Advance Publication

The Journal of Veterinary Medical Science

Accepted Date: 11 Apr 2019
J-STAGE Advance Published Date: 23 Apr 2019

Full paper

Virology

Pathogenesis of oral type I feline infectious peritonitis virus (FIPV) infection: Antibody-dependent enhancement infection of cats with type I FIPV via the oral route

Tomomi Takano[1*], Shinji Yamada[1,2], Tomoyoshi Doki[1], and Tsutomu Hohdatsu[1]

[1] *School of Veterinary Medicine, Kitasato University, Towada, Aomori, 034-8628, Japan.*

[2] *Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Miyagi, 980-8575, Japan.*

* Address for correspondence: Tomomi Takano, *Laboratory of Veterinary Infectious Disease, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan*; *email*: takanot@vmas.kitasato-u.ac.jp Tel: +81-176-23-4371, Fax: +81-176-23-8703

Running head: ADE of cat with oral FIPV infection

ABSTRACT

Feline infectious peritonitis virus (FIPV) causes a severe, immune-mediated disease called FIP in domestic and wild cats. It is unclear whether FIP transmits from cat to cat through the oral route of FIPV infection, and the reason for this includes that FIP is caused by oral inoculation with some FIPV strains (*e.g.*, type II FIPV WSU 79-1146), but is not caused by other FIPV (*e.g.*, type I FIPV KU-2 strain: FIPV-I KU-2). In this study, when cats passively immunized with anti-FIPV-I KU-2 antibodies were orally inoculated with FIPV-I KU-2, FIP was caused at a 50% probability, i.e., FIPV not causing FIP through oral infection caused FIP by inducing antibody-dependent enhancement. Many strains of type I FIPV do not cause FIP by inoculation through the oral route in cats. Based on the findings of this study, type I FIPV which orally infected cats may cause FIP depending on the condition.

Keywords: antibody-dependent enhancement, feline coronavirus, feline infectious peritonitis, oral infection

INTRODUCTION

Feline coronavirus (FCoV) belongs to the genus *Alphacoronavirus*, subfamily *Coronavirinae*, family *Coronaviridae* [7]. FCoV is classified into two serotypes (I and II), based on differences in the amino acid (aa) sequence of Spike (S) protein and antibody neutralization. Furthermore, FCoV exists as two different biotypes: Feline enteric coronavirus (FECV: avirulent FCoV) and Feline infectious peritonitis virus (FIPV: virulent FCoV) [21]. Antibodies against virus enhance viral load and disease severity in some viral infections including FIP. This phenomenon is known as antibody-dependent enhancement (ADE) of viral infection [14]. ADE of FIPV infection can be induced by the presence of sub-neutralizing levels of anti-FIPV S antibodies [6]. Unlike dengue virus infection, ADE was induced by re-infection with the identical serotype virus in FIPV infection [17].

FECV is spread predominantly through the oral route in cats [9]. On the contrary, it is unclear FIPV transmits from cat to cat through the oral route of FIPV infection. It is suggested that the incidence of FIP in cats is dependent on the route of FIPV infection. The incidences of FIP in cats intraperitoneally and oronasally inoculated with the type I FIPV UCD4 strain were 37.5 and 0%, respectively [11]. In a similar fashion, a previous study described that oral inoculation with type I FIPV KU-2 strain (FIPV-I KU-2) cannot lead to FIP in cats, but subcutaneous and intraperitoneal inoculation with FIPV-I KU-2 can lead to FIP [13, 17]. In this study, we investigated whether oral inoculation with FIPV-I KU-2 causes FIP in cats passively immunized with anti-FIPV-I KU-2 antibodies. In addition, we investigated whether ADE of type I FIPV infection can be promoted in vitro using feline macrophages.

MATERIALS AND METHODS

Experimental animals

Anti-FCoV antibody-negative seven specific pathogen free (SPF) cats aged 5-6 months were used. The cats were maintained in a temperature-controlled isolated facility. All experiments were

approved by the President of Kitasato University through the judgment of the Institutional Animal Care and Use Committee of Kitasato University (2004, and 18-050), and performed in accordance with the Guidelines for Animal Experiments of Kitasato University. Sample sizes were determined based on the previous study, and the minimum number of cats was used.

Passive immunization with anti-FIPV antibodies and virus inoculation in cats

Passive immunization with anti-FIPV-I KU-2 antibodies was performed as described before [17]. Briefly, three cats (Ab1, Ab2, and Ab3) were subcutaneously administrated with serum from FIPV-I KU-2-infected healthy cats, and one cat (Ab4) was administrated with IgG purified from FIPV-I KU-2-infected cats-derived ascites by ammonium sulfate precipitation followed affinity purification on a Protein A column. The neutralizing antibody titer against FIPV-I KU-2 was 1:320 in both the serum- and purified IgG. The cats were orally inoculated with FIPV-I KU-2 ($10^{4.3}$ $TCID_{50}$/head) 3 days after passive immunization. As a control, three cats (C1, C2, and C3) without passive immunization were inoculated with the virus (Fig.1A). Cats were euthanized when reaching the humane endpoint or 90 days after inoculation with FIPV.

Neutralization test

The test sera were serially 2-fold diluted in medium and mixed with an equal volume of a virus suspension containing approximately 200 $TCID_{50}$/100 μl and the mixtures were incubated at 37 °C for 60 min. Each mixture was then inoculated into the *Felis catus* whole fetus-4 cells (kindly supplied by Dr. M. C. Horzinek of Utrecht University) in 96-well flat-bottomed plates, and incubation was made in an atmosphere of 5 % CO_2 in air at 37 °C for 3 days. For each serum dilution, tests were duplicated. The neutralizing antibody titer (NT) was expressed as a reciprocal of the highest dilution of the test sera that inhibiting cytopathic effect completely.

ELISA

The ELISA for anti-FCoV antibodies was performed as described by Takano et al.[17]. Briefly, detergent-disrupted, purified FIPV virions were diluted appropriately with carbonate buffer (0.05 M, pH 9.6). A total of 100 *μl* of the dilution was pipetted into each well of a 96-well flat-bottomed plate. The plates were allowed to stand overnight at 4 ℃, washed with PBS containing 0.02 % Tween-20, and 100 *μl* of the test serum sample was then added to each well. Horseradish peroxidase-conjugated goat anti-cat IgG (ICN Pharmaceuticals, Inc., USA) was diluted to optimal concentration with PBS containing 10 % FCS and 0.05 % Tween-20, and 100 μ*l* of dilution was added to each well of plates. After incubation at 37 ℃ for 30 min, 100*μl* of the substrate solution was added to each well and plates were incubated at 25 ℃ for 20 min in a dark room. The substrate solution was prepared by dissolving *o*-phenylenediamine dihydrochloride at a concentration of 0.4 mg/m*l* in 0.1 M citric acid and 0.2 M Na_2HPO_4 buffer (pH 4.8), and 0.2 *μl*/m*l* of 30 % H_2O_2 was then added. The reaction was stopped with 3 N H_2SO_4 solution and the optical density (O.D.) at 492 nm was determined.

RNA Isolation and RT-PCR

RNA was isolated from rectal swab samples by a method reported previously [8]. To synthesize cDNA from FIPV genomic RNA, 1 μ*l* RNA extract and 0.02 mol sense primer for the FIPV nucleocapsid (N) gene (positions 876–895, 5'-CAACTGGGGAGATGAACCTT-3') were added to Ready-to-Go RT-PCR beads (GE Healthcare Life Sciences) and the volume was adjusted to 50 μ*l* with water. The resulting solution was incubated at 42 ℃ for 1 hr to synthesize cDNA. cDNA was amplified by PCR using primers specific for the FCoV N gene (sense primer, positions 876–895, 5'-CAACTGGGGAGATGAACCTT-3'; antisense primer, positions 1644–1663, 5'-GGTAGCATTTGGCAGCGTTA-3'). PCR was performed as reported previously [8].

Inoculation of macrophages with virus

For the macrophages, feline alveolar macrophages, which are used for analysis of ADE of type II FIPV infection [18], were selected. Feline alveolar macrophages were obtained from SPF cats by broncho-alveolar lavage with Hank's balanced salt solution (HBSS) as described previously by Hohdatsu et al. [6]. Feline alveolar macrophages were maintained in RPMI 1640 growth medium supplemented with 10 % FCS, 100 U of penicillin per m*l*, 100 μg of streptomycin per m*l*, and 50 μM 2-mercaptoethanol. Viral suspension (FIPV-I KU-2, $10^{4.3}$ $TCID_{50}$/ m*l*) and IgG purified from FIPV-I KU-2-infected cats-derived ascites (sub-neutralizing titer: final concentration of 1:300) were mixed in an equal volume ratio and allowed to react at 4 °C for 1 hr, and 0.2 m*l* of this reaction solution was used to inoculate feline alveolar macrophages (10^5 cells) cultured in each well of 8-well Lab-Tek Chamber Slide (Thermo Fisher Scientific, Waltham, MA, USA). As controls, IgG alone and virus suspension alone were added to feline alveolar macrophages. After virus adsorption at 37 °C for 1 hr, the cells were washed with HBSS and then added 1m*l* of growth medium. After 48 hr, N protein levels were determined by immunofluorescence assay (IFA), as described previously [15]. For recognizing FIPV-I KU-2 N protein, mAb YN2 (mouse IgG2b) prepared by our laboratory [16] was used. FIPV-infected cells were analyzed using a Leica DM48 microscope and LAS X integrated imaging system (Leica Microsystems, Wetzlar, Germany).

RESULTS

Two cats (Ab2 and Ab4) were euthanized at 15 and 18 day of post FIPV infection (dpi) with FIPV-I KU-2, respectively, when they reached the humane endpoint. These animals showed febrile (>39.5°C), lethargy, anorexia, and jaundice. Upon necropsy, ascites was noted in two cats with FIP and pyogranulomatous lesions were present in the intestine and spleen. Pleural effusion and inflammatory lesions in the lung were noted in Ab2. In passively immunized cats without clinical symptoms after FIPV infection, several 1-2-mm nodules were observed in the intestine, but there was no other lesion. Cats inoculated orally with FIPV-I KU-2 without passive immunization did not develop clinical

symptoms. No FIP-related lesion was noted on necropsy at 90 dpi with FIPV-I KU-2. Fig. 1B shows the survival rate of cats infected with FIPV-I KU-2. The survival rate of cats with passive immunization was lower than that of the cats without passive immunization, and the average survival time after infection with FIPV-I KU-2 was also shorter. We tested for the presence of neutralizing antibodies against FIPV-I KU-2 in cats with passive immunization. In cats with passive immunization, NT was increased to 4-32 fold on the day of virus inoculation (day 3 post passive immunization) (Fig. 2A). NT was maintained at a constant level in cats excluding 2 cats (Ab2 and Ab4) which developed FIP after viral challenge. In cats without passive immunization, NT started to increase on 12 dpi with FIPV-I KU-2, and reached 64-128 fold on 36 dpi (Fig. 2B). Time-course changes in the serum anti-FIPV antibody level were investigated by ELISA using purified soluble FIPV antigen. In cats passive immunization, the ELISA OD value was increased to 0.2–0.8 on the day of virus inoculation (day 3 post passive immunization). As with the NT titer, the ELISA OD value was continuously increased in 2 cats excluding 2 cats developed FIP (Ab2 and Ab4; Fig. 2C). In cats without passive immunization, the ELISA OD value continuously increased 12-18 dpi after inoculation with FIPV-I KU-2, and reached 1.2-1.7 on 24-30 dpi (Fig. 2D). Rectal swab samples from cats were subjected to RT-PCR targeting FCoV N gene. In cats with passive immunization, FCoV N gene was detected day 15 after virus inoculation in Ab3. However, FCoV N gene was not detected in any samples of the other cats (Table 1).

Based on the findings described above, FIPV-I KU-2 was suggested to promote ADE through not only subcutaneous but also oral infection. However, the mechanism of ADE induced by FIPV-I KU-2 infection is unclear. To elucidate this mechanism, it is necessary to promote FIPV-I KU-2-induced ADE in vitro. Therefore, the ADE infection with FIPV-I KU-2 was performed in feline macrophage. No FCoV N protein was detected in macrophages treated with only IgG purified from ascites of FIPV-I KU-2-infected cats (Fig. 3A). In macrophages treated with only FIPV-I KU-2, FCoV N protein was detected in 3.9 ± 2.5% (Mean ± S.D.) of cells (Fig. 3B). In macrophages treated with both purified IgG

and FIPV-I KU-2, FCoV N protein was detected in 35.9 ± 8.1% (Mean ± S.D.) of cells (Fig. 3C), showing that the FIPV-I KU-2 infection rate in macrophages increased in the presence of the antibody.

DISCUSSION

Cats orally infected with FIPV-I KU-2 do not develop FIP. On the basis of this fact, the biotype (phenotype) of the orally inoculated FIPV-I KU-2 is classified as "FECV". However, FIPV-I KU-2 has the genetic characteristics of "FIPV", i.e., the sequence of the S1/S2 site of FIPV-I KU-2 is RSRSS (P1 R→S) [10], and aa at position 1058 has been changed from methionine to leucine [2]. In addition, 178 aa is deleted from 3c protein in FIPV-I KU-2 [3, 22]. Furthermore, no amino acid deletion was noted in 7b protein of FIPV-I KU-2 [7, 19]. It is unclear which of these regions is involved in the pathogenicity of FIPV-I KU-2. It is now possible to prepare recombinant type I FCoV by reverse genetics [4, 20]. It is desired to mutate the regions associated with pathogenicity in FIPV-I KU-2, inoculate cats with these mutants through various routes, and confirm whether the mutant causes FIP.

FCoV N genes were hardly detectable from rectal swab samples of cats infected with FIPV-I KU-2. The reason for this is unclear. We previously confirmed that FCoV N genes were detected from rectal swab samples after FIPV-I KU-2 subcutaneous infection in cats with or without passive immunization of anti-FIPV-I KU-2 antibodies (Takano et al., Unpublished data). On the basis of these facts, we suggested that inoculation routes result in differential patterns of virus shedding in cats infected with FIPV.

Generally, when a cat developed FIP in multi-cat environments, cats living together also develop FIP at a high probability [1]. FIPV excreted from cats with FIP may infect other cats through the oral route. However, orally inoculated type I FIPV mostly does not cause FIP in cats [11]. It has been difficult to explain these contradictory facts. It was clarified that even FIPV not causing FIP through oral infection may cause FIP in anti-type I FCoV-seropositive cats. However, not all anti-FIPV seropositive cats develop FIP. For example, ADE is not promoted and the virus is neutralized in cats

with a high anti- FIPV neutralizing antibody level [6]. FIP also does not develop when cellular immunity is strongly induced after FIPV infection [12]. Based on these findings, to elucidate the clinical state of FIP, it is necessary to analyze the status of immunity in cats after viral infection. However, many recent studies on FIP do not focus on the host but focus on the virus. We suggest that FIP is a "multi-causal disease" involving various risk factors (virulence of FCoV, the status of immunity in host, and the route of virus infection etc.…).

We confirmed that FIP was caused in 50% when cats passively immunized with anti-FIPV-I KU-2 antibodies were inoculated orally with FIPV-I KU-2, i.e., FIPV not causing FIP through oral infection caused FIP by inducing ADE. Moreover, we were able to demonstrate that infection of FIPV-I KU-2 to feline macrophages was enhanced by anti-FIPV-I KU-2 IgG. This study may provide a platform for understanding the mechanism of ADE induced by oral viral infection.

ACKNOWLEDGEMENT

This work was in part supported by MEXT/JSPS KAKENHI Grant Number JP16K08027.

REFERENCES

1. Addie, D., Belák, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., Gruffydd-Jones, T., Hartmann, K., Hosie, M. J., Lloret, A., Lutz, H., Marsilio, F., Pennisi, M. G., Radford, A.D., Thiry, E., Truyen, U. and Horzinek, M. C. 2009. Feline infectious peritonitis: ABCD guidelines on prevention and management. *J. Feline Med. Surg.* **11:** 594-604.
2. Chang, H. W., Egberink, H. F., Halpin, R., Spiro, D. J. and Rottier, P. J. 2012. Spike protein fusion peptide and feline coronavirus virulence. *Emerg. Infect. Dis.* **18:** 1089.
3. Chang, H. W., de Groot, R. J., Egberink, H. F. and Rottier, P. J. 2010. Feline infectious peritonitis: insights into feline coronavirus pathobiogenesis and epidemiology based on genetic analysis of the viral 3c gene. *J. Gen. Virol.* **91:** 415-420.

4. Ehmann, R., Kristen-Burmann, C., Bank-Wolf, B., König, M., Herden, C., Hain, T., Thiel, H. J., Ziebuhr, J. and Tekes, G. 2018. Reverse Genetics for Type I Feline Coronavirus Field Isolate To Study the Molecular Pathogenesis of Feline Infectious Peritonitis. *mBio* **9:** e01422-18.
5. Herrewegh, A.A., Vennema, H., Horzinek, M.C., Rottier, P.J., de Groot, R.J., 1995. The molecular genetics of feline coronaviruses: comparative sequence analysis of the ORF7a/7b transcription unit of different biotypes. *Virology* **212:** 622-631.
6. Hohdatsu, T., Nakamura, M., Ishizuka, Y., Yamada, H. and Koyama, H. 1991. A study on the mechanism of antibody-dependent enhancement of feline infectious peritonitis virus infection in feline macrophages by monoclonal antibodies. *Arch. Virol.* **120:** 207-217.
7. Jaimes, J. A. and Whittaker, G. R. 2018. Feline coronavirus: Insights into viral pathogenesis based on the spike protein structure and function. *Virology* **517:** 108-121.
8. Kaneshima, T., Hohdatsu, T., Satoh, K., Takano, T., Motokawa, K., and Koyama, H. 2006. The prevalence of a group 2 coronavirus in dogs in Japan. *J. Vet. Med. Sci.* **68:** 21-25.
9. Kipar, A., Meli, M. L., Baptiste, K. E., Bowker, L. J. and Lutz, H. 2010. Sites of feline coronavirus persistence in healthy cats. *J. Gen. Virol.* **91:** 1698-1707.
10. Licitra, B. N., Millet, J. K., Regan, A. D., Hamilton, B. S., Rinaldi, V. D., Duhamel, G. E. and Whittaker, G. R. 2013. Mutation in spike protein cleavage site and pathogenesis of feline coronavirus. *Emerg. Infect. Dis.* **19:** 1066.
11. Pedersen, N.C. 1987. Virologic and immunologic aspects of feline infectious peritonitis virus infection. *Adv. Exp. Med. Biol.* **218:** 529-550.
12. Pedersen, N.C., 2014. An update on feline infectious peritonitis: virology and immunopathogenesis. *Vet. J.* **201:** 123-132.
13. Satoh, R., Furukawa, T., Kotake, M., Takano, T., Motokawa, K., Gemma, T., Watanabe, R., Arai. S. and Hohdatsu, T. 2011. Screening and identification of T helper 1 and linear immunodominant antibody-binding epitopes in the spike 2 domain and the nucleocapsid protein of feline infectious

peritonitis virus. *Vaccine* **29:** 1791-1800.

14. Takada, A. and Kawaoka, Y. 2003. Antibody-dependent enhancement of viral infection: molecular mechanisms and in vivo implications. *Rev. Med. Virol.* **13:** 387-398.
15. Takano, T., Endoh, M., Fukatsu, H., Sakurada, H., Doki, T. and Hohdatsu, T., 2017. The cholesterol transport inhibitor U18666A inhibits type I feline coronavirus infection. *Antiviral Res.* **145:** 96-102.
16. Takano, T. and Hohdatsu, T. 2015. Serological Diagnosis of Feline Coronavirus Infection by Immunochromatographic Test. *Methods Mol. Biol.* **1282:** 33-39.
17. Takano, T., Kawakami, C., Yamada, S., Satoh, R. and Hohdatsu, T. 2008. Antibody-dependent enhancement occurs upon re-infection with the identical serotype virus in feline infectious peritonitis virus infection. *J. Vet. Med. Sci.* **70:** 1315-1321.
18. Takano, T., Hohdatsu, T., Toda, A., Tanabe, M. and Koyama, H. 2007. TNF-alpha, produced by feline infectious peritonitis virus (FIPV)-infected macrophages, upregulates expression of type II FIPV receptor feline aminopeptidase N in feline macrophages. *Virology* **364:** 64-72.
19. Takano, T., Tomiyama, Y., Katoh, Y., Nakamura, M., Satoh, R. and Hohdatsu, T. 2011. Mutation of neutralizing/antibody-dependent enhancing epitope on spike protein and 7b gene of feline infectious peritonitis virus: influences of viral replication in monocytes/macrophages and virulence in cats. *Virus Res.* **156:** 72-80.
20. Tekes, G., Hofmann-Lehmann, R., Stallkamp, I., Thiel, V. and Thiel, H. J. 2008. Genome organization and reverse genetic analysis of a type I feline coronavirus. *J. Virol.* **82:** 1851-1859.
21. Tekes, G. and Thiel, H. J. 2016. Feline coronaviruses: pathogenesis of feline infectious peritonitis. *Adv. Virus Res.* **96:** 193-218.
22. Vennema, H., Poland, A., Foley, J. and Pedersen, N. C. 1998. Feline infectious peritonitis viruses arise by mutation from endemic feline enteric coronaviruses. *Virology* **243:** 150-157.

FIGURE LEGENDS

Fig.1 Passive immunization and virus inoculation in cats. (A) Schematic of the passive immunization and virus inoculation schedule. (B) Survival rates of cats inoculated with FIPV-I KU-2.

Fig.2 Anti-FIPV antibody levels after passive immunization and virus inoculation in cats. (A) NT titer in cats inoculated with FIPV-I KU-2 after passive immunization (n=4). (B) NT titer in cats inoculated with FIPV-I KU-2 without passive immunization (n=3). (C) ELISA OD value in cats inoculated with FIPV-I KU-2 after passive immunization (n=4). (D) ELISA OD value in cats inoculated with FIPV-I KU-2 without passive immunization (n=3).

Fig.3 Antibody-dependent enhancement of FIPV-I KU-2 infection in feline macrophages. (A) Macrophages added with a 1:300 dilution of purified IgG from FIPV-I KU-2-infected cats-derived ascites only. (B) Macrophages infected with FIPV-I KU-2 only. (C) Macrophages infected with FIPV-I KU-2 in the presence of purified IgG from FIPV-I KU-2-infected cats-derived ascites. Data represent three independent experiments.

(A)
Cats
With Passive Immunization
Serum or IgG
Subcutaneous injection
FIPV-I KU-2
Oral inoculation
-3
0
Days post inoculation
Cats
Without Passive Immunization
PBS
Subcutaneous injection
FIPV-I KU-2
Oral inoculation
-3
0
Days post inoculation
(B)
Survival (%)
100
80
60
40
20
0
Cats
Without Passive
Immunization
Survival rate: 100%
Cats
With Passive
Immunization
Survival rate: 50%
0
15
30
45
60
75
90
Days post inoculation

Fig.1

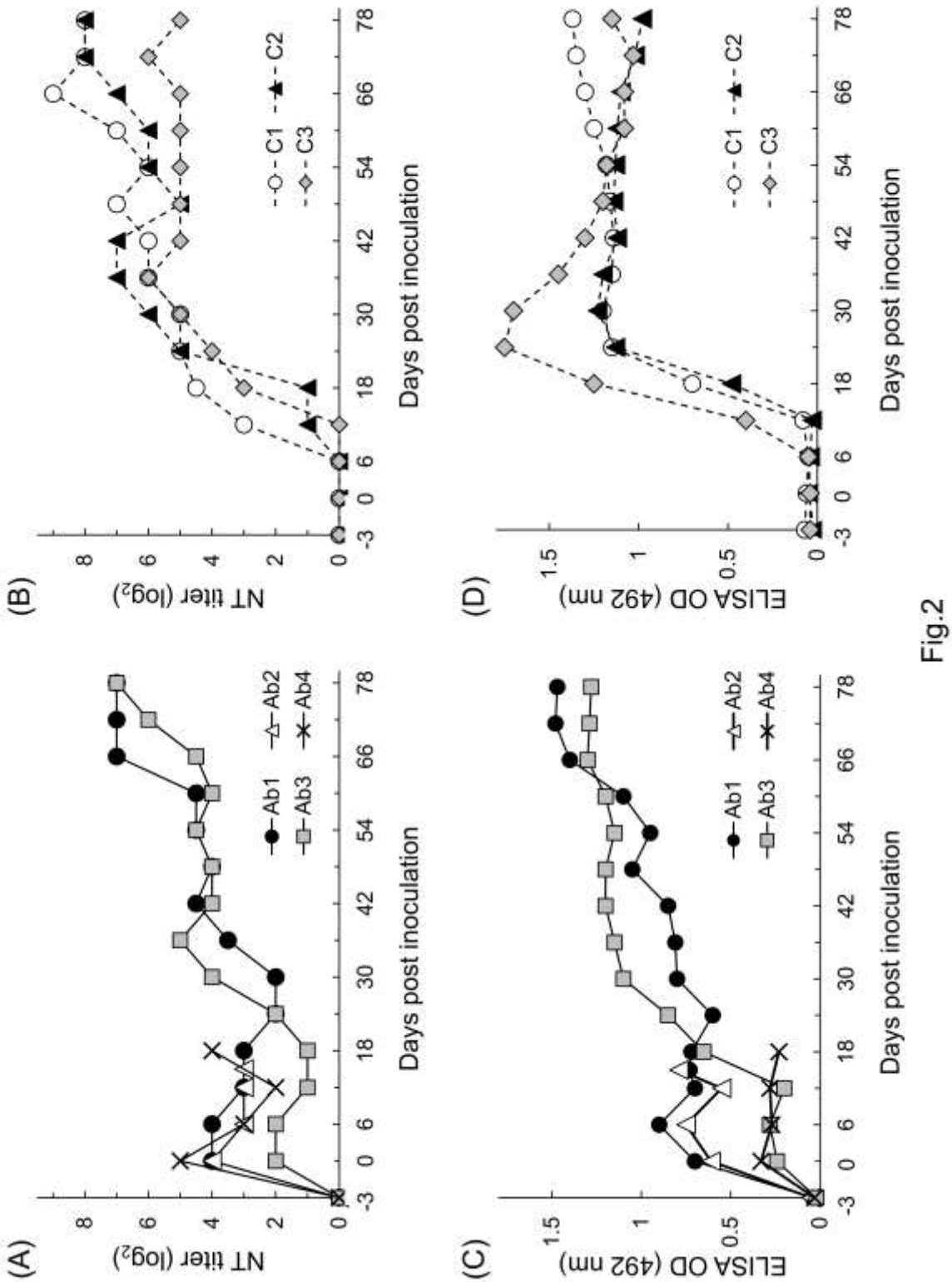
(A)
NT titer (log2)
Days post inoculation
Ab1
Ab2
Ab3
Ab4
(B)
NT titer (log2)
Days post inoculation
C1
C2
C3
(C)
ELISA OD (492 nm)
Days post inoculation
Ab1
Ab2
Ab3
Ab4
(D)
ELISA OD (492 nm)
Days post inoculation
C1
C2
C3

Fig.2

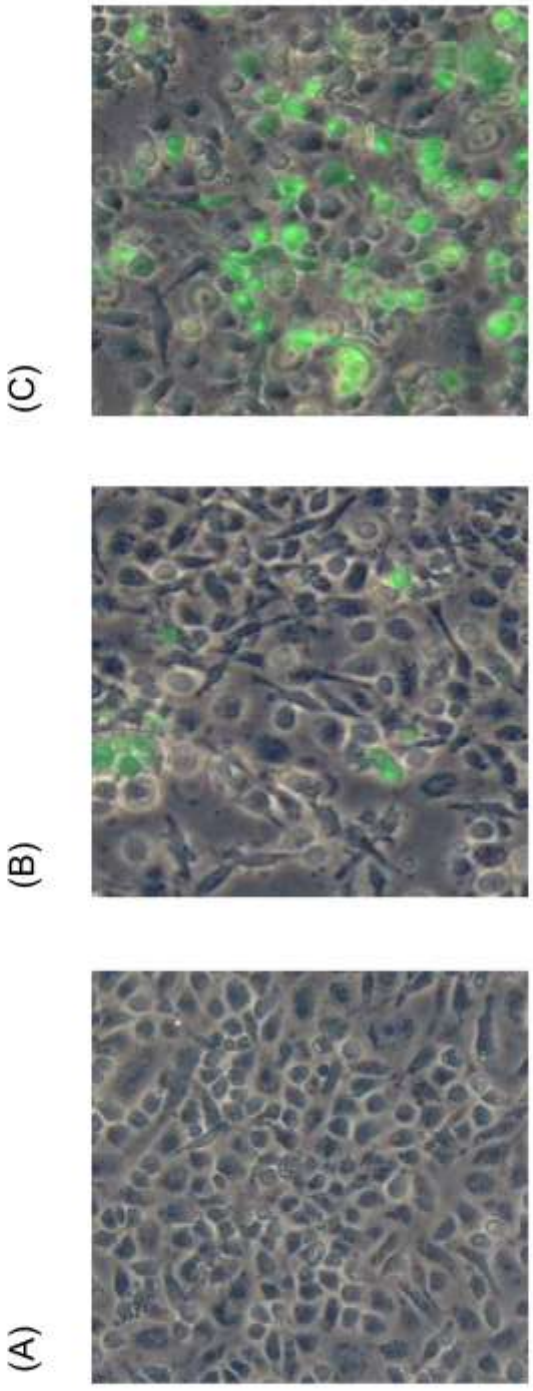

Fig.3

316
317
318
319
320
321
322
323

Table 1. Detection of the FCoV N gene in rectal swab samples of FIPV-I KU-2-infected cats by RT-PCR.

	Day of post inoculation with FIPV-I KU-2					
Cat Number	0	3	6	9	12	15
Ab1	–	–	–	–	–	–
Ab2	–	–	–	–	–	–
Ab3	–	–	–	–	–	+
Ab4	–	–	–	–	–	–
C1	–	–	–	–	–	–
C2	–	–	–	–	–	–
C3	–	–	–	–	–	–

+: FCoV N gene positive; –: FCoV N gene negative.