Eligibility of Feline Calicivirus for a Surrogate of Human Norovirus in Comparison with Murine Norovirus, Poliovirus and Coxsackievirus

TAKAHITO OHMINE1, SEIKA NARAI1, TOSHIKI MATSUBARA1, TOSHIHITO NOMURA1, KOSUKE ODA1, MASAYA FUKUSHI1, TAKASHI IRIE1, TAKAYUKI KOMATSU2, YUKINOBU TOHYA3, AND TAKEMASA SAKAGUCHI1

1Department of Virology, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Hiroshima 734-8551, Japan
2Department of Microbiology and Immunology, Aichi Medical University School of Medicine, Nagakute, Aichi 480-1195, Japan
3Laboratory of Veterinary Microbiology, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, Fujisawa, Kanagawa 252-0880, Japan

Received 24 August, 2017/Accepted 9 January, 2018

Feline calicivirus (FCV) is frequently used as a surrogate of human norovirus. We investigated eligibility of FCV for anti-viral assay by investigating the stability of infectivity and pH sensitivity in comparison with other viruses. We found that infectivities of FCV and murine norovirus (MNV) are relatively unstable in infected cells compared with those of coxsackievirus (CoV) and poliovirus (PoV), suggesting that FCV and MNV have vulnerability. Western blotting indicated that inactivation of FCV was not due to viral protein degradation. We also demonstrated sensitivity of FCV to low pH, the 50% inhibitory pH value being ca. 3.9. Since human norovirus is thought to persist longer, in infectivity and to be a resistant virus, CoV, which is robust and not restrained in use as PoV, may be more appropriate as a test virus for disinfectants, rather than FCV and MNV.

Key words: Norwalk virus / Enterovirus / Virucidal test / Disinfectant.

Human norovirus (HuNoV) that infects from the intestine is a public health problem. Viruses that infect from the intestine retain their infectivity after exposure to the acidic environment of the stomach and to detergents, bile acid, in the duodenum (Green, 2013; Pallansch et al., 2013). They are relatively resistant to physical stress and disinfectant. Feline calicivirus (FCV), which belongs to the family Calciviridae and causes a respiratory infection in cats, is frequently used as a surrogate of HuNoV for virucidal tests of disinfectants. Murine norovirus (MNV) belongs to the same genus as that of HuNoV, and is a relatively new substitute of HuNoV. Poliovirus (PoV) and coxsackievirus (CoV), both of which belong to the family Picornaviridae, are enteroviruses that cause acute poliomyelitis and hand, foot and mouth disease, respectively. These viruses infect from the human intestine and are relatively resistant to disinfectants (Pallansch et al., 2013). In the present study, we investigated the stability of infectivity and pH dependency of FCV and the other viruses in order to consider an appropriate test virus.

Preparations of cells and viruses are as follows. CRFK cells, feline kidney-derived cells, were propagated in Eagle’s minimum essential medium (MEM, Wako Pure Chemical Industries, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS), penicillin G (100 units/ml, Meiji Seika Pharma, Tokyo, Japan) and streptomycin (100 µg/ml, Meiji Seika Pharma). FL cells, human amnion-derived cells, and RAW264.7 cells, mouse macrophage-derived cells, were propagated in Dulbecco’s modified Eagle’s minimum essential medium (DMEM, Wako Pure Chemical Industries) supplemented with 10% FCS, penicillin G and streptomycin. These
pathic effects such as cell rounding, shrinking and detaching on day 1, and they spread to almost all of the cells on day 2 (Fig. 2C). Probably, virus production ended by day 2 and the viruses survived on the following days. CoV and PoV showed cytopathic effects similar to FCV, and the time course of their appearance was similar to that of FCV (data not shown). Cytopathic effects of MNV were not so prominent as the other viruses.

To investigate the time course of FCV infectivities in

three cell lines were originally purchased from ATCC.

FCV F9 strain and MNV S7 strain were propagated in CRFK cells and RAW264.7 cells, respectively. PoV Sabin-1 vaccine strain and CoV group B type 5 were propagated in FL cells. Preparation of the virus solution and the origins of the viruses have been described by Ueda et al. (2013).

Experimental procedures are as follows. The four viruses were inoculated to the susceptible cells in 60-mm dishes at a multiplicity of infection (m.o.i.) of 0.1 (for CoV and PoV) or 0.3 (for FCV and MNV) and maintained in 5 ml DMEM supplemented with penicillin G and streptomycin. DMEM containing 10% FCS was used instead of DMEM for the assay of MNV. A cell dish was frozen every day, and cells with the culture medium in a dish were harvested. After 3 cycles of freezing and thawing, the cell lysate was clarified by low-speed centrifugation, and virus infectivity in the supernatant was measured by a TCID\textsubscript{50} method as described previously (Fig. 1) (Ueda et al., 2013).

Virus titers (infectivities) reached the maximum level on the next day post-infection (day 1). However, the stability of infectivities on the following days differed among viruses (Fig. 1). Infectivity of FCV reached the highest level, more than 10^8 TCID\textsubscript{50}/ml, on day 1. However, infectivity was reduced on day 2 and thereafter at a rate of about 1/10-fold per day (Fig. 1). Infectivity of MNV reached a peak level of more than 10^7 TCID\textsubscript{50}/ml on day 1 and then decreased. In contrast, infectivities of CoV and PoV reached peaks of more than 10^8 TCID\textsubscript{50}/ml on day 1 and the levels were maintained on days 2, 3, 4 and 5. These results indicate that the infectivities of FCV and MNV were unstable in the infected cells, while those of CoV and PoV were stable.

A part of the cells infected with FCV underwent cyto-

pathic effects such as cell rounding, shrinking and detaching on day 1, and they spread to almost all of the cells on day 2 (Fig. 2C). Probably, virus production ended by day 2 and the viruses survived on the following days. CoV and PoV showed cytopathic effects similar to FCV, and the time course of their appearance was similar to that of FCV (data not shown). Cytopathic effects of MNV were not so prominent as the other viruses.

To investigate the time course of FCV infectivities in
detail, CRFK cells were infected with FCV, and the virus was harvested every 4 hours until 48 hours post-infection. Infectivity reached a peak of more than $10^9$ TCID$_{50}$/ml at 36 hours post-infection (Fig. 2A) and then decreased. Infectivity decreased to ca. 1/50 over a period of 12 hours from 36 to 48 hours post-infection (Fig. 2A). Appropriate timing for harvesting infected cells is needed for preparation of a high titer stock of FCV. At 36 hours post-infection, when infectivity had peaked, cytopathic effects such as cell shrinking and rounding were found in almost all of the cells (Fig. 2C).

To investigate the mechanism of FCV inactivation, the VP1 capsid proteins in the samples were analyzed by Western blotting as described previously (Irie et al., 2007). The samples were processed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and proteins were transferred onto a nylon membrane. The membrane was probed with anti-FCV VP1 monoclonal antibody 8G1 (Tajima et al., 1998) and horseradish peroxidase-conjugated anti-mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were detected by using a chemiluminescence substrate for HRP, Luminata Forte Western HRP Substrate (Millipore), followed by analysis using a LumiCube imaging analyzer (Liponics, Tokyo, Japan).

The amount of VP1 proteins increased until the peak titer at 36 hours post-infection, corresponding approximately to the increase in infectivity. However, the amount of VP1 proteins appeared to be constant thereafter despite a decrease in infectivity (Fig. 2A, inlet). A similar experiment was performed and viruses were obtained daily until day 6. During that period, the amounts of VP1 proteins were relatively constant despite a reduction of infectivity (Fig. 1, 2B). These results indicate that virus inactivation was not due to viral protein degradation. Virus integrity to maintain virus infectivity may be disrupted, although the precise mechanism is unknown.

The four viruses were also examined for their pH tolerance. pH buffers ranging from pH 2.0 to 7.2 were prepared by mixing 0.2 M Na$_2$HPO$_4$ and 0.1 M citric acid with different ratios based on the McIlvaine buffer system (McIlvaine, 1921). The buffer for pH 2.0 was composed of only 0.1 M citric acid. Fine adjustments of pH were performed by using an F-52 pH meter (HORIBA, Kyoto, Japan).

A pH buffer (90 μL) was mixed with 10 μL of the virus solution and kept for 1 hour at room temperature. After the treatment, the mixture was neutralized by a 10-fold dilution with DMEM and virus infectivity was measured by a TCID$_{50}$ method. FCV was inactivated at a low pH between 2.0 to 4.0 (Fig. 3A). On the other hand, the infectivities of MNV, CoV and PoV were constant even at a low pH as shown by approximate straight lines in the graph of Fig. 3A.

For FCV, logarithmic values of virus infectivities were plotted against pH values ranging from 2 to 4.5, at which drastic changes of infectivities were observed, and an approximate straight line was drawn (Fig. 3B). On the basis of the line and the original infectivity of FCV, 6.3 x $10^5$ TCID$_{50}$/ml, the pH value of 50% reduction of infectivity was calculated to be 3.901. The results indicate that FCV is sensitive to low pH and can be inactivated at pH 4.0 or lower.

We previously performed virucidal tests for 7 kinds of plant-derived tannins or their derivatives against 12 viruses, which included the exactly same 4 viruses used in this study (Ueda et al., 2013). In the report, it was indicated that CoV and MNV were the most resistant to the tannins among the 12 viruses. PoV was more sensitive, showing slight sensitivity to 3 more tannin reagents, and FCV was far more sensitive. Although sensitivity to tannins was a different aspect from that of the present study, vulnerability of FCV was also shown in the previous
Roles of surrogate viruses for HuNoV are still important. Since the result of a meta-analysis of research papers indicated that HuNoV genomic RNA persists longer than do other surrogate viruses after various treatments (Knight et al., 2016), a robust virus seems to be better for the purpose. For FCV, there have been many reports including this study showing the unstableness of infectivity at a low pH and in other conditions (Cromeans et al., 2014; Park et al., 2010) as was also shown by a meta-analysis of previous papers (Hoelzer et al., 2013). The use of FCV as a surrogate of HuNoV should be avoided. MNV, taxonomically closely related to HuNoV, is known to be sensitive to ethanol (Akasaka et al., 2016; Cromeans et al., 2014; Zonta et al., 2016). Other surrogates including Tulane virus, porcine enteric calicivirus, porcine sapovirus, echovirus 12 and coliphage MS2 are also used (Cromeans et al., 2014; Esselli et al., 2015; Kniel, 2014). As a robust enterovirus, the use of CoV as a surrogate virus could be considered. Since PoV is a target of eradication by WHO and regulated in use by the law in Japan, the use of PoV in virucidal tests should be avoided.

The Ministry of Health, Labor and Welfare of Japan revised the “sanitary management of large-scale cooking facilities manual” in June 2017 (MHLW, 2017). In that manual, some ethanol-containing disinfectants have been nominated as effective disinfectants in addition to conventional disinfectants such as those containing hypochlorite ions. Although ethanol-based disinfectants had been thought to be ineffective for HuNoVs (CDC, 2011), new ethanol-based disinfectants that have extra additives such as organic acids and glycerol fatty acid ester can inactivate FCV (Igimi et al., 2017). However, virucidal tests using only FCV may over-estimate disinfectants, and the use of other viruses must be considered in the future.

In summary, FCV and MNV were unstable in infected cells and FCV was vulnerable to low pH, while CoV and PoV were stable. These features may facilitate the use of CoV as a surrogate virus of HuNoV in virucidal tests.

ACKNOWLEDGEMENTS

We thank Mr. K. Suzuki and Mr. T. Tsuji (Altan Corporation, Japan) for helpful discussions and Reiko Yoshimoto and Ryoko Kawabata for their excellent technical support. We also thank the staff of the Analysis Center of Life Science, Hiroshima University for the use of their facilities.

REFERENCES

Akasaka, T., Shimizu-Onda, Y., Hayakawa, S., and Ushijima, H. (2016) The virucidal effects against murine norovirus and feline calcivirus F4 as surrogates for human norovirus by the different additive concentrations of ethanol-based sanitizers. J. Infect. Chemother., 22, 191-193.

Cromeans, T., Park, G. W., Costantini, V., Lee, D., Wang, Q., Farkas, T., Lee, A., and Vinje, J. (2014) Comprehensive comparison of cultivable norovirus surrogates in response to different inactivation and disinfection treatments. Appl. Environ. Microbiol., 80, 5743-5751.

Centers for Disease Control and Prevention (CDC) (2011) Updated norovirus outbreak management and disease prevention guidelines. MMWR Recomm. Rep., 60, 1-18.

Esseili, M. A., Saif, L. J., Farkas, T., and Wang, Q. (2015) Feline calcivirus, murine norovirus, porcine sapovirus, and Tulane virus survival on postharvest lettuce. Appl. Environ. Microbiol., 81, 5085-5092.

Ettayebi, K., Crawford, S. E., Murakami, K., Broughman, J. R., Karandikar, U., Tenge, V. R., Nell, F. H., Blutt, S. E., Zong, X. L., Qu, L., Kou, B., Opekun, A. R., Burnin, D., Graham, D. Y., Ramani, S., Atmar, R. L., and Estes, M. K. (2016) Replication of human noroviruses in stem cell-derived human enteroids. Science, 353, 1387-1393.

Green, K. Y. (2013) Caliciviridae: the noroviruses. Fields Virology, 6th ed. (Knipe, D. M., and Howley, P. M. eds.), 582-608: Lippincott Williams & Wilkins, Philadelphia.

Hoelzer, K., Fansassel, W., Van Doren, J. M., and Dennis, S. (2013) Virus inactivation on hard surfaces or in suspension by chemical disinfectants: systematic review and meta-analysis of norovirus surrogates. J. Food Prot., 76, 1006-1016.

Igimi, S., Noda, M., and Uema, M. (2017) Research report of inactivation conditions of noroviruses 2017. http://www.mhlw.go.jp/file/06- Seisakujouhou-11130500-Shokuhinzenbu/0000125854.pdf (in Japanese).

Irie, T., Shimazu, Y., Yoshida, T., and Sakaguchi, T. (2007) The YLDL sequence within Sendai virus M protein is critical for budding of virus-like particles and interacts with Alix/AIP1 independently of C protein. J. Virol., 81, 2263-2273.

Kniel, K. E. (2014) The makings of a good human norovirus surrogate. Curr. Opin. Virol., 4, 85-90.

Knight, A., Haines, J., Stals, A., Li, D., Uyttendaele, M., Knight, A., and Jaykus, L. A. (2016) A systematic review of human norovirus survival reveals a greater persistence of human norovirus RT-qPCR signals compared to those of cultivable surrogate viruses. Int. J. Food Microbiol., 216, 40-49.

McIlvaine, T. C. (1921) A buffer solution for colorimetric comparison. J. Biol. Chem., 49, 183-186.

Ministry of Health, Labor and Welfare (MHLW) (2017) Sanitary management of large scale cooking facilities manual. http://www.mhlw.go.jp/file/06-Seisakujouhou-11130500-Shokuhinzenbu/0000125826.pdf (in Japanese).

Palansch, M. A., Oberste, M. S., and Whitton, J. L. (2013) Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. Fields Virology, 6th ed. (Knipe, D. M., and Howley, P. M. eds.), 490-530: Lippincott Williams & Wilkins, Philadelphia.
Ueda, K., Kawabata, R., Irie, T., Nakai, Y., Tohya, Y., and Sakaguchi, T. (2013) Inactivation of pathogenic viruses by plant-derived tannins: strong effects of extracts from persimmon (Diospyros kaki) on a broad range of viruses. *PLoS One* 8, e55343.

Zonta, W., Mauroy, A., Farnir, F., and Thiry, E. (2016) Comparative virucidal efficacy of seven disinfectants against murine norovirus and feline calicivirus, surrogates of human norovirus. *Food Environ. Virol.*, 8, 1-12.

Park, G. W., Barclay, L., Macinga, D., Charbonneau, D., Pettigrew, C. A., and Vinje, J. (2010) Comparative efficacy of seven hand sanitizers against murine norovirus, feline calicivirus, and GII.4 norovirus. *J. Food Prot.*, 73, 2232-2238.

Tajima, T., Yoshizaki, S., Nakata, E., Tohya, Y., Ishiguro, S., Fujikawa, Y., and Sugii, S. (1998) Production of a monoclonal antibody reacted broadly with feline calicivirus field isolates. *J. Vet. Med. Sci.*, 60, 155-160.