ and Ken MAEDA^{1)*}

¹⁾Laboratory of Veterinary Microbiology, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan

²⁾Takizawa Animal Hospital, 2-95-3 Miyahara-cho, Kita, Saitama 331-0812, Japan

³⁾Oosagami Animal Clinic, 1-33-3 Laketown, Koshigaya, Saitama 343-0828, Japan

⁴⁾Tenpaku Animal Hospital, 1-2011 Hirabariminami, Tenpaku, Nagoya City, Aichi 468-0020, Japan

⁵⁾Jinryo Being Animal Hospital, 2-8-3 Jinryo-cho, Kasugai, Aichi 486-0821, Japan

⁶⁾Denenchofu Animal Hospital, 2-1-3 Denenchofu, Ota, Tokyo 145-0071, Japan

⁷⁾Japan Animal Medical Center, 6-22-3 Honmachi, Shibuya, Tokyo 151-0071, Japan

⁸⁾Maruko Mirai Animal Hospital, 3-22-4 Shimomaruko, Ota, Tokyo 146-0092, Japan

⁹⁾Nakamura Pet Clinic, 4-5-30 Sakurabashi, Naka, Okayama 703-8285, Japan

¹⁰⁾Ohashi Animal Hospital, 1950-2 Goudonishi, Iwasaki, Komaki, Aichi 485-0011, Japan

¹¹⁾Verts Animal Hospital, 2-21-5 Naka, Hakata, Fukuoka 812-0893, Japan

¹²⁾Miwa Exotic Animal Hospital, 1-25-5 Komagome, Toshima, Tokyo 170-0003, Japan

¹³⁾Inuyama Animal General Medical Center, 29 Haguroohmishita, Inuyama, Aichi 484-0894, Japan

¹⁴⁾Laboratory of Veterinary Pathology, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuo, Sagami-hara, Kanagawa 252-5201, Japan

(Received 31 January 2016/Accepted 22 February 2016/Published online in J-STAGE 3 March 2016)

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. 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 been 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)

doi: 10.1292/jvms.16-0059; *J. Vet. Med. Sci.* 78(6): 1013-1017, 2016

Epizootic catarrhal enteritis (ECE), a new enteric disease of domestic ferrets (*Mustelo putorius furo*), was first described in the United States in the early 1990s [11]. A novel alphacoronavirus, ferret coronavirus (FRCoV), was detected as the causative agent of ECE in 2000 and designated as ferret enteric coronavirus (FRECV) [11, 12]. Ferrets with ECE show general clinical signs including lethargy, anorexia and vomiting, and characteristic signs with foul-smelling, green mucous-laden diarrhea [12]. FRCoV was also reported as the causative agent of feline infectious peritonitis (FIP)-like disease in 2006, and the virus was designated as ferret systemic

coronavirus (FRSCV) [2-4]. Ferrets with FIP-like disease show characteristic clinical signs of large palpable intra-abdominal masses like dry type of FIP [2-4]. FRCoVs were divided into two genotypes, I and II, based on differences in the spike (S) gene, and it was suggested that genotype I was associated with FIP-like disease and genotype II was with ECE [13]. However, we previously showed that there was no significant relationship between the genotypes of FRCoV and disease in Japan [9]. In addition, genotype I FRCoV was also detected from many asymptomatic ferrets in the Netherlands [6]. The relationship between genotypes of FRCoV and clinical symptoms remains unclear.

Although FRCoV genes were detected in ferrets by reverse transcription-polymerase chain reaction (RT-PCR), there is no method to detect antibodies to FRCoV. We attempted to isolate FRCoV using feline cell lines and our newly established ferret cell line (manuscript in preparation), but the virus has not yet been isolated. Because the nucleocapsid (N) is conserved between coronaviruses and used as an antigen to detect antibody [5, 8], the N protein of FRCoV was one

*CORRESPONDENCE TO: MAEDA, K., Laboratory of Veterinary Microbiology, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan.

e-mail: kmaeda@yamaguchi-u.ac.jp

©2016 The Japanese Society of Veterinary Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <<http://creativecommons.org/licenses/by-nc-nd/4.0/>>.

of the most likely antigen candidates to detect antibody to FRCoV. In this study, an enzyme-linked immunosorbent assay (ELISA) using recombinant N proteins was established and applied to investigate the seroprevalence of FRCoV infection in Japan.

MATERIALS AND METHODS

Samples from domestic ferrets: From animal hospitals in Japan, 9 serum and 26 plasma samples were collected from domestic ferrets between Aug 1st, 2012 and Feb 4th, 2014 and used for ELISA and immunoblot analysis. We analyzed and reported the results for 79 of the feces samples in our previous study [9]. One fecal sample from a ferret in our animal facility was used to amplify the N gene of the FRCoV Yamaguchi-1 strain.

Amplification of N genes: RNA of the Yamaguchi-1 strain was extracted from feces using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. N genes of the Yamaguchi-1 strain was amplified by RT-PCR using TaKaRa RNA LA PCRTM Kit (AMV) Ver. 1.1 (TaKaRa, Otsu, Japan). RT was performed using random 9-mer oligonucleotide primers, and PCR was performed using primer pairs, NF2 (5'-TTA CAT ATG GTA TAA GAA CTA AAC-3') and NR2 (5'-CGA TGT AGG AAC CTT CAA AAT A-3'). PCR products were electrophoresed on a 0.8% gel and extracted using a QIAEX II Gel Extraction Kit (QIAGEN).

Construction of expression plasmids: Yamaguchi-1 strain fragments were amplified using primer pairs, N1F (5'-TGG GAT CCA TGG CTG GAA ACG GAC CAC-3') and N179R (5'-GAC TCG AGT TAG TTA TTG GAT CTA TTG TTG GAC-3') for nt 1–537 encoding a.a. 1–179, and N180F (5'-TGG GAT CCA TTA ACA GTA ACA GTG GTG ATA T-3') and N374R (5'-GAC TCG AGT TAG TTT AGT TCA TCA ATA ATT TCA-3') for nt 538–1125 encoding a.a. 180–374. These forward and reverse primers contained *Bam*HI and *Xho*I sites at the 5'-end, respectively. Fragments were purified using a MinElute PCR purification Kit (QIAGEN) and digested with restriction enzymes, *Bam*HI and *Xho*I. Two fragments of the Yamaguchi-1 strain were electrophoresed on a 0.8% gel and extracted using a QIAEX II Gel Extraction Kit (QIAGEN). Fragments were then cloned into *Bam*HI and *Xho*I sites of the expression plasmid pGEX-6P-1 vector (GE Healthcare, Piscataway, NJ, U.S.A.) using a DNA Ligation Kit Ver. 2.1 (TaKaRa). Plasmids were transformed into *Escherichia* (*E.*) *coli* strain DH5 α (TOYOBO, Osaka, Japan).

Expression and purification of glutathione-S transferase (GST)-fusion proteins: Two N protein fragments, N1-179 and N180-374, were expressed as fusion proteins with GST, GST-N (1-179) and GST-N (180-374), respectively. *E. coli* containing recombinant or control plasmid was cultured in 2 \times yeast extract and tryptone (YT) medium (1.6% tryptone, 1% yeast extract and 0.5% NaCl, pH 7.0) containing 50 μ g ampicillin m ⁻¹. Expression of recombinant proteins was induced by the addition of 1 mM isopropyl β -D-1-thiogalactopyranoside (Wako, Osaka, Japan) for 4 hr. The bacterial cells were suspended in sonication buffer (50 mM

Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol) and lysed using a Multi-beads shocker (YASUI KIKAI, Osaka, Japan). After centrifugation, supernatants were mixed with Triton X-100 at a final concentration of 1% for 30 min and then centrifuged at 20,630 \times g at 4°C for 30 min. The supernatants were collected, mixed with glutathione sepharose 4B beads (GE Healthcare) and incubated at 4°C for 30 min. After centrifugation, beads were washed four times with phosphate-buffered saline (PBS) containing 0.5% Triton X-100 and once with sonication buffer. The beads were mixed with 300 μ l of 10 mM glutathione and incubated at 4°C for 1 hr. After incubation, supernatants were harvested as purified recombinant proteins and used for ELISA and immunoblot analysis. The purified proteins were confirmed to be single bands by coomassie-brilliant blue (CBB) staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

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

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

ELISA: The concentration of purified recombinant proteins was adjusted to 5 μ g m ⁻¹ with adsorption buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6). GST was used as a control at 5 μ g m ⁻¹. One hundred microliters of purified recombinant proteins and GST were added to 96-well microplates (Maxisorp; Nunc, Roskilde, Denmark). After incubation at 37°C for 2 hr, plates were placed at 4°C overnight. The wells were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and then incubated with 200 μ l of 1% Block Ace (Dainippon Pharmaceutical, Osaka, Japan) in PBS at 37°C for 30 min. After washing three times with PBS-T, 100 μ l of diluted sera or plasma were added to duplicate wells and incubated at 37°C for 30 min. Sera or plasma was diluted to 1:100 or 1:500 with PBS-T containing 0.4% Block Ace. Subsequently, wells were washed three times with PBS-T before 100 μ l of peroxidase-conjugated anti-ferret immunoglobulin (ROCKLAND, Limerick, PA, U.S.A.) diluted with PBS-T containing 0.4% Block Ace was added and incubated at 37°C for 30 min. Following three washes with PBS-T, 100 μ l of Horseradish Peroxidase Substrate (BIO-RAD) was added to each well. After incubation at room temperature for 30 min, the enzymatic reaction was stopped by adding 100 μ l of 2% oxalic acid to each well. The absorbance was measured using a spectrophotometer (BIO-RAD) at 415 nm. All results were subtracted from the value for GST, and the cut-off value was arbitrarily set at 0.5.

Immunoblot analysis: Recombinant proteins mixed with

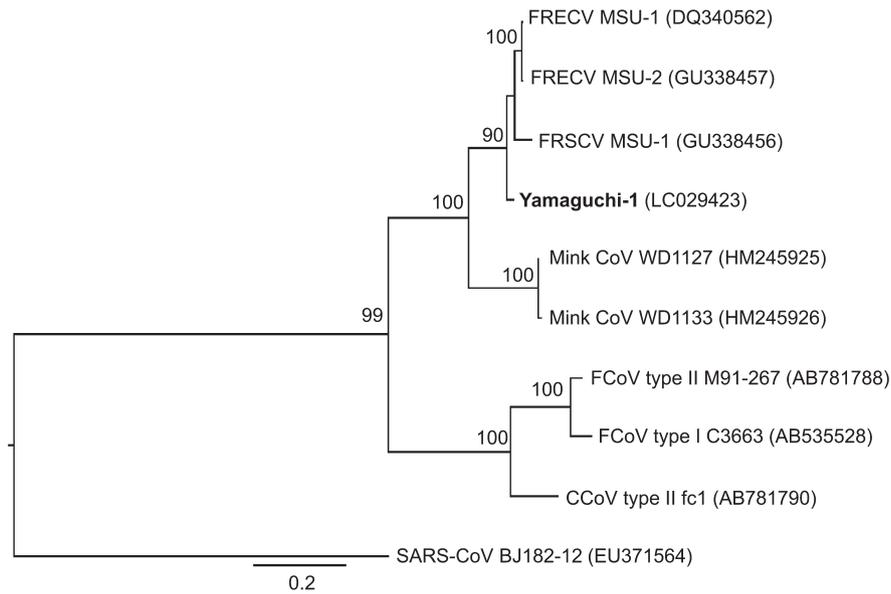


Fig. 1. Phylogenetic tree based on the N protein amino acid sequences. We referred to the following sequences to construct a phylogenetic tree of N proteins: FRECV strain MSU-2 (GU338457), FRECV strain MSU-1 (DQ340562), FRSCV strain MSU-1 (GU338456), mink CoV strain WD1127 (HM245925), mink CoV strain 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.

2 × sample buffer were electrophoretically separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, U.S.A.). After transferring, the membranes were incubated with Tris-buffered saline (TBS) (20 mM Tris-HCl and 150 mM NaCl, pH 7.5) containing 3% gelatin (BIO-RAD) at 37°C for 45 min. After washing three times with TBS containing 0.05% Tween 20 (T-TBS), membranes were incubated with 2 ml of ferret serum or plasma diluted to 1:1,000 in T-TBS containing 1% gelatin (BIO-RAD) at 37°C for 45 min. After three washes with T-TBS, membranes were incubated with 2 ml of peroxidase-conjugated anti-ferret immunoglobulins with T-TBS containing 1% gelatin at 37°C for 45 min. The membranes were washed three times with T-TBS and then three times with TBS. The reaction was visualized using 3,3'-diaminobenzidine tetrahydrochloride (Wako).

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

Phylogenetic analysis: A phylogenetic tree was constructed using the program 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 de-

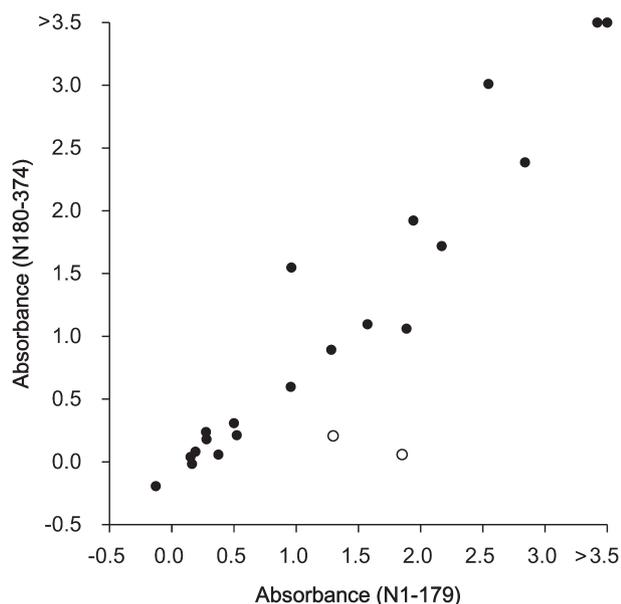


Fig. 2. ELISA using two recombinant proteins, GST-N (1-179) and GST-N (180-374). Seven sera and 15 plasma samples collected from domestic ferrets in Japan were diluted to 1:500. Peroxidase-conjugated anti-ferret immunoglobulin at 1:2,000 was used as the secondary antibody. The absorbance was measured 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 (\circ) indicate ferrets No.10 and No.22 with low reactivities to GST-N (180-374).

ecided 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 differ-

ent 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 GST-N (1-179) was carried out with 1:100 dilutions of nine sera and 26 plasma samples from domestic ferrets in 12 animal hospitals in five prefectures in Japan. The results showed that 31 of the 35 (89%) ferrets were seropositive for FRCoV infection. There was no significant difference between seropositivity and age or sex (Table 1).

DISCUSSION

In this study, we attempted to clarify the seroprevalence of FRCoV in Japan and developed an ELISA using two Yamaguchi-1 strain recombinant N proteins, GST-N (1-179) and GST-N (180-374). More ferret serum samples recognized GST-N (1-179) than GST-N (180-374) (Fig. 2). In addition, identities of N (1-179) between Yamaguchi-1 and the other FRCoVs (96.6–98.3%) were higher than those of N (180-374) (90.7–93.8%) (data not shown). Therefore, we selected GST-N (1-179) as the ELISA antigen for our serosurvey. Surprisingly, we found that 89% (31/35) of domestic ferrets were seropositive to this antigen by ELISA (Table 1). There are reports of FRCoV gene detection in 56%–61% of ferrets in Japan and the Netherlands [6, 9]. These data indicate that FRCoV has already spread within the ferret population and that many ferrets may be persistently infected with FRCoV.

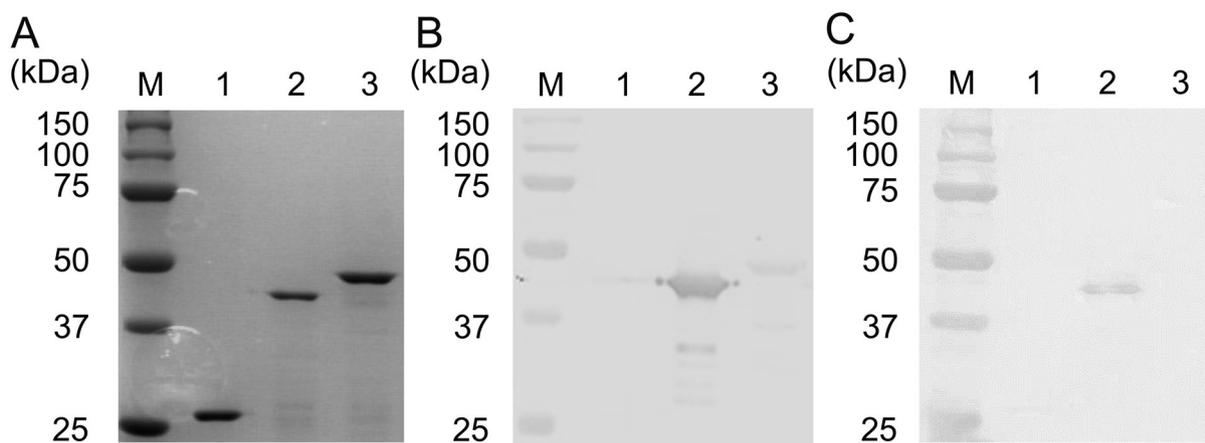


Fig. 3. Immunoblot analysis using recombinant proteins. Three purified proteins, GST (lane 1), GST-N (1-179) (lane 2) and GST-N (180-374) (lane 3), were used as antigens. The result of CBB staining after SDS-PAGE analysis is shown in A. Immunoblot analysis was performed using plasma of ferret No.48 (B)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

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%

However, there was no significant difference between seropositivity and symptoms, age or sex. Further studies are required to clarify the pathogenesis of FRCoV in ferrets.

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

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

ACKNOWLEDGMENTS. This experiment was supported by grants from the Ministry of Health, Labour and Welfare of Japan (H24-Shinko-Ippan-006; H25-Shinko-Ippan-008) and by JSPS KAKENHI Grant Number 15H04599.

REFERENCES

- Andrew, R. 2009. FigTree v1.4 computer program and documentation distributed by the author. Available: (<http://tree.bio.ed.ac.uk/software/figtree/>).
- 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. [[Medline](#)] [[CrossRef](#)]
- 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. [[Medline](#)] [[CrossRef](#)]
- 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. [[Medline](#)] [[CrossRef](#)]
- 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. [[Medline](#)] [[CrossRef](#)]
- 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. [[Medline](#)]
- Ronquist, F. and Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574. [[Medline](#)] [[CrossRef](#)]
- 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**: 181–186. [[Medline](#)] [[CrossRef](#)]
- Terada, Y., Minami, S., Noguchi, K., Mahmoud, H. Y., Shimoda, H., Mochizuki, M., Une, Y. and Maeda, K. 2014. Genetic characterization of coronaviruses from domestic ferrets, Japan. *Emerg. Infect. Dis.* **20**: 284–287. [[Medline](#)] [[CrossRef](#)]
- 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. [[Medline](#)] [[CrossRef](#)]
- 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. *J. Am. Vet. Med. Assoc.* **217**: 526–530. [[Medline](#)] [[CrossRef](#)]
- Wise, A. G., Kiupel, M. and Maes, R. K. 2006. Molecular characterization of a novel coronavirus associated with epizootic catarrhal enteritis (ECE) in ferrets. *Virology* **349**: 164–174. [[Medline](#)] [[CrossRef](#)]
- Wise, A. G., Kiupel, M., Garner, M. M., Clark, A. K. and Maes, R. K. 2010. Comparative sequence analysis of the distal one-third of the genomes of a systemic and an enteric ferret coronavirus. *Virus Res.* **149**: 42–50. [[Medline](#)] [[CrossRef](#)]