

ajava

Asian Journal of Animal and Veterinary Advances



Academic
Journals Inc.

www.academicjournals.com

Pronase/diethylaminoethyl Dextran Supplementation Enhances Growth of Feline Infectious Peritonitis Virus Serotype I Strains in Cell Culture

¹Satoru Yamazaki, ²Kazuhiro Ikeda, ¹Kazuei Matsubara, ³Norio Hirano, ¹Hideharu Taira and ⁴Masaki Imai

¹The United Graduate School of Agriculture Sciences, Iwate University, 3-18-8 Ueda, Morioka 020-8550, Japan

²Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical University, 1397-1 Yamane, Hidaka 350-1241, Japan

³The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu, 501-1193, Japan

⁴Influenza Virus Research Center, National Institute of Infectious Diseases, Tokyo 208-0011, Japan

Corresponding Author: Masaki Imai, Influenza Virus Research Center, National Institute of Infectious Diseases, Tokyo 208-0011, Japan Tel: +81-42-561-0771 Fax: +81-42-561-6149

ABSTRACT

Feline Infectious Peritonitis (FIP) is a lethal systematic disease caused by FIP Virus (FIPV). FIPV is divided into two serotypes which differ in their growth characteristics in tissue cultures; serotype II viruses replicate efficiently, whereas viruses in serotype I do not replicate or produce plaques. Here, the effects of proteases on plaque formation by serotype I viruses were examined. The incorporation of trypsin or pronase in an agar overlay medium of *Felis catus* whole fetus (Fcfw-4) cells had substantially no effect on plaque formation by serotype I viruses. However, addition of diethylaminoethyl (DEAE)-dextran to trypsin-or pronase-containing overlay greatly enhanced plaque formation by serotype I strains. In addition, serotype I viruses from clinical specimens could produce plaques only with an overlay containing both pronase and DEAE-dextran. These findings suggested that both pronase and DEAE-dextran are required for optimal plaque formation by serotype I viruses.

Key words: Clinical specimens, *Felis catus* whole fetus cells, feline infectious peritonitis virus, plaque formation, protease, DEAE-dextran

INTRODUCTION

Feline Infectious Peritonitis (FIP) is a chronically progressive and immunologically mediated disease of domestic and feral cats. The causative agent of this disease is feline coronaviruses (FCoVs). Clinically, the disease is categorized as either wet or dry. Wet FIP is an effusive disease in which vascular permeability and vasculitis causes protein-rich fluid to accumulate in the peritoneal and/or pleural spaces, whereas dry FIP is a non-effusive disease associated with clinical signs of convulsions, paralysis and uveitis caused by the formation of granulomas and pyogranulomas of the Central Nervous System (CNS) and eyes. Both types of FIP are caused by infection with FIP virus (FIPV) which is an enveloped, single-stranded, positive-sense RNA virus of the genus *Alphacoronavirus* (order *Nidovirales*, family *Coronaviridae*) (Addie and Jarrett, 2006; Battilani *et al.*, 2010; Chang *et al.*, 2011).

FIPVs are classified into two distinct serotypes (serotype I and serotype II) on the basis of genetic differences in their spike (S)-protein (Benetka *et al.*, 2004; Herrewegh *et al.*, 1995). Serologic analyses have reported that FIPV serotype I was the predominant circulating serotypes and was responsible for 80-90% of FIPV infections (Hohdatsu *et al.*, 1992; Lin *et al.*, 2009). However, the replication and pathogenesis of serotype I FIPVs remain still poorly understood, because of its relatively poor growth in tissue cultures.

Previous studies have shown that proteolytic enzymes enhance the infection of some coronaviruses, including Mouse Hepatitis Virus (MHV), Severe Acute Respiratory Syndrome (SARS) coronavirus and human coronavirus 229E, in cultured cells (Sturman *et al.*, 1985; Matsuyama *et al.*, 2005; Kawase *et al.*, 2009). Here, the effects of proteases on the replication of the serotype I strain of FIPV were assessed in cell cultures. The data suggest that proteases are required for optimal plaque formation by serotype I viruses.

MATERIALS AND METHODS

Cells and viruses: *Felis catus* whole fetus (Fcwf-4) cells were grown in Eagle's Minimal Essential Medium (MEM) supplemented with 5% Fetal Bovine Serum (FBS) and 5% Tryptose Phosphate Broth (TPB). The cells were cultured at 37°C with 5% CO₂. UCD-1 (serotype I), Yayoi (serotype I) and HR (serotype II) strains of FIPV were provided by Hohdatsu *et al.* (1992), Hayashi *et al.* (1981) and Kai *et al.* (1987), respectively. Fcwf-4 cells grown in T25 flasks were infected with the serotype I or serotype II strains and cultured in MEM containing 5% TPB, 20 µg mL⁻¹ diethylaminoethyl (DEAE)-dextran (GE Healthcare Bio-Science AB) and 0.5 µg mL⁻¹ pronase (40,000 units g⁻¹; Kaken Pharmaceutical) at 37°C. At 48 h post-infection, the cells were collected, frozen at -80°C, thawed and homogenized in a Potter-Elvehjem tissue grinder.

Clinical specimens: Pleural effusion fluid or cerebrospinal fluid samples were obtained from three cats with suspected FIP between 2002 and 2005 in a Japanese veterinary clinic after informed consent was obtained from the cat owners. Homogenates of Fcwf-4 cells inoculated with clinical samples were prepared as described above. Serum samples were obtained for diagnostic purpose from the three cats and were sent to Dainippon Sumitomo Pharma Co. Ltd. in Japan. The presence of anti-FCoV Immunoglobulin G (IgG) antibody was examined by Enzyme-linked immunosorbent assay (ELISA) as previously described by Soma and Takahashi (2000).

Plaque assays: Fcwf-4 cells grown in 60 mm dishes were washed three times with FBS-free MEM and then inoculated with 0.2 mL of cell homogenates. The cells were incubated at 37°C for 90 min in non-supplemented MEM or in MEM supplemented with 0.25 µg mL⁻¹ trypsin (Becton, Dickinson and Company), 0.5 µg mL⁻¹ pronase, 20 µg mL⁻¹ DEAE-dextran, 20 µg mL⁻¹ DEAE-dextran and 0.25 µg mL⁻¹ trypsin, or 20 µg mL⁻¹ DEAE-dextran and 0.5 µg mL⁻¹ pronase. The cells were overlaid with 5 mL of agar medium consisting of 1% Bacto agar and 5% TPB in MEM and incubated at 37°C for 3 days. The cultures were then overlaid with agar medium containing neutral red dye (1:5000 dilution) and plaque numbers were counted under a microscope. The infectious virus titer was expressed in Plaque-forming Units (PFUs).

Reverse transcription (RT)-PCR and nested PCR: Plaques of infected cells were picked and processed for RNA isolation. Total RNA was extracted with RNeasy Mini Kit (Qiagen) according to

the manufacturer's instructions. RT-PCR and nested PCR were performed as previously described (Benetka *et al.*, 2004). Primer sets used in the detection of FIPV RNA were as follows:

- **Serotype I PCR:** 5'-TTGACCTTGACTGGCTCAAC-3' and 5'-CGTCCACAGAGATGCCAATA-3'
- **Serotype I nested PCR:** 5'-TCTGCATACCACTGTTACTG-3' and 5'-GAGATGCCAATAGACTTGAC-3'
- **Serotype II PCR:** 5'-AGGTTGTTGTGGATGCATAG-3' and 5'-ACGGTCAAGTTCGTC AAGTA-3'
- **Serotype II nested PCR:** 5'-AGGAAGTTGTTGTCCTCTA-3' and 5'-GTCCATACAAGACCTGTAAT-3'

Nested amplification products were visualized via 1% agarose gel electrophoresis and stained with ethidium bromide.

RESULTS AND DISCUSSION

Effect of proteases and DEAE-dextran on plaque formation by laboratory strains of FIPV: To determine whether the presence of protease in an agar overlay medium enhances plaque formation by serotype I FIPV strains in cell culture, Fcwf-4 cells were infected with virus and virus titer in the homogenate was measured by plaque assay in the presence or in the absence of trypsin or pronase. Even in the absence of trypsin or pronase in the overlay, UCD-1 and Yayoi strains of serotype I produced detectable plaques in monolayer cultures of Fcwf-4 cells (Fig. 1a, b). Addition of trypsin or pronase to the overlay did not result in a further increase in numbers of plaques produced by the serotype I viruses.

A study reported that along with trypsin, DEAE-dextran is required for optimal plaque formation by neonatal Calf Diarrhea Virus (Matsuno *et al.*, 1977). As shown in Fig. 1a, addition of DEAE-dextran to the overlay medium resulted in slight increase in numbers of plaque by UCD-1 and Yayoi strains. However, when DEAE-dextran was present in the trypsin-containing overlay, a striking increase in numbers of plaque by UCD-1 and Yayoi strains was observed in comparison with the overlay without any supplement (Fig. 1b; 6.7- and 6.0-fold, respectively). Similarly, addition of DEAE-dextran to the pronase-containing overlay increased dramatically the number of plaques by UCD-1 and Yayoi strains (Fig. 1b; 12.7- and 8.1-fold, respectively). For comparison, we examined the effect of supplements on plaque formation by HR strain of serotype II. As shown in Fig. 1a and b, no noticeable effect on plaque-forming ability of HR strain was observed with trypsin, DEAE-dextran, or their combination. Notably, addition of pronase to the overlays decreased plaque numbers. The reason is unclear; however, the surface proteins of the serotype II virus strain may be digested with pronase, resulting in reduced viral infectivity. Taken together, these results suggested that along with proteases, DEAE-dextran might be required for plaque formation by serotype I strains of FIPV but not by serotype II strains.

Effect of proteases and DEAE-dextran on plaque formation by FIPV isolates from clinical specimens: To assess whether serotype I FIPVs from clinical samples produce plaques in the presence of supplements, samples were collected from three cat patients with a diagnosis of FIP. Information about the patients is summarized in Table 1. By using ELISA, we confirmed that case 1 and case 3 patients had antibodies against FCoV. We collected cerebrospinal fluid or pleural effusion fluid specimens from the patients for virus isolation by plaque assays on Fcwf-4 cells. For

case 1 patient, plaques could be discerned in all of the overlay media tested (Fig. 1c). Importantly, addition of DEAE-dextran to the overlays containing trypsin or pronase increased dramatically the number of plaques produced (10- or 20-fold, respectively) when compared with the overlay without any supplement, while addition of trypsin alone, pronase alone, or DEAE-dextran alone enhanced minimally (2.0-5.4-fold). For case 2 and case 3 patients, on the other hand, plaques could be detected only in the overlay containing both pronase and DEAE-dextran. Thus, these data suggest that addition of both pronase and DEAE-dextran to the overlay medium may be essential for plaque formation by serotype I FIPVs in cultures of Fcwf-4 cells.

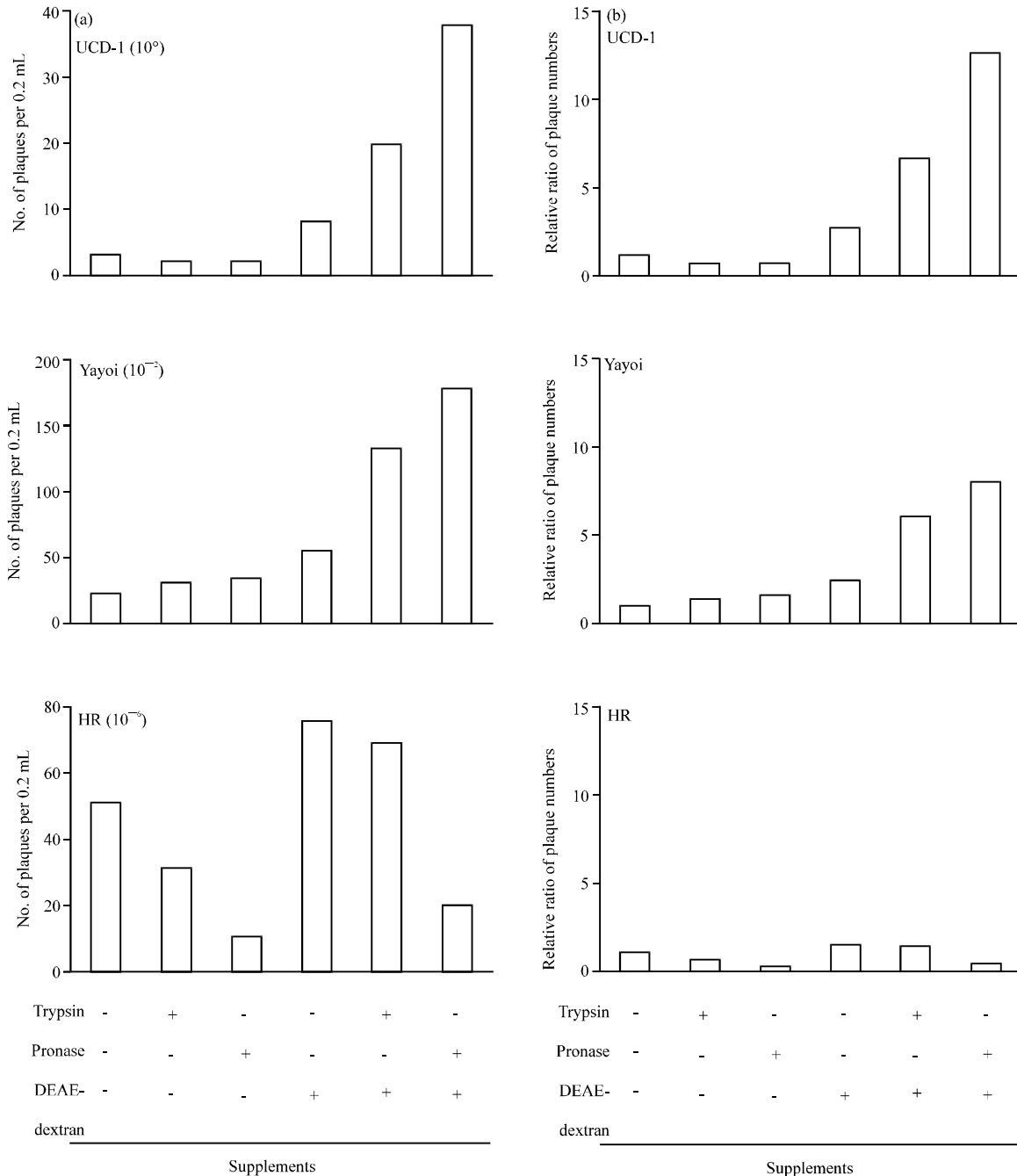


Fig. 1: Continue

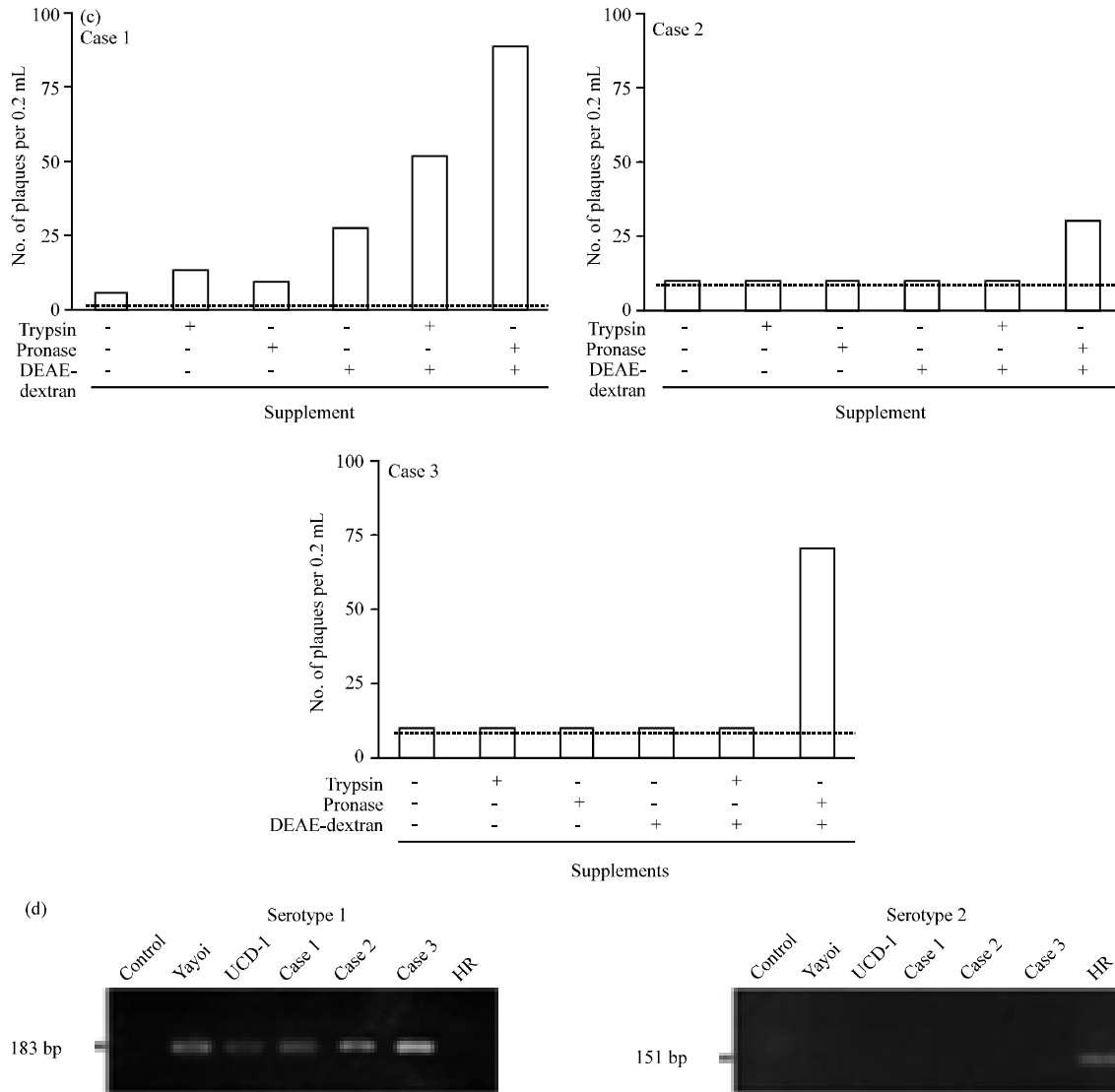


Fig. 1(a-d): Effect of supplements in the overlay; (a) The number of plaques in the homogenates of cells, The numbers in parentheses are the dilutions of homogenates at which the number of plaques was counted, (b) Relative ratio of the number of plaques and (c) The number of plaques in the original undiluted homogenates of cells inoculated with clinical specimens, The lower limit of detection for the plaque assays is indicated with a dashed line and (d) Identification of serotype I (upper panel) and serotype II (lower panel) FIPVs by nested PCR, Fcwf-4 cells were inoculated with serial ten-fold dilutions of cell homogenates and incubated under MEM containing 1% agar in the presence (+) or absence (-) of supplements. At 3 days post-inoculation, the cultures were overlaid with agar medium containing neutral red dye, and plaques were counted under a microscope

To identify the serotypes of each virus isolated from case 1, case 2 and case 3 patients, plaques were picked and were subjected to RT-nested PCR for the S protein gene of FIPVs (Fig. 1d). The

Table 1: Patient information

Case	Age	Sex	Clinical symptom	*Antibody titer	Specimen
1	4 month	Male	Convulsions	3200	Cerebrospinal fluid
2	4 year 7 month	Male	Dyspnea	<400	Pleural effusion
3	1 year 8 month	Male	Paralysis	1600	Cerebrospinal fluid

*IgG antibody to FCoV in serum was detected by ELISA

serotype I-specific primer set amplified all of the plaque-purified clones derived from the clinical sample as well as the UCD-1 and Yayoi strains (upper panel), whereas the serotype II-specific primer set amplified only the HR strain (lower panel), indicating that all of the viruses isolated from cats showing clinical signs of FIP were serotype I FIPVs.

Previous studies reported that DEAE-dextran enhances the adsorption of viruses onto cells and their penetration into cells. For example, it is well established that DEAE-dextran enhances cell infection by poliovirus and SV40 virus (McCutchan and Pagano, 1968; Vaheri and Pagano, 1965). These studies suggest that DEAE-dextran may promote the adsorption and penetration of FIPV serotype I strains in Fcwf-4 cells.

Trypsin cleaves peptide bonds C-terminal to lysine and arginine residues, except where followed proline, while pronase is proteolytic enzymes that are produced in the culture supernatant of *Streptomyces griseus* and contains at least ten proteolytic components; five serine-type proteases, two Zn²⁺ endopeptidase, two Zn²⁺-leucine aminopeptidase and one Zn²⁺ carboxypeptidase (Narahashi *et al.*, 1968; Yamskov *et al.*, 1986). Because pronase has both exo- and endo-peptidase activities, it has a broad specificity, cleaving nearly all peptide bonds (Nomoto *et al.*, 1960). In this study, we observed that in the presence of DEAE-dextran, pronase increased the number of PFUs produced by serotype FIPV I strains derived from clinical specimens. We therefore speculate that S-protein cleavage by pronase may enhance the propagation of serotype I FIPV strains when DEAE-dextran is present to facilitate viral adsorption onto cells. Further investigations in terms of the putative activation of S-protein by pronase are needed.

Despite the fact that FIP is predominantly caused by serotype I strains of FIPV, a practicable plaque assay for this serotype has not been established yet. Therefore, limited information exists about the mechanisms of the replication and pathogenesis of serotype I FIPVs. Although, the role of proteases and DEAE-dextran in the replication of serotype I FIPVs has not been defined, our results indicated that inclusion of both protease and DEAE-dextran in the overlay medium was necessary for optimal plaque formation by serotype I FIPVs. The findings suggest that the present plaque assay system is a useful tool for studying the life style of serotype I FIPVs.

ACKNOWLEDGMENT

The authors express their thanks to Paul Langman, Ph.D., Iwate University, Morioka, Japan for his kind assistance with English usage.

REFERENCES

- Addie, D.D. and O. Jarrett, 2006. Feline Coronavirus Infections. In: Infectious Diseases of the Dog and Cat. 3rd Edn., Greene, C.E. (Ed.). Saunders Elsevier, St Louis, MO, USA., pp: 88-102.
- Battilani, M., A. Balboni, M. Bassani, A. Scagliarini, S. Paltrinieri and S. Prospero, 2010. Sequence analysis of the nucleocapsid gene of feline coronaviruses circulating in Italy. New. Microbiol., 33: 387-392.

- Benetka, V., A. Kubber-Heiss, J. Kolodziejek, N. Nowotny, M. Hofmann-Parisot and K. Mostl, 2004. Prevalence of feline coronavirus types I and II in cats with histopathologically verified feline infectious peritonitis. *Vet. Microbiol.*, 99: 31-42.
- Chang, H.W., H.F. Egberink and P.J. Rottier, 2011. Sequence analysis of feline coronaviruses and the circulating virulent/avirulent theory. *Emerg. Infect. Dis.*, 17: 744-746.
- Hayashi, T., T. Yanai, M. Tsurudome, H. Nakayama, Y. Watabe and K. Fujiwara, 1981. Serodiagnosis for feline infectious peritonitis by immunofluorescence using infected suckling mouse brain sections. *Jpn. J. Vet. Sci.*, 43: 669-676.
- Herrewegh, A.A.P.M., H. Vennema, M.C. Horzinek, P.J.M. Rottiera and R.J. de Groot, 1995. The molecular genetics of feline coronaviruses: Comparative sequence analysis of the ORF7a/7b transcription unit of different biotypes. *Virology*, 212: 622-631.
- Hohdatsu, T., S. Okada, Y. Ishizuka, H. Yamada and H. Koyama, 1992. The prevalence of types I and II feline coronavirus infections in cats. *J. Vet. Med. Sci.*, 54: 557-562.
- Kai, K., Y. Kaneda, N. Goto and M. Kanoe, 1987. Isolation and characterization of a new and high titer strain of feline infectious peritonitis virus. *Jpn. J. Vet. Sci.*, 49: 1105-1111.
- Kawase, M., K. Shirato, S. Matsuyama and F. Taguchi, 2009. Protease-mediated entry via the endosome of human coronavirus 229E. *J. Virol.*, 83: 712-721.
- Lin, C.N., B.L. Su, C.H. Wang, M.W. Hsieh, T.J. Chueh and L.L. Chueh, 2009. Genetic diversity and correlation with feline infectious peritonitis of feline coronavirus type I and II: A 5-year study in Taiwan. *Vet. Microbiol.*, 136: 233-239.
- Matsuno, S., S. Inouye and R. Kono, 1977. Plaque assay of neonatal calf diarrhea virus and the neutralizing antibody in human sera. *J. Clin. Microbiol.*, 5: 1-4.
- Matsuyama, S., M. Ujike, S. Morikawa, M. Tashiro and F. Taguchi, 2005. Protease-mediated enhancement of severe acute respiratory syndrome coronavirus infection. *Proc. Natl. Acad. Sci.*, 102: 12543-12547.
- McCutchan, J.H. and J.S. Pagano, 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. *J. Nat. Can. Inst.*, 41: 351-357.
- Narahashi, Y., K. Shibuya and M. Yanagawa, 1968. Studies on proteolytic enzyme (Pronase) of *Streptomyces griseus* K-1. Separation of exo and endopeptidase of Pronase. *J. Biochem.*, 64: 427-437.
- Nomoto, M., Y. Narahashi and M. Murakami, 1960. A proteolytic enzyme of *Streptomyces griseus*. Substrate specificity of *Streptomyces griseus* protease. *J. Biochem.*, 48: 906-915.
- Soma, T. and M. Takahashi, 2000. Antibody testing against feline infectious peritonitis virus by a modified ELISA. *J. Environ. Dis.*, 9: 1-5.
- Sturman, L.S., C.S. Ricard and K.V. Holmes, 1985. Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: Activation of cell-fusing activity of virions by trypsin and separation of two different 90K cleavage fragments. *J. Virol.*, 56: 904-911.
- Vaheri, A. and J.S. Pagano, 1965. Infectious poliovirus RNA: A sensitive method of assay. *Virology*, 27: 434-436.
- Yamakov, I.A., T.V. Tichonova and V.A. Davankov, 1986. Pronase-catalyzed hydrolysis of amino acid amides. *Enz. Microbial. Tech.*, 8: 241-244.