ORIGINAL RESEARCH

Inactivation of Non-Enveloped Viruses and Bacteria by an Electrically Charged Disinfectant Containing Meso-Structure Nanoparticles via Modification of the Genome

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Introduction: A previous study demonstrated the virucidal effect of an electrically charged disinfectant (CAC-717), which contains meso-structure nanoparticles, on enveloped viruses (influenza viruses). However, the effect of CAC-717 on other microorganisms and the mechanisms by which CAC-717 inactivates the microorganisms remain unclear. In this study, CAC-717 was further evaluated in terms of its biocidal and virucidal activity as well as its effect on bacterial and viral nucleic acids.

Methods: The inactivation effects of CAC-717 against various microorganisms [non-enveloped virus, feline calicivirus (FCV); bacteria, *Salmonella enterica* and *Escherichia coli*] were investigated by comparing the viral titer of the medium tissue culture infectious dose ($TCID_{50}$) and the *D* value (estimated treatment time required to reduce the number of microorganisms by 90%). Furthermore, the effects of CAC-717 on viral and bacterial genomic RNA/DNA were examined using a polymerase chain reaction (PCR).

Results: Treatment of an equal volume of CAC-717 with cell lysate infected with a non-enveloped virus, feline calicivirus (FCV), reduced the $TCID_{50}$. Viral titer dropped below the detection limit after 2 min of treatment. The *D* value of FCV was 0.256 min (average of multiple endpoint *D* values) and endpoint *D* value was 0.341 min. The *D* value for *E. coli* and *S. enterica* was 0.290 min and 0.080 min (average of multiple endpoint *D* values), respectively and the endpoint *D* value was 0.545 min and 0.054 min, respectively. In addition, PCR showed the inhibition of nucleic acid amplification of the RNA and DNA genome of FCV and bacteria, respectively.

Conclusion: Our findings suggest that CAC-717 inactivates viruses and bacteria by modifying the viral and bacterial nucleic acids.

Keywords: class I disinfectant, *E. coli*, feline calicivirus, food safety, meso-structure, *Salmonella*

Introduction

The development of the food industry and expansion of distribution channels in recent years has led to many more people consuming foods made in large production facilities. Consequently, the incidence of serious food poisoning involving large numbers of people traced to a single source is becoming more common. For example, ingestion of food contaminated with *Escherichia coli* O157:H7 or other serotypes can lead to life-threatening complications including hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS).[1–4]

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Salmonella enterica causes gastroenteritis and typhoid fever,[5] mostly due to ingestion of contaminated poultry and eggs. As well as a source of food, chicken eggs have been widely used for the production of vaccines in recent years. A technology that can disinfect *Salmonella* without heating is therefore needed. Furthermore, some non-enveloped viruses are commonly associated with food poisoning. For example, norovirus, which is resistant to alcohol disinfectants, is a foodborne agent for infectious gastroenteritis, where oysters and other shellfish are an important vehicle for transmission.[6]

Chlorine bleach (sodium hypochlorite) is generally recommended for the inactivation of a broad range of microorganisms including norovirus.[7,8] However, application of this chemical generates toxic gases that can also act as an irritant and cause metal corrosion. Chemical disinfectants other than chlorine bleach may also be used, although they each have limitations in terms of safety and/or efficiency. For this reason, a safe and effective disinfectant against a broad range of microorganisms is required.

Recently, we generated a meso-structure comprising a fine particle structure of about $50 \sim 500$ nm, primarily composed of carbon and calcium from mineral components derived from plants and soil.[9] An electrically charged disinfectant (CAC-717) was then made by placing this meso-structure in water.[9] CAC-717 has been demonstrated to inactivate enveloped viruses such as influenza viruses.[9] Although free alkaline ions are present in CAC-717, upon contact with human and animal tissue the pH is almost physiological (i.e. neutral pH).[9] Consequently, CAC-717 does not cause irritation in animal eyes.[9] Therefore, by comparison with current chemical disinfectants, such as chlorine bleach, CAC-717 could be a safer alternative. Due to its potential effectiveness, CAC-717 could be useful in reducing the risk of food poisoning in the food and dairy industries. However, it remains unclear whether CAC-717 is effective against a broad range of microorganisms. Moreover, the mechanism(s) of action of CAC-717 need to be established.

In this study, the inactivation effects of CAC-717 against various microorganisms [non-enveloped virus, feline calicivirus (FCV); bacteria, *S. enterica*, and *E. coli*] were investigated. Furthermore, we examined the effects of CAC-717 on viral and bacterial genomic RNA/DNA to gain insight into the likely mechanism of inactivation.

Materials and Methods
Study Design
To enhance food safety, key hygiene control at each step in the processing of food is recommended wherever possible. In particular, the development of safe and efficient disinfectants that can be used to minimize microbiological risk is an essential requirement. These considerations prompted us to design and produce a novel disinfectant CAC-717, which was previously reported to have inactivating activity against influenza virus without irritation and corrosion. Here, we have extended this work by addressing a number of additional issues as follows. Firstly, we aimed to assess the effectiveness of CAC-717 by analyzing the inactivation of FCV and bacteria such as *E. coli* and *S. enterica*. We also investigated changes to the viral and bacterial nucleic acids induced by CAC-717 treatment to elucidate its potential mechanism of viral and bacterial inactivation.

Synthesis of the Electrically Charged Disinfectant (CAC-717)
CAC-717 (FDA/USA Regulation No. 880.6890 Class I disinfectant, Japan Patent No. 5778328)[10,11] was synthesized as described previously.[9] Briefly, mineral water containing calcium hydrogen carbonate was subjected to a continuous electric field at a voltage of 2×10^4 V for 48 h using a Teflon insulation-coated electrostatic field electrode (N-800N, Mineral Activation Technical Research Center, Omuta, Fukuoka, Japan; Japan Patent No. 5864010).[12] The obtained solution was CAC-717 as a 1x solution. The 1x CAC-717 was used in all the assays described in this study. The CAC-717 contains 6.9 mM calcium hydrogen carbonate particles with a mesoscopic structure and a pH of approximately 12.5.[9,13]

Viruses and Bacteria
The FCV F9 strain [VR-782; ATCC (American Type Culture Collection (ATCC), Manassas, VA, USA)], *Escherichia coli* HST04 (Takara Bio Inc., Otsu, Japan), and *S. enterica* serovar Abony NCTC 6017 (Microbiologics Inc., St Cloud, MN, USA) were used as test microorganisms.

Preparation of Bacterial Suspensions
E. coli on Luria-Bertani (LB) agar medium (Nacalai Tesque Inc., Kyoto, Japan) incubated at 37ºC for 24 h or *S. enterica* in buffered peptone water (Merk & Co., Kenilworth, NJ, USA) incubated at 35ºC for 24 h were collected and suspended in 1 mL of distilled water to obtain a bacterial suspension.

Preparation of FCV-Infected Cell Lysate

Crandell-Rees feline kidney-cell (CRFK) cells (CCL-94; ATCC) were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FCS) (JRH Biosciences Inc., Saint Louis, MO, USA). Penicillin-streptomycin (PS) was added to MEM as 100 units/mL of penicillin, 100 μg/mL of streptomycin (Nakalai Tesque Inc.). CRFK cells (3×10^5 cells) were grown in 100 mm dishes and adsorbed with FCV at a MOI (multiplicity of infection) of 0.1 for 1 h. The medium was exchanged with 2% FCS and PS-supplemented MEM medium and cultured at 37°C under 5% CO_2 for 3 days. The cultured FCV–infected cells were suspended in phosphate buffered saline (PBS) (Life Technologies, Carlsbad, CA, USA), centrifugally washed three times, and then freeze-thawed at −80°C. Thereafter, the collected cells were disrupted by passage through a 28 G injection needle and suspended in PBS to prepare an FCV–infected cell lysate.

Evaluation of CAC-717 Efficiency Against Viruses and Bacteria

A 10 μL aliquot of microorganism suspension (FCV, *E. coli*, or *S. enterica*) was mixed with 10 μL of 1× CAC-717. The mixture was incubated at 25°C for 0, 0.5, 1, 2, 5 or 10 min. Samples were quickly diluted with 1 mL of distilled water for *S. enterica* and *E. coli* or with 400 μL of PBS for FCV. Then, FCV, *S. enterica* and *E. coli* were subjected to various assays described below.

Counting of Viable Bacterial Number

The number of colony forming units (CFU) was calculated after culturing for 24 h at 37°C using LB agar medium for *E. coli*[14] or culturing for 48 h at 35°C using sheet medium (Sanita-kun; JNC Corporation, Tokyo, Japan) for *S. enterica* in accordance with the supplier's instructions.[15]

Scanning Electron Microcopy (SEM)

CAC-717 was treated with 2% glutaraldehyde/0.1 M phosphate buffer for at least 14 h at 4°C followed by treatment with 2% osmium tetroxide solution for 2 h, and then dehydration with 30–100% ethanol at room temperature. The samples were subsequently subjected to drying with *tert*-butyl alcohol before depositing a coat of osmium plasma. SEM was performed using a JSM-7500F electron microscope (JEOL Ltd., Tokyo, Japan) at 5 kV and ×100,000 magnification.

Calculation of the Viral Titer of FCV

Samples were diluted 10-fold with PBS and added to CRFK cells (7.5×10^3 cells/well) seeded on a 96-well microtiter plate. Cells were then cultivated at 37°C under 5% CO_2 for 3 days. A cytopathic effect (CPE) was observed and $TCID_{50}$ (median tissue culture infectious dose) was calculated from the CPE based on the Behrens-Kärber method.[16]

DNA Extraction of Bacteria and Polymerase Chain Reaction (PCR)

Bacterial DNA of *E. coli* and *S. enterica* was extracted by heat treatment at 95 ° C for 15 min. Bacterial 16S rDNA PCR kit fast (800) (Takara Bio Inc.) was used for amplification of *E. coli* 16S rDNA according to the manufacturer's guidelines,[17] while One shot PCR (Takara Bio Inc.) was used to amplify the invasion protein gene (*invA*) of *S. enterica* as described previously.[18] After DNA gel electrophoresis, band intensities were calculated using image data analysis software (ImageJ version 1.52a; National Institute of Health, Bethesda, MD, USA). Band intensities were determined as an average field intensity value after adjustment of images to a standard threshold value. The band intensity at 0 min in *E. coli* or *S. enterica* was set to 100% and comparison made with the corresponding bands at 0.5, 1, 2, 5, and 10 min.

Viral RNA Extraction of FCV and Real-Time PCR

Viral RNA of FCV was extracted using a QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted into 60 μL of nuclease-free water and transcribed with PrimeScriptII 1st strand cDNA Synthesis kit (Takara Bio Inc.) using random primers to generate the cDNA. The following temperature regime was employed: 65°C for 5 min; 4°C for 5 min; 42°C for 60 min. The resultant cDNA was analyzed by real-time PCR using SYBR Premix Ex Taq II (Tli RNase H plus) (Takara Bio Inc.) according to the manufacturer's instructions. The real-time PCR components included SYBR Premix Ex Taq II as well as forward and reverse gene primers targeted the open reading frame 1 (ORF1) region of FCV (83 bp) encoding the nonstructural protein of FCV (GenbankM86379). The primers had the following sequences: Forward primer (FCV-F1): 5′-TAATTCG GTGTTTGATTTGGCCTGGGCT-3′; Reverse primer

(FCV-R1): 5′-CATATGCGGCTCTGATGGCTTGAAAC
TG-3′. Real-time PCR was performed using a Thermal
Cycler Dice Real Time System (Takara Bio Inc.) as
described previously.[19] The cycling program included
initial denaturation at 95°C for 30 sec followed by 40
cycles of 95°C for 30 sec and 60°C for 30 sec. Each
reaction was carried out more than three times and the
results were analyzed using Thermal Cycler Dice
Realtime System Single software (Takara Bio Inc.).
The relative DNA levels of each sample were compared
with serially diluted viral cDNA and estimated using
the standard curve of diluted viral cDNA versus absor-
bance. PCR specificity was verified by dissociation
curve analysis of the amplified DNA fragments of step 1
(95°C/15 sec), step 2 (60°C/30 sec), and step 3
(95°C/15 sec).

DNA Sequencing

The amplified products obtained by PCR or real-time PCR
were verified by direct DNA sequencing after gel extraction
using a QIAquick Gel Extraction Kit (Qiagen) or after sub-
cloning into Takara T-Vector pMD20 (Takara Bio Inc.),
respectively. Sequencing was performed on an ABI373OXL
Genetic Analyzer (Applied Biosystems, Foster City,
CA, USA).

Indirect Immunofluorescence Assay

Detection of FCV in FCV–infected CRFK cells was per-
formed by indirect immunofluorescence assays. After
incubation with samples for 1 day, cells were fixed with
4% paraformaldehyde for 20 min and cold methanol for 10
min. Cells were blocked with 3% bovine serum albumin
(BSA) for 30 min and incubated with anti-FCV antibody
(ab33990 [FCV1-43]; Abcam, Cambridge, UK) at 1:100
dilution in PBS for 1 h at 37°C. Cells were subsequently
labeled with fluorescein isothiocyanate (FITC)-labeled
donkey anti-mouse IgG (Jackson ImmunoResearch
Laboratories, Inc., West Grove, PA, USA) at 1:200 dilu-
tion by PBS for 30 min at 37°C. The stained cell mono-
layer was visualized at a magnification of x20 under
fluorescence microscopy (Biozero BZ-8100; Keyence,
Osaka, Japan) with excitation of 480/30 nm and emission
of 510 nm. Exposure times were held constant at 4 sec
throughout the entire experiment. Quantification was per-
formed using ImageJ version 1.52a software (National
Institute of Health). Fluorescent images were adjusted to
a standard threshold value. Green intensities in the image
were given an average field intensity value. The intensity

at 0 min was taken as 100% and then compared with that
at 1, 2, and 5 min.

Calculation of the Average of Multiple Endpoint D Values

The endpoint was considered the first point at which all
replicates showed no growth (<10 CFU/mL or <10
$TCID_{50}$/mL). The D value was calculated according to
the treatment time required to lower the number of micro-
organisms to 1/10. The following formula, which is mod-
ified from a previous report,[20] was used to calculate the
D value.

$$D \text{ value} = \Delta\log_{10} N / \Delta t$$

where $\Delta\log_{10}N$: logarithm of the change in the number of
microorganisms, Δt: change in time, N: number of
microorganisms.

Endpoint D values were calculated using the same
formula for the D value between the endpoint and 0 min.
The average of multiple endpoint D values was calculated
using the same formula for endpoint D value between each
time point and 0 min, and then an average was taken.[20]

Statistical Analysis

All data were collected and assembled in an Excel spread-
sheet for statistical analysis. The statistical analysis of
significant difference was performed by non-repeated mea-
sured analysis of variance (ANOVA) followed by
Bonferroni correction. The analysis was carried out using
the GraphPad Prism 7 software (GraphPad Prism Software
Inc., La Jolla, CA, USA). The obtained results are the
mean ± standard error of mean (SEM) of experiments
conducted at least in triplicate.

Results

Firstly, SEM analysis of CAC-717 was performed, which
showed it to contain aggregated nanoparticles (approximately
50 nm in size) (Figure 1). The change of FCV infectivity after
treatment with an equal volume of CAC-717 was also inves-
tigated. A 10 μL aliquot of virus-infected cell lysate was mixed
with 10 μL of CAC-717 for 0, 1, 2, or 5 min (Figure 2). After
treatment, the suspension was recovered with 400 μL of PBS
and the $TCID_{50}$ determined. The results showed that the initial
viral titer of FCV (0 min) was $7.26 \times 10^5 \pm 2.70 \times 10^5$
$TCID_{50}$/mL. CAC-717 treatment caused a significant decrease
in viral titer: $1.09 \times 10^2 \pm 1.04 \times 10^2$ $TCID_{50}$/mL at 1 min; below
the detection limit (less than 10 $TCID_{50}$/mL) at 2 and 5 min.
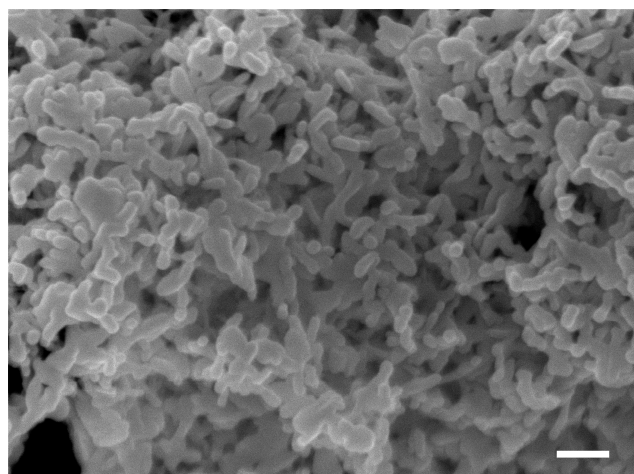From the data, the D value, which is the time required to

Figure 1 Electron microscopic image of the meso-structure in CAC-717 After treatment with osmium tetroxide, CAC-717 was subjected to scanning electron microscopy (SEM) analysis (JSM-7500F; JEOL Ltd., Tokyo, Japan). The scale bar indicates 100 nm.
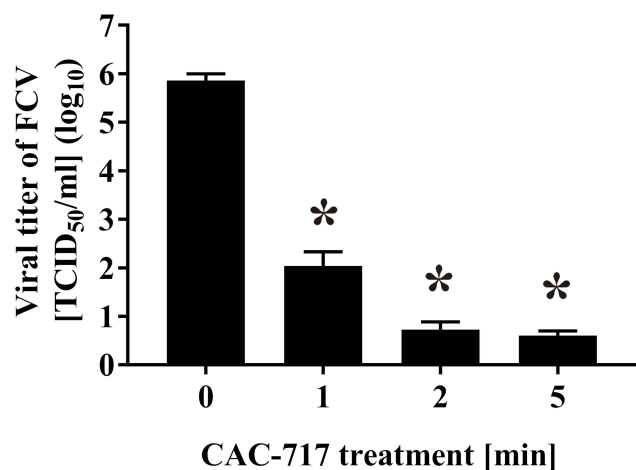

Figure 3 Immunofluorescent assay showed a reduction of FCV infectivity after CAC-717 treatment. The FCV–infected cell lysate was subjected to CAC-717 treatment for the indicated time (min) at 25°C. Recovered lysate was added to Candell-Rees feline kidney (CRFK) cells and after culturing for 1 day the cells were fixed with glutaraldehyde and analyzed by an immunofluorescent assay using anti-FCV antibody. Uninfected control (0 min) was included. Phase contrast images and the results of the immunofluorescent assay for FCV (Green) (exposure time 4 sec) are shown. The scale bars indicate 100 μm.

Figure 2 Treatment with CAC-717 reduced the viral titer of feline calicivirus (FCV). The FCV–infected cell lysate was exposed to an equal volume of CAC-717 for the indicated time (min) at 25°C. Viral titer of FCV [$TCID_{50}$ (median tissue culture infectious dose)/mL] was calculated after CAC-717 treatment as described in Materials and Methods. Zero for virus titer means below the detectable limit. Differences where *p<0.05 versus control (0 min) were considered significant when verified by the non-repeated measured analysis of variance (ANOVA) followed by the Bonferroni correction using GraphPad Prism7 software (GraphPad Prism Software Inc., La Jolla, CA, USA).

achieve 90% reduction of viral titer, was calculated. The average of multiple endpoint D values was used to estimate the D value as described in Materials and Methods, which was 0.256 min, while the endpoint D value was 0.341 min.

Furthermore, an immunofluorescent assay using anti-FCV antibody against FCV capsid protein was performed after incubation of CRFK cells with CAC-717-treated FCV (Figure 3). Proliferation of FCV in CRFK cells was observed after incubation with untreated cell lysate (0 min) for 24 h. However, reduced FCV proliferation was observed in
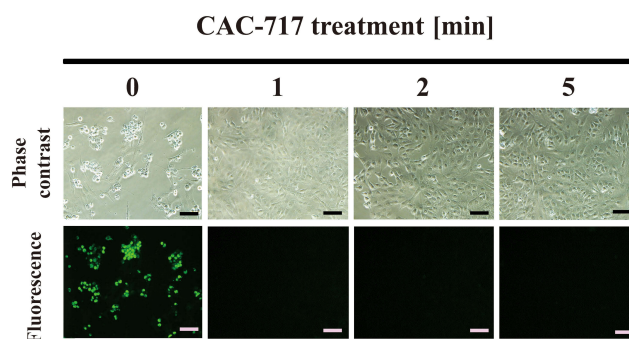
CRFK cells after incubation with cell lysate exposed to CAC-717 treatment in a treatment time-dependent manner. The average field intensity value in the green fluorescent image at 0 min was set to 100%, while those at 1, 2, and 5 min were 0.17%, 0.08%, and 0.01%, respectively. These findings suggested that CAC-717 treatment decreased infectivity of FCV.

In addition, the effect of CAC-717 on viable cell number of bacteria, such as *E. coli* and *S. enterica*, was examined (Figure 4). Viable cell number after CAC-717 treatment was determined as described in Materials and Methods. The viable cell number of *E. coli* at 0 min was $1.52×10^9 ± 0.35×10^9$ CFU/mL, but there was a significant decrease to $4.00×10^8 ± 1.58×10^8$ CFU/mL at 1 min, $7.50×10^6 ± 2.50×10^6$ CFU/mL at 2 min, and undetectable at 5 min (Figure 4A). Similarly, a bactericidal effect of CAC-717 on *S. enterica* was evaluated. Viable cell number of *S. enterica* was $2.14×10^7 ± 0.12×10^7$ CFU/mL at 0 min, but $2.00×10^5 ± 0.58×10^5$ CFU/mL at 1 min, and undetectable at 2 and 5 min (Figure 4B). Overall, the average of the multiple endpoint D values for *E. coli* and *S. enterica* were 0.290 min and 0.080 min, respectively. In addition, the endpoint D values for *E. coli* and *S. enterica* were 0.545 min and 0.054 min, respectively.

Moreover, the effect of CAC-717 treatment on DNA of *E. coli* and *S. enterica* was examined. PCR was used to amplify *E. coli* 16S rDNA and *S. enterica* invA to assess DNA damage caused by CAC-717 treatment for 0, 0.5, 1, 2, 5, and 10 min (Figure 5). The amplified band of *E. coli* 16S rDNA (800 bp) and *S. enterica* invA DNA (378 bp) was slightly less intense at 0.5 min and 1 min compared to 0 min and markedly less intense at 2, 5, and 10 min. Analysis using
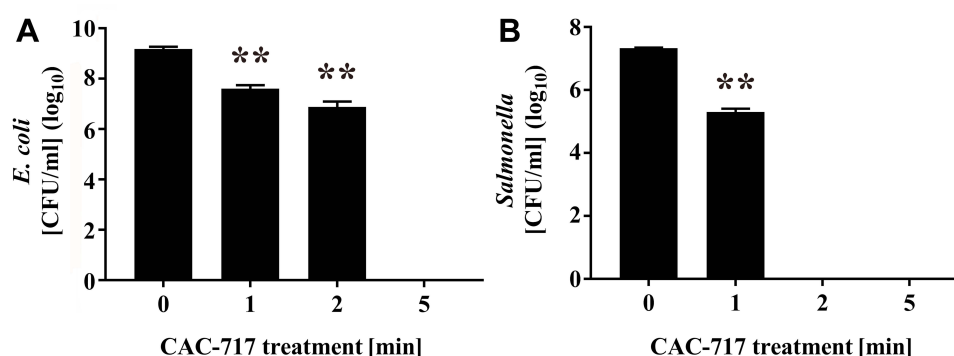
Figure 4 Decrease in viable numbers of *Escherichia coli* and *Salmonella enterica* after CAC-717 treatment. An aliquot of *E. coli* or *S. enterica* culture was mixed with an equal volume of CAC-717 and incubated for the indicated time at 25°C. After treatment, *E. coli* or *S. enterica* was resuspended in distilled water and spread on LB (Luria-Bertani) agar medium for *E. coli* or sheet medium (Sanita-kun; JNC Corporation, Tokyo, Japan) for *S. enterica*. The bacteria on the medium were then cultured for 1 day at 37°C for *E. coli* and 2 days at 35°C for *S. enterica*. The number of colonies on each plate were then counted. Colony forming units (CFU) per mL of (**A**) *E. coli* and (**B**) *S. enterica* treated with CAC-717 for the indicated time at 25°C are shown. Differences where **$p<0.01$ versus control (0 min) were considered significant when verified by the non-repeated measured ANOVA followed by the Bonferroni correction using GraphPad Prism7 software. Zero $\log_{10}$ CFU/mL means below the detectable limit.
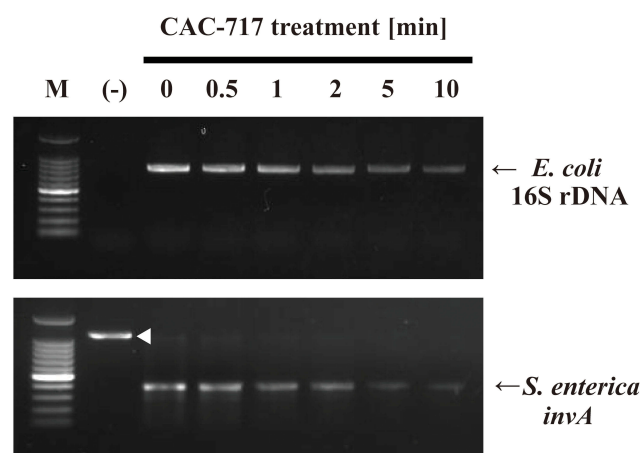

Figure 5 Damage to the bacterial genomic DNA caused by CAC-717 treatment. *E. coli* and *S. enterica* were subjected to CAC-717 treatment for the indicated time (min) at 25°C. Extracted genomic DNA was then analyzed to compare the levels of intact genomic DNA by polymerase chain reaction (PCR) using Takara bacteria 16S rDNA PCR kit Fast(800) and One shot PCR for *Salmonella* as described in Materials and Methods. An arrowhead indicates the internal control, while arrows indicate the amplified PCR products for 16S rDNA in *E. coli* and *invA* in *S. enterica*, respectively.

ImageJ software supported the results, which showed that band intensities were 100% at 0 min, 95.74% at 0.5 min, 90.04% at 1 min, 65.77% at 2 min, 31.38% at 5 min, and 4.13% at 10 min in *E. coli*. The corresponding values for *S. enterica* were 100% at 0 min, 99.90% at 0.5 min, 44.46% at 1 min, 38.75% at 2 min, 0% at 5 min, and 0% at 10 min. In addition, the obtained band of *E. coli* 16S rDNA was gel extracted and direct sequence analysis performed. As a result, the obtained DNA sequence of *E. coli* 16S rDNA was 99% identical (*N*=4) to that of *E. coli* strain HST 04, complete genome (ie identical to Genbank Accession number CP 013952), indicating that the amplified PCR product

was derived from *E. coli* DNA. The 378-bp PCR product from *S. enterica* using the same PCR kit was previously confirmed to correspond to *invA* of *Salmonella enterica* subsp. *enterica* (identical to Genbank accession number EU348369) by DNA sequencing.[18]

To further investigate the effect of CAC-717 treatment on virus, biochemical changes to the viral genome of FCV were analyzed (Figure 6). Real-time PCR using sequence-specific primers for FCV showed the detection of intact FCV RNA by PCR amplification in the untreated FCV sample (0 min). Dissociation curve analysis of the reaction products confirmed the specificity of the reaction. Reduced levels of intact FCV
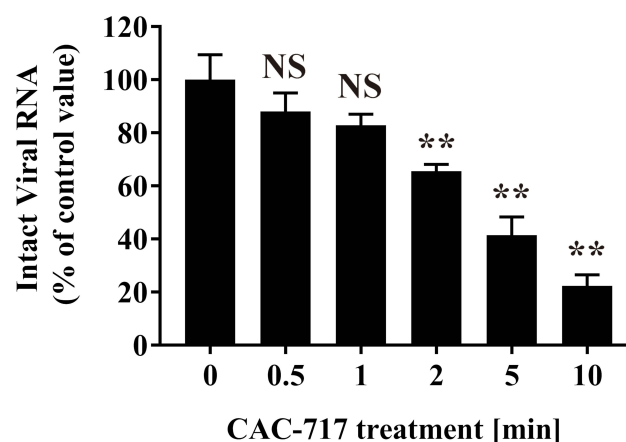

Figure 6 Damage to FCV genomic RNA caused by CAC-717 treatment. FCV–infected cell lysate was subjected to CAC-717 treatment for the indicated time (min) at 25°C. Extracted viral RNA was then analyzed to compare the levels of intact viral RNA by real-time PCR using primers for FCV as described in Materials and Methods. Differences where **$p<0.01$ versus control (0 min) were considered significant when verified by the non-repeated measured ANOVA followed by the Bonferroni correction using GraphPad Prism7 software. NS: no significant difference compared with control (0 min).

RNA were found in CAC-717 treated FCV for 0.5 and 1 min by comparison with the untreated FCV ($100.00 \pm 9.45\%$ at 0 min; $88.09 \pm 6.92\%$ at 0.5 min; $82.89 \pm 4.11\%$ at 1 min), but the difference was not significant. However, a significant decrease was observed in intact FCV RNA after CAC-717 treatment for 2 min ($65.56 \pm 2.55\%$), 5 min ($41.41 \pm 4.00\%$), and 10 min ($22.35 \pm 2.42\%$).

Discussion

A previous study showed that influenza virus could be inactivated by CAC-717.[9] The present results show that CAC-717 efficiently inactivates both *E. coli* and *S. enterica* as well as FCV. The findings from this study together with the previous study[9] indicate that CAC-717 might effectively inactivate a broad range of microorganisms. Moreover, during the submission process for the present manuscript, CAC-717 was shown to inactivate mouse norovirus and human norovirus.[13]

FCV infectivity after CAC-717 treatment for 1 min decreased by more than 3 $\log_{10}$, satisfying the criteria listed by the US Environmental Protection Agency (US-EPA) for a virucidal agent (at least $\log_{10}$ reduction of FCV viral titer).[21] Therefore, CAC-717 treatment is considered to be an effective virucidal method. The average of multiple endpoint D values for FCV after CAC-717 treatment was 0.256 min. The inactivation efficiency of FCV by CAC-717 was good even in comparison to sodium hypochlorite (675 ppm), where the D value after treatment at room temperature was 1.563 min[22] and with heat treatment at 56°C was 6.7 min.[23] This observation further confirms that CAC-717 is an efficient method for the inactivation of FCV, which is a non-enveloped virus. These findings are consistent with a recently published study using a non-enveloped virus, mouse norovirus and human norovirus, which were efficiently inactivated by CAC-717.[13] In addition, previous studies showed that the infectious titer of influenza virus, which is an enveloped virus, decreased after treatment with CAC-717.[9] Taken together, these results suggest that the inactivating effect of CAC-717 on viruses is unaffected by the presence or absence of an envelope.

In the case of *Salmonella*, the average of multiple endpoint D values with sodium hypochlorite (4 ppm) when used as a fungicide for chicken eggs mixed with *Salmonella enterica* was 0.195 min.[24] However, CAC-717, with an average of multiple endpoint D values of 0.080 min, exerts a more powerful bactericidal effect than sodium hypochlorite.

Moreover, CAC-717 treatment causes damage to the FCV genomic RNA as well as to bacterial genomic DNA of *E. coli* and *S. enterica*. These findings infer that damage to the viral and bacterial genome, such as the mutation of nucleic acids, is involved in the reduction of infectivity. Furthermore, during submission of the present manuscript, the effect of CAC-717 on human norovirus RNA has been reported,[13] which supports the overall conclusion of this study.

CAC-717 contains minerals forming a mesoscopic structure. The structure of the mineral elicits a bactericidal effect by electrolysis via self-discharge and hydrogen occlusion.[9] Indeed, this characteristic may be related to the emission and absorption of terahertz waves when minerals extracted from plants and soil components possess a mesoscopic structure. Far-infrared radiation from mesoscopic structures appear to function as small "nanobatteries".[9] The terahertz radiation is thought to be absorbed by biomolecules such as proteins and carbohydrates as well as nucleic acids. The far-infrared radiation seems to, at least in part, contribute to the inactivation mechanisms of CAC-717. However, the precise damage to the nucleic acids as well as other biomolecules remains to be determined.

The mechanisms of action of CAC-717 are apparently different from any antibiotics, suggesting that CAC-717 may be effective in inactivating antibiotic resistant strains of harmful bacteria.[25–28] However, further studies using various antibiotic-resistant bacteria would be required to confirm the effect of CAC-717 on these strains.

Bacterial and fungal toxins, such as Shiga toxins and aflatoxins, are known to cause food poisoning. If CAC-717 is effective at inactivating these toxins it could be used for cleaning and toxin removal as well as for disinfection. Consequently, CAC-717 could be a novel advanced disinfectant that can simultaneously inactivate pathogens and remove their associated toxins. However, further studies on CAC-717 are required to fully understand the inactivation mechanisms and evaluate the usefulness of CAC-717.

Previous observations using influenza virus indicated that the addition of 10% BSA to CAC-717 had no effect on viral inactivation.[9] The effect of CAC-717 treatment on FCV in the presence of serum was further examined (Fig. S1). FCS (JRH Biosciences Inc., Saint Louis, MO, USA) at a concentration of 0%, 5%, 10%, 20%, 50% was mixed with FCV–infected cell suspension and subjected to CAC-717 treatment. After the treatment, infectivity was unchanged among 0-50% FCS in PBS (Fig. S1A) and CAC-717 (Fig. S1B). Thus, the FCV–inactivating effect of CAC-717

was maintained even in the presence of substantial amounts of serum proteins.

By using CAC-717, safe disinfection without irritation and corrosion may be possible. As CAC-717 is derived from plants and soils, safety of this disinfectant is thought to be high. Indeed, our previous study involved a toxicity test of CAC-717 using Organisation for Economic Co-operation and Development (OECD) guidelines for testing chemicals No.405; test for acute eye irritation[29] and tests based on International Organization for Standardization (ISO).[30,31] None of these tests using CAC-717 indicated any harmful effects. These findings suggest CAC-717 could make a valuable contribution as a safe and efficient disinfectant.

Conclusion

The present study suggests that CAC-717 can efficiently inactivate bacteria and non-enveloped viruses. In addition to an inactivation effect on enveloped viruses as shown in a previous study, CAC-717 might potentially be useful for the inactivation of a broad range of harmful bacteria and viruses. Furthermore, the current study shows CAC-717 damages nucleic acids of microorganisms. Optimizing the conditions of reaction and production of CAC-717 will inevitably enhance the efficiency of this disinfectant.

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Disclosure

K.F. and R.O. are employed by the Mineral Activation Technical Research Center and Santa Mineral Co., Ltd., respectively. The authors declare that they have no other conflicts of interest with the content of this article.

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