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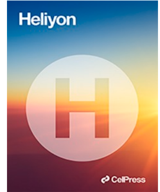

Research article

Compound C inhibits the replication of feline coronavirus

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ABSTRACT

Feline Coronavirus (FCoV) is a viral pathogen of cats and a highly contagious virus. Cats in a cattery can be infected by up to 100%, and even household cats are infected by 20–60%. Some strains of FCoV are known to induce a fatal disease in cats named Feline Infectious Peritonitis (FIP). However, no effective treatments are available. We demonstrated that compound C (dorsomorphin) can potentially inhibit feline coronavirus replication. Compound C treatment decreased the FCoV-induced plaque formation and cytopathic effect in FCoV-infected cells. Compound C treatment also significantly reduced the amount of viral RNA and viral protein in the cells in a dose-dependent manner. Our findings suggest that compound C is potentially useful for feline coronavirus-related diseases.

1. Introduction

Feline coronaviruses (FCoVs) are positive-sense single-stranded RNA viruses belonging to the *Coronaviridae* family [1]. The infection of FCoV in cats is widespread. FCoV antibodies are found in up to 100% of cats in catteries and also 20–60% of pet cats [1–5]. Feline enteric coronavirus (FECV) and Feline Infectious Peritonitis Virus (FIPV) belong to Feline coronavirus. FECV is an avirulent strain and endemic in cats. However, FIPV is a virulent strain responsible for a fatal feline infectious peritonitis (FIP) disease [6,7]. FIPV can be formed from FECV by sporadic mutation of spike protein, and FIPV acquires macrophage tropism [8]. FECV infects and replicates mainly in the mature intestinal epithelial cells [9,10], and virulent FIPV efficiently infects and replicates in the macrophages [11–15]. Macrophage infection of FIPV results in the spread of the virus throughout the body and the antigen can be detected in various organs, including the liver, spleen, and kidney [16]. Although FIPV infection results in fatal cat diseases, FIPV infections do not cause epidemics and occur only sporadically [17].

AMP-dependent protein kinase (AMPK) is well-known to be an evolutionarily conserved energy sensor protein, and compound C/dorsomorphin is known to inhibit AMPK [18,19]. AMPK has an essential role in cell growth and survival, and activation of AMPK is also involved in virus replication. The infection of the virus can affect the activity of AMPK [20]. Moreover, the activation of AMPK can be beneficial or detrimental in various virus replications [21]. Compound C treatment showed many AMPK inhibitory effects however AMPK also inhibits several kinases including Src, ALKs, and Erk 8 [18,22].

Because FIPV is responsible for the fatal disease of cats, medicines for FIPV are required. However, efficient medicines for FIPV have yet to be made available. The medicine which can inhibit the replication of FCoV will help reduce FCoV-related diseases. In 2019, the

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appearance of SARS-CoV-2 caused a severe problem in the world, and the biology of coronavirus replication has been intensively studied [23]. Here, we examined whether FCoV replication is affected by the treatment of compound C, known as an AMPK inhibitor [19]. We found that compound C treatment interferes with the replication of FCoV and the expression of FCoV genes.

2. Materials and methods

2.1. Cell culture and infection

Crandell feline kidney (CRFK) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Welgene, Seoul, Korea) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA.) and 1% antibiotic-antimycotic solution (Welgene). We purchased CRFK cells from the Korean Cell Line Bank (KCLB, Seoul, Korea) and the cells in a humidified 5% CO2 incubator at 37 °C. We used CRFK cells to propagate FECV in Eagle's Minimum essential medium (MEM, Welgene) supplemented with 2% FBS and 1% penicillin-streptomycin solution (Welgene). CRFK cells were susceptible to FECV infection at a Multiplicity of Infection (MOI) of 0.01, resulting in a cytopathic effect (CPE). The FECV virus was obtained from ATCC (Rockville, MD, USA).

2.2. Cell viability assay

Cell viability was measured using a MTT assay. CRFK cells were seeded in a 24-well plate. Twelve hours later, cells were infected with the indicated concentrations of compound C as an antiviral agent for 72 h. A final concentration of 0.5 mg/mL of the MTT solution was added and incubated for an additional 2 h. MTT was obtained from Thermo Fisher Scientific (Cleveland, OH, USA). Compound C was purchased from Abcam (Cambridge, MA, USA).

2.3. Quantitative RT-PCR

We used Quantitative RT-PCR to measure the level of FCoV RNA in cells and media. Cells and media were collected, and total RNA was prepared using Trizol (Thermo Fisher Scientific). cDNA was synthesized and subjected to RT-PCR using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). FECV RNA dependent RNA polymerase (RdRp) gene was amplified using the forward primer 5′- AAG TGT GAC CGC GCT TTA CC -3′ and reverse primer 5′-AAC TCA TTG GAG AGG CGG TAG -3'. FECV nucleocapsid (N) gene was amplified using the forward primer 5′- ATG GCC ACA CAG GGA CAA C -3′ and reverse primer 5′- GAT CCT TGG TCG AGG GTG ATG -3'. The feline GAPDH gene was amplified using the forward primer 5′- AAT TCC ACG GCA CAG TCA AGG -3′ and reverse primer 5′- CAT TTG ATG TTG GCG GGA TC -3'.

2.4. Plaque forming assay

Twenty well plates of confluent CRFK cells were infected (200 μl) with compound C treatment and incubated for 1 h. Then each well was covered with a mixture of 0.6% agar and 2 × MEM and incubated for 72 h. Three days post-infection (DPI), cells were washed with PBS and fixed with 4% Paraformaldehyde solution in PBS over 5 h. After discarding the Paraformaldehyde solution, cells were stained with Crystal Violet overnight. After that, the given numbers of plaques were calculated by the formula: PFU/mL = number of plaques per well × dilution factor/the inoculated volume in mL.

2.5. $TCID_{50}$ assay

Tissue culture infectious dose at 50% ($TCID_{50}$) assay was used to measure the infectious titers of FECV. CRFK cells were grown to reach over 80% confluency in 96-well plates. Serial 2-fold dilutions (10^{-2} to 9.77×10^{-6}) of FECV in MEM were added and were assayed by inoculation of 100ul sample per well for 3 days. CPE was checked at 3 days post-infection (DPI). Cells were washed with PBS and fixed with 4% Paraformaldehyde solution in PBS for 30 min. After discarding the Paraformaldehyde solution, cells were stained by Crystal Violet for 1 h and then washed 3 times with PBS. The titer was calculated using the modified Kärber method [24]. The viral titers expressed as $TCID_{50}$ and following formula was used for calculation of the $TCID_{50}$ per mL: Log 10($TCID_{50}$/mL) by improved Kärber Method = log (dilution giving highest CPE) – log (dilution factor) x (Σ infected rate at each dilution - 0.5)

2.6. Western blotting

For Western blot analysis, an equal amount of cell lysates and media were subject to Western blot. FECV proteins were detected with anti-FECV antibody (1:5000 dilution) using an enhanced chemiluminescence (ECL) system (Dogen, Seoul, Korea). Western blot data were acquired using a Chemidoc MP imaging system (Bio-Rad). Mouse polyclonal anti-FECV antibodies were generated using the inactivated FECV particles in mice, as described previously [25].

2.7. Statistical analysis

The results of cell viability assay, Western blot, quantitative RT-PCR, $TCID_{50}$ assay, and plaque forming assay were analyzed using the 2-tailed Student's t-test using Microsoft Excel software (Microsoft, Redmond, WA, USA) and GraphPad Prizm software (GraphPad,

Boston, MA, USA). Statistical significance was set at $p < 0.05$. For the calculation of half inhibitory concentration (IC_{50}) and half cytotoxic concentration (CC_{50}), the AAT Bioquest website program was used (https://www.aatbio.com/tools/ic50-calculator).

3. Results

3.1. Compound C treatment reduces feline enteric coronavirus (FECV) plaque formation and alleviates FECV-induced cytotoxicity

Compound C inhibits AMPK activity and we examined whether compound C treatment inhibits the replication of FECV. Plaque formation assay is a gold standard to measure viral replication, and we used plaque formation assay to measure the effect of compound C treatment [26]. We treated compound C up to 10 μM because cell viability was not significantly reduced up to 10 μM and the calculated half cytotoxic concentration (CC_{50}) was 132.33 μM (Fig. 1A). We used CRFK cells for FECV infection and compound C treatment decreased the number of plaques. The average virus titer in control was 4.2×10^5 PFU/ml, and the virus titer in compound C (10 μM) was 1.2×10^5 PFU/ml (Fig. 1B). The calculated half inhibitory concentration (IC_{50}) was 6.81 μM (Fig. 1B and C). Therefore, the selectivity index (CC_{50}/IC_{50}) based on plaque formation assay was 19.4. In addition, compound C treatment also decreases the size of plaques (Fig. 1B). These results indicate that compound C treatment inhibits FECV replication. FECV infection results in cytopathic effects, and we examined whether compound C treatment alleviates FECV-induced cytotoxicity. FECV infection showed cytopathic effects, including cell detachment, and compound C treatment decreased the cytopathic effect in FECV-infected cells (Fig. 1D). We also measured the effect of compound C treatment on the virus-infected cell viability and increased cell viability (Fig. 1E). These results indicate that compound C treatment reduces FECV-induced cytotoxicity.

3.2. Compound C treatment decreases FECV protein expression

Because compound C treatment decreased plaque formation, we examined whether compound C treatment decreased the viral protein expression. Because an anti-FECV antibody was not available, we produced polyclonal anti-FECV antibodies in mice, and the antibody detected the multiple viral proteins in FECV-infected cells (Fig. 2A). Next, we examined the FECV protein expression in CRFK

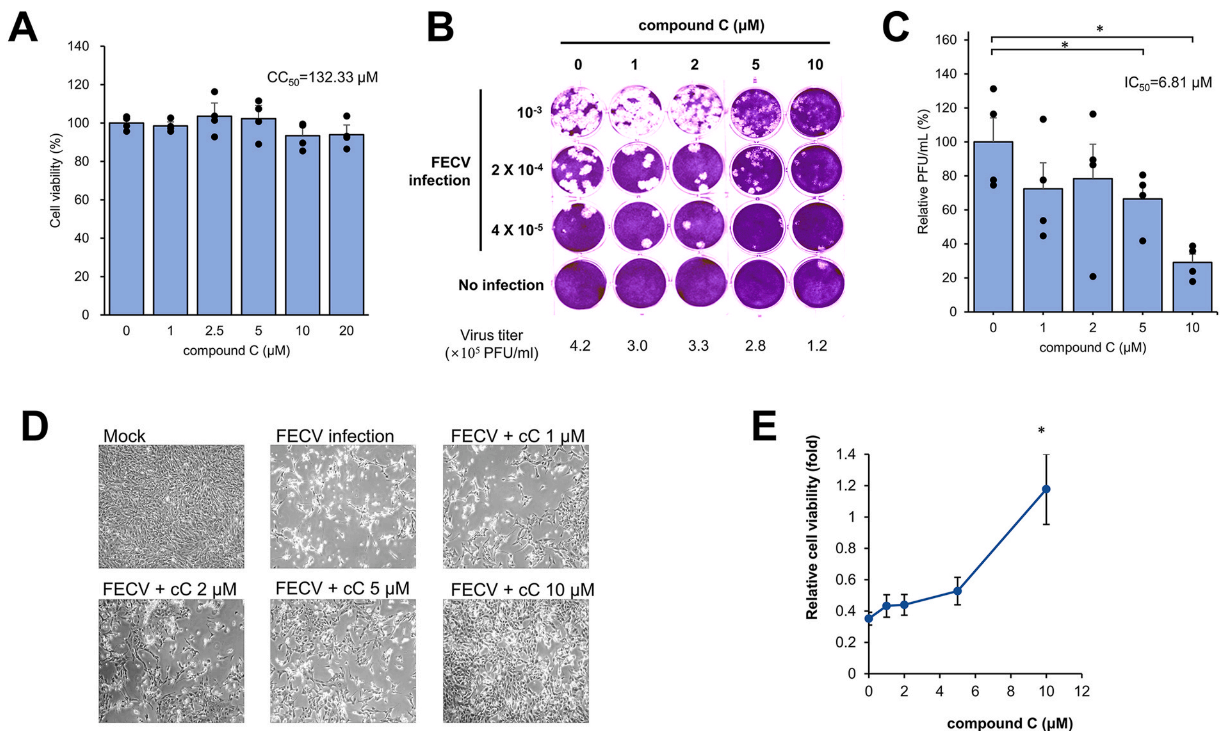

Fig. 1. Compound C treatment reduces FECV-induced plaque formation and alleviates FECV-induced cytopathic effect in CRFK cells. (A) CRFK cells were treated with the indicated concentration of FECV. Cell viability was measured using an MTT assay. (B) CRFK cells were infected with the indicated dilution of FECV and incubated with indicated concentrations of compound C for 3 days. Plaques are enumerated and used for the calculation of virus titers in PFU/ml. (C) The graph shows the viral titer of FECV (PFU/mL). Error bars represent standard deviation ($n = 4$). Control vs. compound C treatment, $*p < 0.05$. (D) CRFK cells were infected and treated with the indicated concentrations of compound C for 3 days. Cells were observed by light microscopy. Morphology of FECV-infected CRFK cells showed CPE characterized by clumped cells and detachment. (E) Compound C treatment reduced FECV-induced cell death. Cell viability was measured by MTT assay. Control vs. compound C treatment, $*p < 0.05$. The IC_{50} and CC_{50} were calculated using AAT Bioquest online calculator.

cells upon compound C treatment which significantly decreased FECV protein expression (Fig. 2B and C). We also calculated the half inhibitory concentration (IC_{50}) and calculated IC_{50} for compound C was 1.36 μM (Fig. 2D). These results also support that compound C treatment decreases FECV production in the infected cells.

3.3. *Compound C treatment decreases FECV RNA expression*

To confirm that compound C treatment decreases FECV replication, we examined the expression of FECV RNA. FECV RNA includes FECV mRNA as well as the FECV genome. We evaluated the level of FECV RNA in the infected cells and conditioned media. We used quantitative RT-PCR to measure the amount of FECV RNA and used RNA dependent RNA polymerase (RdRp) gene and nucleocapsid (N) gene. Compound C treatment decreased the level of FECV RdRp RNA both in cell lysates and conditioned media (Fig. 3A). In addition, N gene expression was also down-regulated by compound C in a dose-dependent manner (Fig. 3B). We also calculated the half inhibitory concentration (IC_{50}) and calculated IC_{50} for compound C was 2.13–2.59 μM (Fig. 3C). Interestingly, compound C treatment decreased the level of FECV RNA in the media, indicating that the FECV virion particles in the media were significantly reduced by compound C treatment (Fig. 3). These results indicate that compound C inhibited FECV replication.

3.4. *Compound C treatment decreases FECV titers*

To measure FECV titers in compound *C*-treated cells, we used Tissue Culture Infectious Dose ($TCID_{50}$) assays. Serially diluted media from the FECV-infected cell culture were used for virus titration. We found that compound C treatment decreased $TCID_{50}$ in the media indicating that compound C treatment decreased FECV titers (Fig. 4A and B). These results demonstrate that compound C treatment decreases the FECV replication in the infected cells.

4. Discussion

In this report, we demonstrated that compound C treatment reduces the replication of feline coronavirus (FCoV). We used feline enteric coronavirus (FECV) as a model system for feline infectious peritonitis virus (FIPV). Because FIPV is derived from FECV by

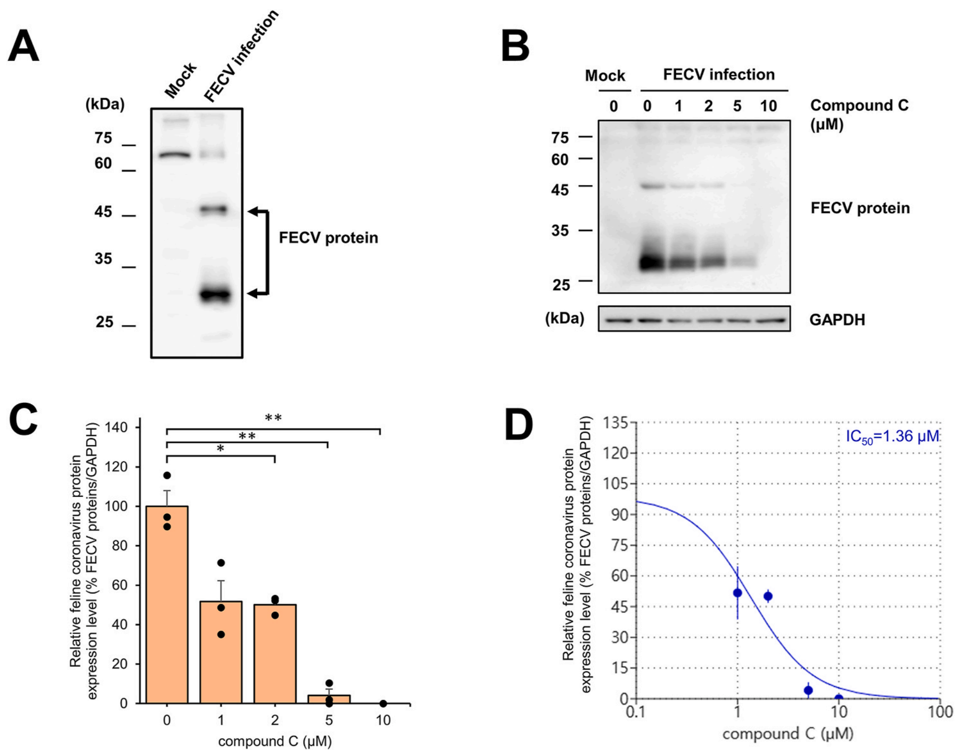

Fig. 2. Compound C treatment inhibits FECV protein expression. (A) Polyclonal anti-FECV antibody was produced in mice. CRFK cells were infected with either Mock of FECV, and equal cell lysates were subject to Western blot with anti-FECV antibody. (B) CRFK cells were infected with FECV and treated with the indicated concentrations of compound C for 3 days. The cell lysates were subject to Western blot with anti-FECV antibody. (C) Bar graphs show the relative FECV protein expression level (FECV protein/GAPDH). (D) IC_{50} of compound C was calculated and shown in the graph. The error bars represent the standard deviation (n = 3). Arrowheads represent FECV proteins. *$p < 0.05$, **$p < 0.01$. AAT Bioquest was used to calculate IC_{50} and plot the three-parameter logistic regression curve.

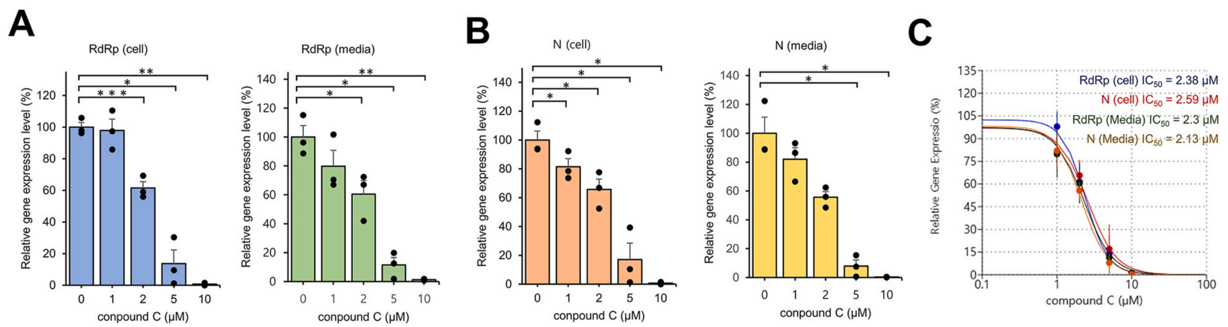

Fig. 3. Compound C treatment reduced the RNA expression of FECV. CRFK cells were infected with FECV and treated with the indicated concentration of compound C for 3 days. Quantitative RT-PCR analyzed the expression of FECV RNA. (A) Expression levels of RdRp were evaluated in CRFK cells (cell) or conditioned media (media) by quantitative RT-PCR. The graph shows the average and standard deviation (n = 3). (B) Expression levels of N mRNA were evaluated in CRFK cells (cell) or conditioned media (media) by quantitative RT-PCR. The graph shows the average and standard deviation (n = 3). (C) IC_{50} of compound C was calculated and shown in the graph. Control vs. compound C treatment, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. GraphPad Prism was used to calculate IC_{50} and plot the three-parameter logistic regression curve.

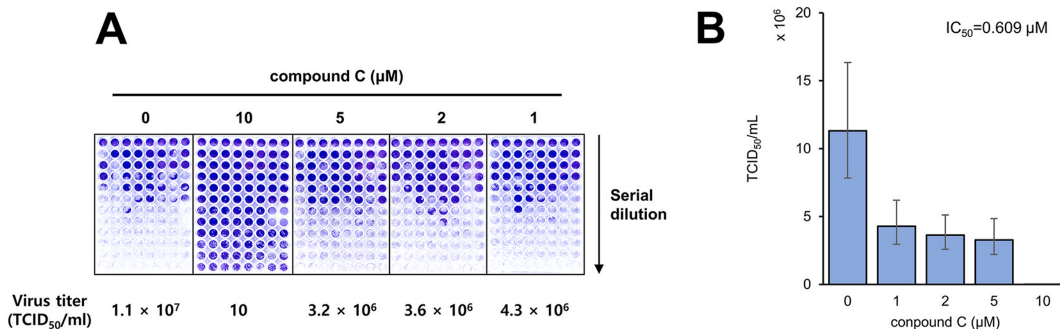

Fig. 4. Compound C treatment reduces viral titers of FECV. (A) Representative images of $TCID_{50}$ assay. CRFK cells were infected with FECV and treated with indicated concentrations of compound C for 3 days. (B) The FECV titers ($TCID_{50}$/mL) were calculated with the modified Kärber method. Error bars represent 95% confidence interval (CI). The IC_{50} was calculated using AAT Bioquest online calculator.

sporadic mutation, FECV and FIPV share many common biological characteristics. Therefore FECV, an avirulent form of FCoV, is a good and safe model to develop the medicine for FIPV. When effective medicines for FECV are developed, the next step will be the validation of the effect *in vivo*, and the cats with FIPV will be helpful to validate the effects *in vivo*.

We demonstrated the inhibitory effect of compound C for FECV using several methods. Initially, we used plaque formation assay to validate the inhibitory effect of compound C for FECV (Fig. 1). Plaque formation assay is a gold standard for enumerating the virus, and reducing plaques indicates a decrease in virus infection [26]. Virus infection reduces cell viability, and compound C treatment decreases FECV- induced cell death (Fig. 1). In addition, we examined the gene expression of FECV upon compound C treatment using Western blot and quantitative RT-PCR (Figs. 2 and 3). Significantly, the proper antibody for FECV was unavailable, and we generated the polyclonal antibodies against FECV using FECV whole virus (Fig. 2). The antibodies can be further used for FECV research.

Compound C is known to be an inhibitor of AMP-dependent kinase (AMPK), however, several reports showed that compound C also inhibits a number of kinases [22]. Because the specific AMPK inhibitor is unavailable now, compound C is frequently used to inhibit AMPK. Several reports indicate that the activity of AMPK is involved in the replication of various viruses, and the inhibition of AMPK can contribute to the inhibition of coronavirus replication. However, the inhibition of other kinases by compound C treatment may contribute to inhibiting FECV replication. Thus, the mechanism of compound C on FECV can be more complex, and further research will be required to elucidate the mechanism of compound C on FECV inhibition. This research will help develop more efficient coronavirus medicines without significant side effects.

Data availability statement

Data will be made available on request.

Funding statement

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CRediT authorship contribution statement

Yeonjeong Park: Writing – original draft, Investigation. **Chansoo Kim:** Writing – original draft, Investigation. **Yea-in Park:** Investigation. **Siyun Lee:** Investigation. **Jaeyeon So:** Investigation. **Rackhyun Park:** Methodology. **Junsoo Park:** Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27641.

References

[1] S. Sharif, S.S. Arshad, M. Hair-Bejo, A.R. Omar, N.A. Zeenathul, A. Alazawy, Diagnostic methods for feline coronavirus: a review, Vet. Med. Int. 2010 (2010) 809480.
[2] D.D. Addie, O. Jarrett, A study of naturally occurring feline coronavirus infections in kittens, Vet. Rec. 130 (1992) 133–137.
[3] A.A.P.M. Herrewegh, M. Mähler, H.J. Hedrich, B.L. Haagmans, H.F. Egberink, M.C. Horzinek, P.J.M. Rottier, R.J. de Groot, Persistence and evolution of feline coronavirus in a closed cat-breeding colony, Virology 234 (1997) 349–363.
[4] D.D. Addie, Clustering of feline coronaviruses in multicat households, Vet. J. 159 (2000) 8–9.
[5] N.C. Pedersen, R. Sato, J.E. Foley, A.M. Poland, Common virus infections in cats, before and after being placed in shelters, with emphasis on feline enteric coronavirus, J. Feline Med. Surg. 6 (2004) 83–88.
[6] M. Delaplace, H. Huet, A. Gambino, S. Le Poder, Feline Coronavirus Antivirals: A Review, 2021. Pathogens.
[7] J. Leibowitz, G. Kaufman, P. Liu, Coronaviruses: propagation, quantification, storage, and construction of recombinant mouse hepatitis virus, Current Protocols in Microbiology 21 (2011), 15E.11.11-15E.11.46.
[8] P.J. Rottier, K. Nakamura, P. Schellen, H. Volders, B.J. Haijema, Acquisition of macrophage tropism during the pathogenesis of feline infectious peritonitis is determined by mutations in the feline coronavirus spike protein, J. Virol. 79 (2005) 14122–14130.
[9] N.C. Pedersen, J.W. Black, J.F. Boyle, J.F. Evermann, A.J. McKeirnan, R.L. Ott, Pathogenic differences between various feline coronavirus isolates, Adv. Exp. Med. Biol. 173 (1984) 365–380.
[10] N.C. Pedersen, J.F. Boyle, K. Floyd, A. Fudge, J. Barker, An enteric coronavirus infection of cats and its relationship to feline infectious peritonitis, Am. J. Vet. Res. 42 (1981) 368–377.
[11] G.A. Dean, T. Olivry, C. Stanton, N.C. Pedersen, In vivo cytokine response to experimental feline infectious peritonitis virus infection, Vet. Microbiol. 97 (2003) 1–12.
[12] N.C. Pedersen, Morphologic and physical characteristics of feline infectious peritonitis virus and its growth in autochthonous peritoneal cell cultures, Am. J. Vet. Res. 37 (1976) 567–572.
[13] N.C. Petersen, J.F. Boyle, Immunologic phenomena in the effusive form of feline infectious peritonitis, Am. J. Vet. Res. 41 (1980) 868–876.
[14] J.M. Ward, Morphogenesis of a virus in cats with experimental feline infectious peritonitis, Virology 41 (1970) 191–194.
[15] R.C. Weiss, F.W. Scott, Pathogenesis of feline infetious peritonitis: pathologic changes and immunofluorescence, Am. J. Vet. Res. 42 (1981) 2036–2048.
[16] N.C. Pedersen, Virologic and immunologic aspects of feline infectious peritonitis virus infection, Adv. Exp. Med. Biol. 218 (1987) 529–550.
[17] M.C. Horzinek, A.D.M.E. Osterhaus, The virology and pathogenesis of feline infectious peritonitis, Arch. Virol. 59 (1979) 1–15.
[18] X. Liu, R.R. Chhipa, I. Nakano, B. Dasgupta, The AMPK inhibitor compound C is a potent AMPK-independent antiglioma agent, Mol. Cancer Therapeut. 13 (2014) 596–605.
[19] M. Jang, R. Park, H. Kim, S. Namkoong, D. Jo, Y.H. Huh, I.S. Jang, J.I. Lee, J. Park, AMPK contributes to autophagosome maturation and lysosomal fusion, Sci. Rep. 8 (2018) 12637.
[20] J. Mankouri, M. Harris, Viruses and the fuel sensor: the emerging link between AMPK and virus replication, Rev. Med. Virol. 21 (2011) 205–212.
[21] M.S. Bhutta, E.S. Gallo, R. Borenstein, Multifaceted Role of AMPK in Viral Infections, 2021. Cells.
[22] J. Vogt, R. Traynor, G.P. Sapkota, The specificities of small molecule inhibitors of the TGFß and BMP pathways, Cell. Signal. 23 (2011) 1831–1842.
[23] K. Miller, M.E. McGrath, Z. Hu, S. Ariannejad, S. Weston, M. Frieman, W.T. Jackson, Coronavirus interactions with the cellular autophagy machinery, Autophagy 16 (2020) 2131–2139.
[24] C. Lei, J. Yang, J. Hu, X. Sun, On the calculation of TCID(50) for quantitation of virus infectivity, Virol. Sin. 36 (2021) 141–144.
[25] A.S. Tung, S.-T. Ju, S. Sato, A. Nisonoff, Production of large amounts of antibodies in individual Mice 1, J. Immunol. 116 (1976) 676–681.
[26] A. Au - Baer, K. Au - Kehn-Hall, Viral Concentration Determination through Plaque Assays: Using Traditional and Novel Overlay Systems, JoVE, 2014 e52065.