—Original—

Evaluation of Four Antiseptics Using a Novel Murine Norovirus

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Abstract: We isolated a novel murine norovirus (MNV), MT30-2 strain, from feces of conventional mice in Japan to evaluate the virucidal activity of four antiseptics. The MNV MT30-2 strain was inactivated by as little as 0.2% (w/v) povidone-iodine (PVP-I) and 0.1% (w/v) sodium hypochlorite (NaOCl) treatment as determined by a novel plaque assay. Importantly, PVP-I reduced the MNV titer by 4 log₁₀ within 15 s of exposure. The other two antiseptics, benzethonium chloride (BEC) and chlorhexidine gluconate (CHG), did not reduce the MNV titer even when treatment lasted for 60 s. When the virus titer was reduced by PVP-I or NaOCl treatment, the amount of MNV RNA was not reduced, indicating that the presence of viral RNA was not related to the virucidal activity of the antiseptics. PVP-I and NaOCl will be useful in controlling the spread of MNV, which is a common problem in mice colonies. In this study, we isolated a novel MNV and newly revealed that two antiseptics (PVP-I and NaOCl) were able to inactivate MNV at low concentrations and in a short contact time.

Key words: antiseptics, murine norovirus, plaque assay, quantitative RT-PCR

Introduction

Noroviruses belong to a group of single-stranded RNA, nonenveloped viruses in the Caliciviridae family that are widely transmitted among humans or animals. At present, five major genogroups (GI–GV) of noroviruses have been designated, with GI, GII, and GIV infecting humans, GIII infecting bovines, and GV infecting murines [17]. Murine norovirus (MNV), prototype MNV-1, was first identified in 2003 in immunocompromised mice lacking recombination-activating gene 2

(RAG2), signal transducer, and activator of transcription 1 (STAT1) (RAG2/STAT1^{−/−}) [9]. Human norovirus (HuNoV) cannot grow in cell culture [5]. On the other hand, MNV is the only norovirus capable of replicating in both cell culture and small animals [17, 18]. Infection of MNV in a normal mouse shows no visible signs, but MNV can induce a clinical disease with a high mortality in congenitally immunodeficient mice [13]. The major infection route of MNV is a fecal–oral route, and shedding of MNV continues for at least eight weeks post infection [6–8]. Accordingly, MNV is one of the most

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transmittable and prevalent pathogens in laboratory mice today [6, 8, 12], making the prevention of the spread of MNV an important issue.

In a previous study using the MNV CW1 strain [18], the antiseptic effect of ethanol (EtOH) against MNV was examined [1], and 60% (v/v) EtOH was shown to reduce the MNV infectious titer by 4 log₁₀ within 30 s of exposure; however, 30% (v/v) EtOH did not reduce the titer. Moreover, 1% (w/v) povidone-iodine (PVP-I) and 0.26% (w/v) sodium hypochlorite (NaOCl) have been shown to inactivate the MNV CW1 strain, although the efficacies of these antiseptics against other MNV strains are unknown.

In this study, to clarify the virucidal activity of antiseptics against another MNV strain, we isolated a novel MNV, strain MT30-2, from conventional mice in Japan. Using the MT30-2 strain, the virucidal activity of PVP-I was examined. PVP-I has been used as an external treatment for humans and has a broad spectrum of virucidal activity against enveloped and nonenveloped DNA and RNA viruses [10]. We compared the virucidal activity of PVP-I with those of NaOCl, benzethonium chloride (BEC), and chlorhexidine gluconate (CHG) using plaque assays. Furthermore, we quantified MNV MT30-2 RNA copies by quantitative reverse transcription polymerase chain reaction (RT-PCR) and compared the results with virus titers determined in the plaque assay.

Materials and Methods

Mice

Six-week-old male conventional ICR mice were purchased from a conventional Japanese mouse breeder (Chiba, Japan). The mice were kept in a temperature- and light-controlled environment with standard food and water given *ad libitum*. All experiments were approved by the Animal Care and Use Committee of Meiji Seika Pharma Pharmaceutical Research Center.

Cells and virus infection

RAW264.7 cells, a mouse macrophage cell line, were purchased from DS-Pharma (Osaka, Japan) and cultured in high-glucose Dulbecco's modified Eagle medium + GlutaMAX™-I (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 25 mM HEPES and 10% fetal

bovine serum (FBS) at 37°C in 5% CO₂. MNV was inoculated onto a monolayer of RAW264.7 cells, and cultured in DMEM supplemented with 25 mM HEPES and 2% FBS (DMEM-2% FBS) at 37°C in 5% CO₂. Three to 5 days later, the cultured medium was centrifuged at 15,000 g for 5 min at 4°C, and the supernatant was used as the virus stock.

Isolation of MNV

Two stool samples per mouse were collected, homogenized in PBS, and then centrifuged at 15,000 g for 15 min at room temperature. The supernatant was passed through a 0.22-μm-pore-size filter, diluted in DMEM-2% FBS, and used to inoculate RAW264.7 cells. Four days later, the culture medium and RAW264.7 cells were frozen at -80°C. After thawing, the culture medium and cells were centrifuged at 8,000 g for 5 min at 4°C, and the supernatant was used for further passage. A total of nine passages followed by two successive plaque purifications were performed. Plaque assays were performed as previously described [18] with RAW264.7 cells.

MNV inactivation experiment

Different antiseptics were used to inactivate the MNV. PVP-I (ISODINE solution 10%) was obtained from Meiji Seika Pharma (Tokyo, Japan). NaOCl (Purelox-S) was purchased from Oyalox (Tokyo, Japan). BEC (Hyamine solution 10%) was purchased from Daiichi Sankyo (Tokyo, Japan). CHG (Hibitane gluconate 20%) was purchased from Dainippon Sumitomo Pharma (Osaka, Japan). Inactivation was induced by adding 100 μl of the diluted antiseptic [0.4 or 2% (w/v) PVP-I, 0.2 or 0.4% (w/v) NaOCl, 0.2% (w/v) BEC, 1% (w/v) CHG] to 100 μl of virus stock solution. The final volume of 200 μl reaction mixture was incubated for 15, 30, 45 or 60 s at room temperature prior to neutralization. Neutralization control samples were used as 0-s incubation samples. To stop the effect of PVP-I, 2 μl of 1 M sodium thiosulfate was added to the reaction mixture. To stop the effect of NaOCl, 10 μl of 1 M sodium thiosulfate was added to the reaction mixture, followed by 10 μl of 1 M HEPES (pH 7.4). The neutralized solutions obtained from the above treatment were then 10-fold diluted with DMEM-2% FBS. For the neutralization control, PVP-I and NaOCl were first neutralized with sodium thiosulfate

and diluted with DMEM-2% FBS before being added to the virus stock. To stop the effect of BEC and CHG, the reaction mixtures were 100-fold diluted with DMEM-2% FBS. The neutralization controls for BEC and CHG were prepared by diluting the virus stock with DMEM-2% FBS without adding antiseptics. The virus titer and copy numbers of MNV RNA in the reaction mixture were measured by plaque assays and quantitative RT-PCR, respectively.

RT-PCR

Viral RNA was extracted from the fecal suspension or supernatant using QIAamp Viral RNA Mini Kits (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. An RT-PCR primer pair (forward, 5'-TTTGGAACAATGGATGCTGA-3'; reverse, 5'-TAGGGTGGTACAAGGGCAAC-3') was designed against the conserved capsid sequences of MNV-1 (GenBank accession No. AY228235), MNV-2 (DQ23041), MNV-3 (DQ223042), and MNV-4 (DQ223043). RT-PCR was performed as previously described [7].

Quantitative RT-PCR

Viral RNA was prepared from the reaction mixture using QIAamp Viral RNA Mini Kits. A forward primer (5'-CAGATCACATGCTTCCCACAT-3'), reverse primer (5'-CCAGAGACCACAAAAGACTCATCA-3') and probe (5'-FAM -CCCATCAACTCCCTCTTCTTGA-3'-TAMRA) were designed against the conserved sequences of MNV. Quantitative RT-PCR was performed using an EZ RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) and ABI Prism 7700 Sequence Detection System (Applied Biosystems). The following RT-PCR parameters were used: 50°C for 2 min, 60°C for 30 min, 95°C for 5 min, and then 50 cycles of denaturation (94°C, 15 s), annealing (60°C, 30 s), and extension (72°C, 30 s). The standard curve was generated with serial dilutions of MNV-1 RNA encoding the capsid region synthesized by an AmpliScribe T7 Transcription Kit (Epicentre, Madison, WI, USA).

Sequence analysis of viral RNA

Viral RNA was purified from MNV-infected RAW cells with a Purelink Viral RNA/DNA Kit (Invitrogen), and cDNA was synthesized using SuperScript III reverse

transcriptase (Invitrogen) and an oligo (dT) primer. Genome-specific sequences were amplified with an Expand Long kit (Roche Applied Science, Indianapolis, IN, USA), and PCR products were sequenced directly with a Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) on an ABI 3730XL DNA analyzer using MNV1-specific primers. When these primers failed, additional sequence-specific primers were designed and used for sequencing. The termini of the MNV genomes were obtained using GeneRacer (Invitrogen), and sequencing was carried out with sequence-specific primers. The nucleotide and protein sequences of ORF1, ORF2, or ORF3 were aligned using BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and employed for subsequent phylogenetic analyses. A phylogenetic tree with 1,000 bootstrap replications was constructed by the neighbor-joining method. The distance of nucleotide substitutions per site was calculated by Kimura's two-parameter method and was visualized using the NJplot software (<http://pbil.univ-lyon1.fr/software/njplot.html>). Nucleotide and amino acid sequences were analyzed with GENETYX-MAC version 12.2.6 (Genetyx, Tokyo, Japan).

Nucleotide sequence accession number

The MNV nucleotide sequence determined in this study was deposited in DDBJ under the accession number AB601769.

Results

Isolation and genetic characterization of a novel MNV

To identify MNV-infected mice, fecal samples were collected from eight mice, and RT-PCR was performed using a novel set of primers designed from conserved sites on MNV genomes. The MNV gene was detected in all eight samples (Fig. 1A). A plaque became visible after nine passages with RAW264.7 cells (Fig. 1B). After two rounds of plaque purification, MNV was isolated as a single plaque and designated as MNV MT30-2. The complete nucleotide sequence was determined, and homology searches with other MNV strains showed the MNV MT30-2 strain to be genetically similar to MNV CR3/2005/USA [15] (Fig. 2).

Fig. 1. Detection of MNV gene and MNV plaques. (A) Agarose gel electrophoresis of MNV-specific RT-PCR products (421 bp). (B) Plaques of RAW264.7 cell-adapted MNV.

Table 1. Virucidal effect of PVP-I and NaOCl compared by real-time RT-PCR and plaque assay

Disinfectant	Treatment time (s)	RNA copy no. (log copy no./ml)	Plaque no. (log PFU/ml)
1% (w/v) PVP-I	0	6.69	6.38
	60	6.65	<2.0
0.2% (w/v) NaOCl	0	6.66	6.13
	60	6.30	3.13

Fig. 2. Phylogenetic tree of the Noroviruses. The phylogenetic tree was generated using the neighbor-joining method on the basis of an alignment of the entire amino acid sequence of the capsid genes. Numbers in parentheses indicate GenBank accession Nos.

Virucidal activity of antiseptics against MNV

To determine the efficacy of different antiseptics (PVP-I, NaOCl, BEC, and CHG) against the MT30-2 strain,

we determined the virus titer using a plaque assay after each antiseptic was added to the virus. Both 0.2 and 1% (w/v) PVP-I reduced the titer by 4 log₁₀ within 15 s (Fig. 3A). Both 0.1 and 0.2% (w/v) NaOCl reduced the titer by 1.6 and 3 log₁₀ in 60 s, respectively (Fig. 3B). In contrast, 0.1% (w/v) BEC and 0.5% (w/v) CHG resulted in only a slight reduction in the titer after 60 s of treatment (Fig. 3C). These results indicated that PVP-I and NaOCl efficiently and rapidly inactivated MNV, whereas both BEC and CHG were ineffective against MNV.

Relationship between plaque assay and quantitative RT-PCR

The effectiveness of antiseptics against a noncultivable virus is often measured by quantitative RT-PCR. Thus, we quantified MNV MT30-2 RNA copies in samples treated for 60 s with 1% (w/v) PVP-I or 0.2% (w/v) NaOCl and compared the values with the virus titers. We found the number of MNV MT30-2 RNA copies was only slightly reduced when the virus titer was greatly reduced (Table 1), i.e., the quantitative RT-PCR results did not necessarily reflect the plaque assay results.

Fig. 3. Virucidal activity of antiseptics against MNV. Each point represents the geometric mean titer (log PFU/ml) of two samples. (A) Effect of PVP-I. Solid squares and solid diamonds indicate the 1% (w/v) and 0.2% (w/v) PVP-I-treated groups, respectively. (B) Effect of NaOCl. Open squares and open diamonds indicate the 0.2% (w/v) and 0.1% (w/v) NaOCl-treated groups, respectively. (C) Effect of BEC and CHG. Open circles and open triangles indicate the 0.1% (w/v) BEC and 0.5% (w/v) CHG-treated groups, respectively.

These results coincided with those in a previous report using MNV CW1 [1].

Discussion

In the present study, we described the isolation of a novel MNV, strain MT30-2. Sequence analysis showed that MT30-2 was genetically similar to MNV CR3/2005/USA (Fig. 2). Using the MT30-2 strain, we showed that two antiseptics (PVP-I and NaOCl) were able to inactivate the virus at low concentrations and in a short contact time (Fig. 3A and 3B). The other two antiseptics, BEC and CHG, which showed virucidal activity against several human viruses [10], appeared to be ineffective against MNV (Fig. 3C). PVP-I and NaOCl also showed strong virucidal activity against MNV CW1 [1], a genetically separated strain from our work (Fig. 2), suggesting that these two antiseptics potentially possess virucidal activity against various MNV strains. EtOH also reduced the MNV infectious titer [1]. However, previous reports demonstrated that EtOH did not show virucidal activity over a short time frame against Feline calicivirus (FCV), another antiseptics-resistant virus that, like MNV, belongs to the *Caliciviridae* family [4, 11]. On the other hand, PVP-I and NaOCl are effective for FCV [3, 4, 11, 16]. Accordingly, PVP-I and NaOCl might be effective against not only other MNV strains but also other related noroviruses including HuNoV.

Since the infectivity of HuNoV is very strong, only a few viral particles induce acute gastroenteritis [2, 14]. Because it is thought that MNV also has strong infectivity the virus needs to be inactivated as completely possible to prevent the spread of MNV. PVP-I, which showed strong virucidal activity in a short time frame against MNV, would be useful for preventing MNV transmissions by human hand washing in laboratories.

In this study, we found that the results of quantitative RT-PCR were not consistent with those of the virucidal assay (Table 1), and the detection of viral RNA was not necessarily related to the virus titers, demonstrating the importance of conducting infectivity assays when evaluating the efficacy of antiseptics. The efficacy of antiseptics may be underestimated if quantitative RT-PCR is used for evaluation, as reported in a previous paper [1]. Thus, interpretation of the results of quantitative

RT-PCR needs further consideration.

In conclusion, our data demonstrate that a novel MNV isolate, MT30-2, is a useful tool to evaluate the effectiveness of antiseptics and that PVP-I as well as NaOCl is the most effective antiseptics for disinfection and infection control of MNV.

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