Food and Environmental Virology (2019) 11:238–246
https://doi.org/10.1007/s12560-019-09381-5

ORIGINAL PAPER

Co-culture with *Enterobacter cloacae* does not Enhance Virus Resistance to Thermal and Chemical Treatments

Wenjun Deng[1] · Giselle Almeida[1] · Kristen E. Gibson[1]

Received: 21 October 2018 / Accepted: 15 March 2019 / Published online: 26 March 2019

Abstract

Human noroviruses (hNoV) are the primary cause of foodborne disease in the USA. Most studies on inactivation kinetics of hNoV and its surrogates are performed in monoculture, while the microbial ecosystem effect on virus inactivation remains limited. This study investigated the persistence of hNoV surrogates, murine norovirus (MNV) and Tulane virus (TuV), along with Aichi virus (AiV) under thermal and chemical inactivation in association with Gram-negative (*Enterobacter cloacae*) bacteria. Thermal inactivation of viruses in co-culture with *E. cloacae* revealed no protective effects of bacteria. At 56 °C, AiV with and without bacteria was completely inactivated by 10 min with decimal reduction values (*D*-values) of 41 and 43 s, respectively. Similar results were also observed for TuV. Conversely, MNV with bacteria was completely inactivated by 10 min while MNV alone remained stable up to 30 min at 56 °C. Both MNV and TuV were slightly more stable than AiV at 63 °C with TuV detection up to 2 min without bacteria. For chemical inactivation on stainless steel surfaces, viruses alone and in association with bacteria were treated with 1000 ppm sodium hypochlorite. Virus association with bacteria had no significant effect ($p > 0.05$) on virus resistance to bleach inactivation compared to virus alone. Specifically, exposure to 1000 ppm bleach for 5 min resulted in an average of 3.86, 2.14, and 0.94 $\log_{10}$ PFU/ml reductions for TuV, MNV, and AiV without bacteria, respectively. Reductions in TuV, MNV, and AiV were 3.50, 1.88, and 0.61 log10 PFU/ml when associated with *E. cloacae*, respectively.

Keywords Norovirus · Tulane virus · Murine norovirus · Thermal inactivation · Sodium hypochlorite · Surface

Introduction

Human noroviruses (hNoVs) are single-strand RNA viruses that cause acute gastroenteritis in humans (Ahmed et al. 2014). As a genus in the family *Caliciviridae*, noroviruses are further divided into at least seven genogroups (GI–GVII) among which GI, GII and GIV are infectious to humans (Chhabra et al. 2018). According to the World Health Organization (WHO), globally hNoV contributed to an estimated 120 million out of 550 million diarrheal illnesses in 2010 (Havelaar et al. 2015). Since its discovery in 1972, the study of hNoVs have been hindered by the lack of an animal model or cell culture system. Therefore, researchers use surrogates such as feline calicivirus (FCV), Tulane viruses (TuV), and murine norovirus (MNV) to elucidate the mechanism of replication, persistence, and inactivation of hNoVs (Wobus et al. 2006; Doultree et al. 1999; Tian et al. 2013). Briefly, FCV, TuV, and MNV are also within the *Caliciviridae* family and belong to the genus *Vesivirus, Recovirus*, and *Norovirus*, respectively. Recently, Jones et al. (2014) successful cultivated hNoV in human B cells with the promotion of *Enterobacter cloacae* as well as other enteric bacteria. Later, Ettayebi et al. (2016) reported that the cultivation model of stem cell-derived human enteroids supported hNoV growth when supplemented with bile. Interestingly, the authors did not observe the same benefits of a bacterial co-factor as was reported by Jones et al. (2014). Additional studies with other human enteric viruses have yielded results similar to Jones et al. (2014) (Kuss et al. 2011; Robinson et al. 2014). Through the attachment to certain types of polysaccharide on bacteria surfaces, poliovirus showed increased virion heat stability, bleach resistance, and binding efficiency to host cell virus receptors (Robinson et al. 2014). These initial

✉ Kristen E. Gibson
keg005@uark.edu

[1] Division of Agriculture, Department of Food Science, University of Arkansas, 2650 N Young Ave, Fayetteville, AR 72704, USA

findings have led to an increase in investigations on the interactions of human enteric viruses and bacteria.

Recently, several studies have investigated the interaction between hNoV and bacteria. Miura et al. (2013) reported that human norovirus-like particles (hNoVLP) could bind to the histo-blood group antigen (HBGA)-like substances in the extracellular polymeric substances (EPS) of *Enterobacter* sp. SENG-6. The HBGA is one of hNoV binding receptors, which presents on cellular surfaces such as intestinal epithelial cells or in secreted biological fluids in human (Harrington et al. 2004). HBGA contains ABH and Lewis antigens, and different genotypes of hNoV specifically recognize certain pattern profiles (Miura et al. 2013). Some hNoV surrogates can also bind to HBGAs. For instance, TuV can recognize type-A and type-B HBGAs as well as sialic acids while MNV and FCV only recognize the latter (Farkas et al. 2010; Li et al. 2017; Tan et al. 2015; Zhang et al. 2015). After Miura et al. (2013), Li et al. (2015) further reported the binding of hNoVLP to 11 HBGA-expressing bacteria and investigated the protective effect of these bacteria when heat stresses were applied to hNoVLPs. As a result, the hNoVLP showed better antigen integrity and mucin-binding ability with the presence of HBGA-expressing *Escherichia coli* during thermal treatment compared to non-HBGA expressing bacteria. Based on their report, another group of researchers compared the effect of HBGA-expressing and non-HBGA expressing *E. coli* on TuV replication under heat denaturation. However, in this study, the viral replication showed no significant difference regardless of the HBGA expression status of the bacteria (Li et al. 2017). In addition to HBGA binding, other potential types of interaction between hNoV and bacteria remain undetermined. Almand et al. (2017) characterized the binding specificity and efficiency of hNoV with select gut microbiota and found that hNoV bound to all tested bacteria with high efficiency while the surrogate TuV bound to only some of the gut bacteria. Since the authors did not further investigate the binding mechanisms, it is unclear whether the association of hNoV and bacteria was via HBGA interactions or not.

The present study focused on understanding the effect of bacteria on hNoV surrogates under inactivation procedures. The inactivation of hNoV includes traditional methods such as heat, chemical disinfection, pH, UV radiation as well as emerging new techniques such as high hydrostatic pressure (Cromeans et al. 2014). The inactivation effects of these methods on hNoV, and its surrogates have been well documented (Cromeans et al. 2014; Hoelzer et al. 2013; Kingsley et al. 2017). However, it is now clear that the association of hNoV and bacteria could certainly impact the effectiveness of applied inactivation strategies (Li et al. 2015; Robinson et al. 2014). In reality, bacteria and viruses can be both present in a given environment, especially within food processing and preparation environments. Therefore, in the present study, the effect of bacteria on the resistance of hNoV surrogates (TuV, MNV) and Aichi virus (AiV)—a cultivable human enteric viruses—under thermal and chemical (i.e., bleach) inactivation was evaluated.

Materials and Methods

Virus Production, Propagation, and Titration

TuV was kindly provided by Dr. Jason Jiang (Cincinnati Children's Hospital Medical Center, Cincinnati, OH). The viruses were produced in LLC-MK2 cells (ATCC CCL-7; American Type Culture Collection, Manassas, VA) which were grown in M199 medium (Corning, Manassas, VA) supplemented with 10% Fetal Bovine Serum (FBS), 1% Penicillin–Streptomycin and 1% Amphotericin B. TuV infection of MK2 cells occurred at 5% CO_2 and 37 °C with rocking for 1 h at a multiplicity of infection (MOI) of 0.1. After the initial hour, infected cells were covered with 2% maintenance media composed of Opti-MEM (Gibco Life Technology, Paisley, Scotland, UK) supplemented with 2% FBS, 1% penicillin–streptomycin and 1% Amphotericin B and further incubated for 5 days. After complete cytopathic effect (CPE), the infected cells were frozen and thawed three times followed by centrifugation at 3000×*g* 4 °C for 15 min. Finally, viruses were filtered by 0.22 µm filter (VWR, Radnor, MA). For titration, 2 ml of MK2 cells were seeded in six-well plates at a concentration of 8×10^5 cells/well and incubated overnight. Five-hundred microliter of tenfold serial diluted samples were added to each well and rocked at 5% CO_2 and 37 °C for 1 h. After removing samples, 2 ml of 3% low melting temperature agarose (VWR, Rockland, ME) was added to the cell monolayer in each well. Plates were then incubated at 5% CO_2 and 37 °C for 3 days. Two milliliters of 0.01% neutral red (Sigma–Aldrich, St. Louis, MO) diluted in 1 × phosphate-buffered saline (PBS) were added to each well; then, plates were further incubated at 5% CO_2 and 37 °C for 1 h. After removing the staining solution, plaques were counted under light.

Aichi virus was kindly provided by Dr. Pierre Pothier at Dijon University Hospital in Dijon, France. Viruses cultured in Vero cells (ATCC CCL-81) that were grown in Minimum Essential Medium (Corning, Manassas, VA) supplemented with 10% FBS, 1% penicillin–streptomycin and 1% non-essential amino acids (NEAA) solution. Aichi virus was propagated at 5% CO_2 and 37 °C with continuous rocking for 3 h at a MOI of 0.1 and further incubated for 3 days. After complete CPE, cells were frozen and thawed three times followed by centrifugation at 3000×*g* for 30 min at 4 °C. In the end, viruses were filtered by 0.22-µm filter. For AiV titration, Vero cells were seeded in six-well plates at a concentration of 2×10^6 cells/well and

incubated overnight. Prepared plates were inoculated with AiV stock, and after 3 h infection, cell monolayers were covered by 2 ml of 1.5% agarose (IBI Scientific, Peosta, IA). Following a 3-day incubation, another layer of 2 ml 1.5% agarose with 0.01% neutral red was added for staining. After 1 h of incubation at 5% CO_2 and 37 °C, plaques were counted under light.

MNV type 1 was kindly provided by Dr. Kellogg Schwab (Johns Hopkins Bloomberg School of Public Health, Baltimore, MD). MNV was propagated in RAW 264.7 (mouse leukemic monocyte macrophage cell line, ATCC TIB-71) that were cultured in Dulbecco modified Eagle medium (Corning, Manassas, VA) supplemented with 10% FBS, 1% penicillin–streptomycin, 1% L-glutamine, 1% NEAA and 1% HEPES. The infection of MNV was at 5% CO_2 37 °C rocking for 1 h with MOI of 0.1 and further incubated for 3 days. After complete CPE, cells were frozen and thawed three times followed by centrifugation at 5000×*g* for 20 min at 4 °C. In the end, viruses were filtered by 0.22 µm filter. For MNV titration, RAW cells were seeded with 2×10^6 cells per well. After 1 h infection, cell monolayers were covered by the agarose overlay (1:1 ratio mixture of 6% low melting temperature agarose and overlay medium). The overlay medium was 2 × MEM supplemented with 10% FBS, 1% penicillin–streptomycin, 1% HEPES and 13% DI water. After 2-day incubation, plates were stained and visualized as described for TuV.

Bacteria Cultivation

Enterobacter cloacae (ATCC 13047) stock at − 80 °C were streaked on Nutrient Agar (Difco, Sparks, MA) plates and incubated at 30 °C for 24 h. From agar plate, a single bacterial colony was selected, inoculated in 50 ml centrifuge tubes containing 10 ml Nutrient Broth medium, and incubated overnight in a shaker at 200 rpm and 30 °C.

Thermal Inactivation in Water Bath

E. cloacae was cultured overnight, and 0.4 ml were normalized to an optical density at 600 nm (OD_{600}) of 0.4 (~ 10^9 CFU/ml). Bacteria were then centrifuged at 8000×*g* for 5 min and washed twice with PBS. Viruses diluted in PBS (10^5 PFU/ml) were added to the bacterial pellet (0.4 ml) at a volume ratio 1:1 and mixed by vortexing. The virus–bacteria mixtures were then exposed to heat stress in a water bath at testing temperatures of 56, 63, and 72 °C. At each time point (0, 0.5, 1, 5, 10, and 30 min), samples were taken out and placed on ice followed by virus titration. Virus only groups were also subjected to the same treatments in parallel.

Bleach Inactivation on Stainless Steel Surface

The concentration of overnight cultured *E. cloacae* was diluted to approximately10^5 cfu/ml. An aliquot of 200 µl bacteria was washed twice with PBS by centrifugation at 10,000×*g* for 15 min. After the second wash, bacterial pellets were resuspended with 200 µl virus stock at 10^6 pfu/ml concentration. Bacteria and virus were allowed to associate for 1 h at room temperature. Three bacteria–virus mixture combinations (i.e., TuV + EC, MNV + EC, and AiV + EC) were obtained for each experimental replication. Bleach at 1000 ppm concentration (pH 7.5) was prepared by diluting commercially available household-use Clorox (active ingredient 8.25% sodium hypochlorite) in 0.1M sodium phosphate buffer. A neutralizer solution (MEM with 0.1% sodium thiosulfate and 10% FBS) was also prepared as described previously (Arthur and Gibson 2015; Cromeans et al. 2014).

A stainless steel (SS) surface was pretreated with 0.1% Tween 80, 70% ethanol and deionized water, then autoclaved. In a biological safety cabinet, 50 µl of each bacteria-virus mixture was pipetted on the SS surface to air-dry for approximately 30 min. The treatment groups were then covered with 100 µl of 1000 ppm bleach, while the negative control group received 100 µl of neutralizer solution. After a 5-min contact time, treatment and negative control groups received 300 µl of neutralizer solution. To inactivate any remaining bacteria, 100 µl of 11 mg/ml imipenem (Sigma–Aldrich, St. Louis, MO) were pipetted into each sample. Preliminary experiments were carried out to confirm that bacteria can be completely inactivated by imipenem. Bacteria at 10^5 CFU/ml was the highest concentration that could be used in order to be fully inactivated by 100 µl of 11 mg/ml imipenem (data not shown). Additionally, neutralization of the active chlorine with the neutralizer solution was confirmed using active chlorine test strips for sanitizers and disinfectants (Bartovation, New York, NY). The samples were then recovered from the SS surface by cell scraper (VWR, Radnor, MA) and pipetting multiple times. In parallel, the same treatments were applied to virus-only samples. Immediately following experiments, virus concentrations were determined by plaque assays as described for virus stock titration.

Statistical Analysis

In this study, a random complete block design (RCBD) was used for experimental design. To analyze the effect of bacteria presence and treatment time length on different virus survival under thermal treatment, a three-way ANOVA was performed in JMP Pro 14 (SAS Institute, Cary, NC). The

bacterial effect on viruses under bleach treatment was analyzed with one-way ANOVA. $P < 0.05$ was considered as statistically significant.

Results

Thermal Inactivation of Viruses with *E. cloacae* Present

The thermal inactivation of TuV, MNV, and AiV with *E. cloacae* (EC) present was measured at 56, 63, and 72 °C. The *D*-values of virus only and virus + EC groups at 56, 63 and 72 °C are summarized in Table 1. At 56 °C, the presence of *E. cloacae* did not increase the heat resistance of tested viruses (Fig. 1). At several time points, virus alone survived even better than virus + EC groups. Specifically, TuV showed significantly higher heat resistance than TuV + EC at 0.5 and 1 min ($p < 0.05$). Similarly, MNV alone survived better than MNV + EC at 5 and 10 min ($p < 0.05$), and AiV survived better than AiV + EC ($p < 0.05$) at 1 min. At the other time points, the virus alone group did not show any significant difference compared to the corresponding EC present groups. Comparatively, among three surrogates, AiV was overall more heat sensitive than MNV and TuV. At 0.5 and 1 min, TuV showed the highest heat resistance. However, there were no significant differences between TuV and MNV at 5 min and after. Notably, MNV survived and remained infective even at 10 min (2 $\log_{10}$ PFU/ml), whereas other viruses were undetectable.

When exposed to 63 °C (Fig. 2), AiV was inactivated within 0.5 min (not shown in Fig. 2) while MNV and TuV survived ≥ 1 min. Similar to what was observed at 56 °C, virus alone groups still showed better heat resistance than virus + EC at 63 °C. At 0.5, 1 and 2 min, the survival of TuV alone was significantly higher than TuV + EC. Also, TuV was the only detectable virus at 2 min. At 72 °C, MNV, MNV + EC, AiV, AiV + EC and TuV + EC were all under the detection limit within 0.5 min. Only 1 $\log_{10}$ PFU/ml of TuV survived at 0.5 min (data not shown).

Bleach Inactivation of Viruses on Stainless Steel (SS) Surface in the Presence of *E. cloacae*

The investigation of the bacterial effect on the resistance of viruses under 1000 ppm chlorine bleach inactivation was carried out on stainless steel (SS) surface (Fig. 3). To avoid bacterial interference on the plaque assay, bacteria starting concentrations were set at 10^5 CFU/ml to allow a complete inactivation by imipenem (antibiotic). Overall, the presence of *E. cloacae* showed neither significant enhancement nor decrease on the survival of viruses. The reductions of virus and virus + bacteria groups were at very similar levels. Regardless of bacteria, AiV showed the highest chlorine bleach resistance among the tested viruses, with only 0.94 $\log_{10}$ PFU/ml reduction after a 5-min exposure to 1000 ppm chlorine bleach while TuV was the most susceptible with 3.86 $\log_{10}$ PFU/ml reduction in infectious virus.

Discussion

After the initial reports on the binding of hNoV to enteric bacteria, questions regarding the role of bacteria in hNoV inactivation arose (Almand et al. 2017; Li et al. 2015; Miura et al. 2013). Heating is an important and traditional way of food processing for inactivation of pathogens, while bleach at 1000 ppm is the recommended minimum concentration for hNoV inactivation on environmental surfaces (Barclay et al. 2014). Our study demonstrated the survival of hNoV surrogates in the presence of gram-negative bacterium *E. cloacae*. In contrast to our initial hypothesis, the results indicated that the bacteria–virus co-culture did not enhance the survival of surrogates under heat (56, 63, and 72 °C) and bleach treatment.

Virus heat inactivation studies in the presence of bacteria have also been reported previously. Li et al. (2017) studied the protective effect of HBGA-expressing *E. coli* O86:H2 and non-HBGA-expressing *E. coli* K-12 on TuV under heat-denaturation at 56 °C. The authors showed that TuV was able to bind to both types of *E. coli* strains through different binding effects; however, neither of the bindings

Table 1 *D*-values of virus alone and virus + *E. cloacae* exposed to varying temperatures

Temperature (°C)	Decimal reduction values (min)					
	AiV		MNV		TuV	
	Virus only	*E. cloacae*	Virus only	*E. cloacae*	Virus only	*E. cloacae*
56	2.38 ± 0.03	2.45 ± 0.03	7.62 ± 0.03*	2.53 ± 0.01*	1.84 ± 0.01	2.19 ± 0.02
63	–	–	0.41 ± 0.01	0.44 ± 0.01	1.24 ± 0.02*	0.41 ± 0.01*
72	–	–	–	–	0.18 ± 0.01*	0.09 ± 0.01*

AiV Aichi virus, *MNV* murine norovirus, *TuV* Tulane virus

*Indicates there is a significant difference in *D*-values between virus and virus + bacteria groups, statistical comparison used student *t* test ($p < 0.05$)

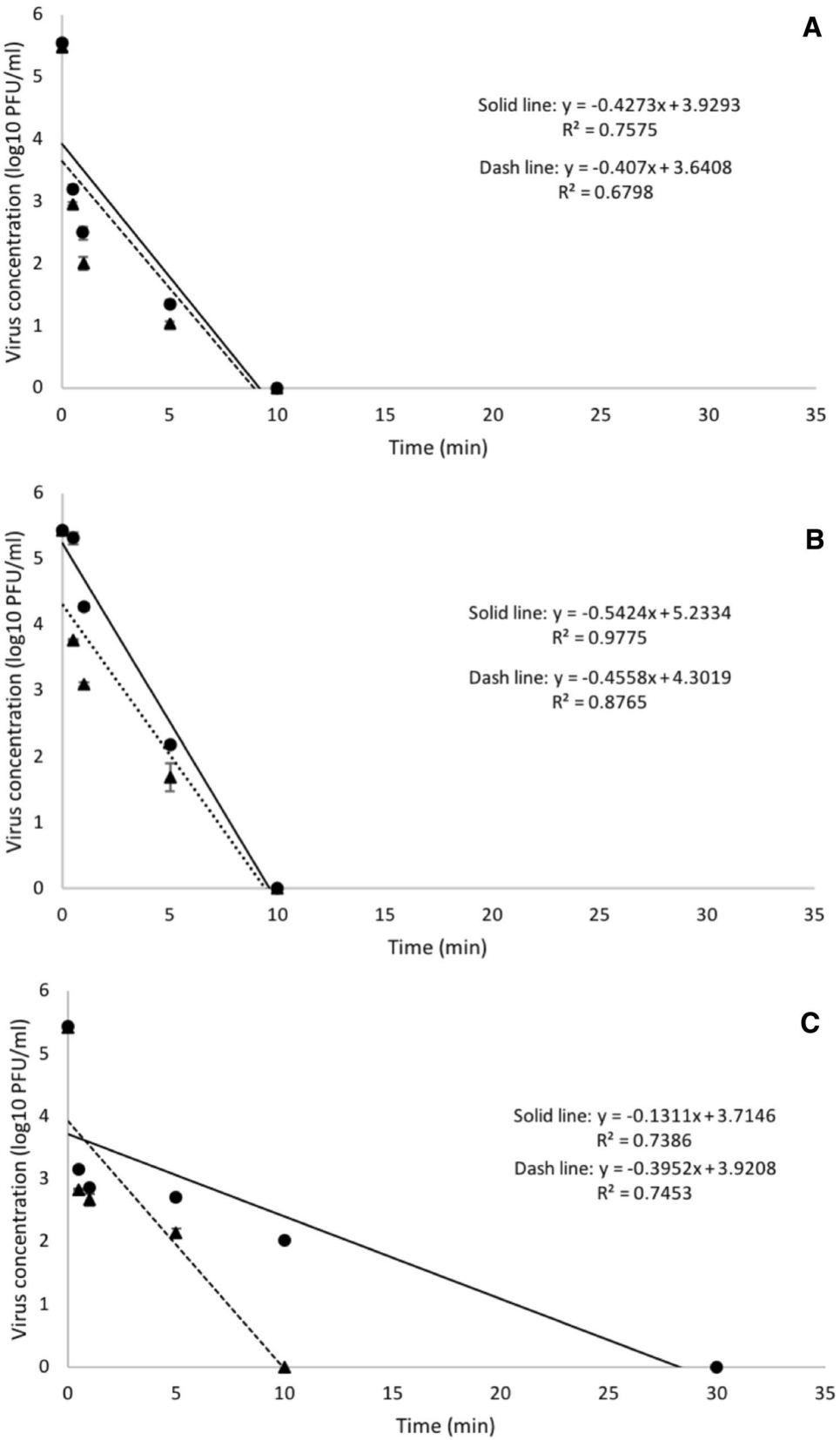

Fig. 1 Thermal stability of viruses alone and in co-culture with *E. cloacae* at 56 °C. Scatter plots indicate mean value of virus concentration for AiV (**a**), TuV (**b**) and MNV (**c**) under conditions with virus alone (round, solid line) versus virus in co-culture with *E. cloacae* (triangle, dash line) at different time points. Regression lines were fitted, and regression equations are presented. The co-cultures of virus and bacteria were in 1:10,000 ratio. Each error bar is constructed using 1 standard deviation from the mean

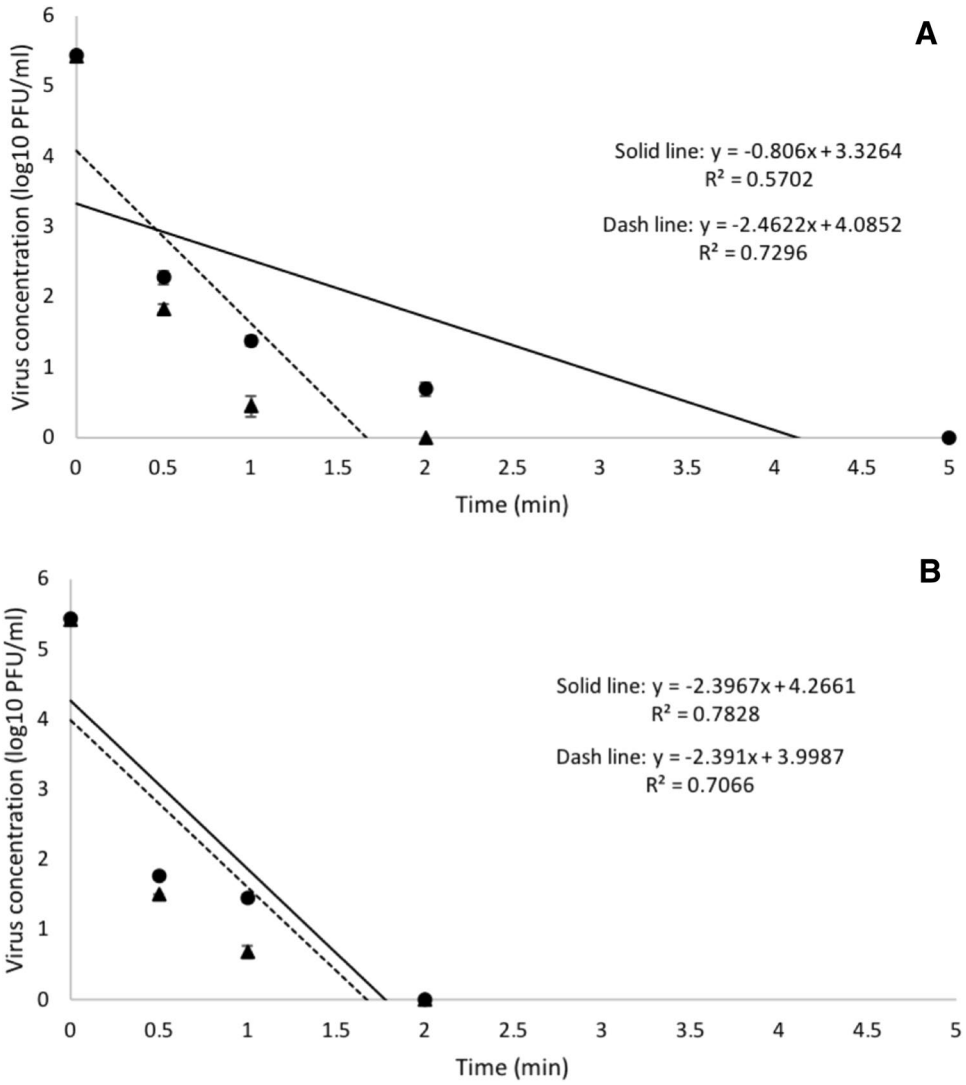

Fig. 2 Thermal stability of viruses alone and in co-culture with E. cloacae at 63 °C. Scatter plots indicate mean value of virus concentration for TuV (**a**) and MNV (**b**) under conditions with virus alone (round, solid line) versus virus in co-culture with *E. cloacae* (triangle, dash line) at different time points. Regression lines were fitted, and regression equations are presented. The co-cultures of virus and bacteria were in 1:10,000 ratio. Each error bar is constructed using one standard deviation from the mean

conveyed protection to TuV when compared to TuV alone. In contrast, Li et al. (2015) reported that incubation with HBGA-expressing *E. coli* significantly protected the immunoreactivity of hNoVLP GI.1 and GII.4 after exposure to heat at 90 °C compared to that of VLP alone or when incubated with non-HBGA expressing bacteria. Unfortunately, the inherent differences between surrogates and VLP as well as the distinct experimental setups do not allow for direct comparison. In fact, the comparison among different studies has always been difficult to make due to differences in experimental designs or manipulations. For instance, the present study showed > 5 $\log_{10}$ PFU/ml reduction of TuV after 10 min at 56 °C which is in accordance with the findings of Cromeans et al. (2014). Meanwhile, Tian et al. (2013) reported 3.5 $\log_{10}$ PFU/ml reduction of TuV infectivity. The latter two studies used $TCID_{50}$ assay for virus quantification.

Interestingly, the virus + bacteria groups during heat inactivation (56 and 63 °C) showed either slightly lower or significantly lower virus concentrations than virus alone groups at all tested time points. One explanation is that bacteria presence changes the susceptibility of the host cell to viruses and thereby affected virus quantification results. The report from Li et al. (2017) demonstrated that incubation with bacteria reduced the replication and/or attachment of TuV 48 h post-infection in LLC-MK2 cells ($p < 0.05$). Another study by Bellinghausen et al. (2016) indicated that certain inactivated bacteria cells could affect the host cell immune responses and could actually induce the immune response against a subsequent viral infection. However, Bellinghausen and co-authors did not observe this bacteria-induced enhancement on the host immune system for all tested bacteria types. In contrast, our preliminary study on virus and *E. cloacae* cell components (i.e., bacterial cell lysate) indicated that the presence of bacteria cell components led to very little effect on virus quantification.

The bleach inactivation experiments evaluated the effect of *E. cloacae* on virus survival during exposure to 1000 ppm chlorine bleach on SS surface. The virus and bacteria

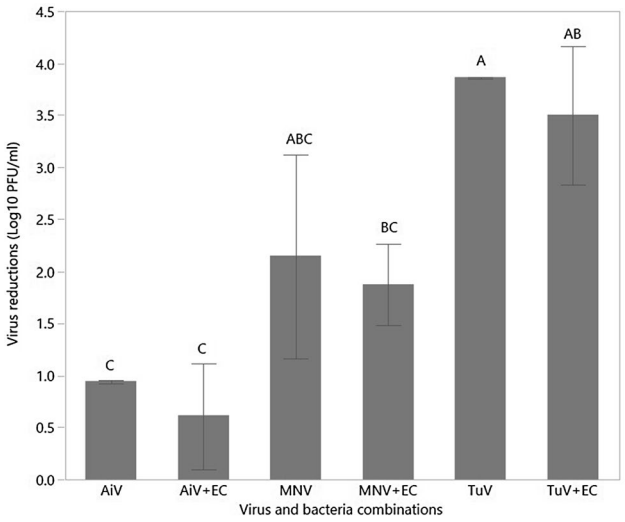

Fig. 3 Bleach inactivation of hNoV surrogates alone and in co-culture with *E. cloacae*. The *x*-axis indicates the experimental conditions, and *y*-axis indicates the $\log_{10}$ reduction of virus on a stainless steel surface after treatment with 1000 ppm bleach for 5 min. The suspensions of virus and bacteria were in 10:1 ratio and allowed to associate for 1 h at room temperature prior to inoculation of the SS surface. EC stands for *E. cloacae*. Each error bar is constructed using 1 standard deviation from the mean. Different letters (A–C) indicate significant differences ($p < 0.05$) among virus log reductions

mixtures were dried on stainless steel surface before bleach treatment to more closely mimic a food processing or preparation environment. Chlorine bleach is a commonly used disinfectant for elimination of hNoV in public and institutional settings along with food preparation environments, and it has been intensively characterized for its effectiveness against hNoV and its surrogates (Diggs et al. 2008; D'Souza and Su 2010). However, it is unclear whether bleach could still effectively inactivate hNoV with bacteria present, considering the complicity of binding activity between virus and bacteria (Almand et al. 2017; Miura et al. 2013). In this study, the co-culture with bacteria did not significantly affect the resistance of viruses, but rather resulted in large variations in virus reduction. For instance, for TuV and AiV, the bleach inactivation of pure viruses on surfaces was consistent across replications, whereas virus reductions in association with bacteria demonstrated large standard deviations. It is possible that the presence of bacteria—though inactivated by antibiotics—could negatively affect the plaque assay; however, preliminary work did not indicate any deleterious effects. Besides *E. cloacae*, additional preliminary experiments also investigated a gram-positive bacterium *Bacillus cereus* in bleach inactivation; however, no significant effect was observed (data not shown).

While the most appropriate variables and study design were considered, several potential limitations of the present study have been identified. First, the bacteria and virus co-culture were not further characterized with respect to the specific binding activities. We did not determine how many viruses were binding to bacteria by heat inactivation, and moreover, whether heat promoted more bindings or broke the existing interaction between virus and bacteria. Second, the design of the bleach inactivation experiments on surfaces did not consider the matrix such as food residuals which could lead to different results. For instance, as reported by Yamaoka et al. (2016), an environment rich in organic matter significantly decreased the effectiveness of sodium chlorite on the inactivation of tested foodborne pathogens including hNoV surrogates such as FCV. Similarly, the thermal inactivation experiment suspension did not include any nutrients or food matrix either. Besides, gram-negative bacteria were selected in our study since the sialylated lipopolysaccharides on gram-negative bacteria surface are commonly involved in hNoV binding (Almand et al. 2017). In fact, both gram-positive and gram-negative bacteria can provide binding sites for hNoV. Future investigation should involve more bacteria types, especially gram-positive bacteria into the experiment design. Moreover, in reality the bacteria present in the food or environment likely exist at much lower concentrations as well as including a greater variety of bacterial genus and species which would result in different virus–bacteria interactions. Lastly, though bacteria–virus associations were indirectly observed, we did not further characterize the specific binding sites and mechanisms. It is therefore not clear which and how many types of binding occurred during this study.

Despite the close genetic relatedness with hNoV, the surrogates of hNoV vary in their biological, biophysical, and biochemical characteristics (Cromeans et al. 2014). Though not belonging to the Norovirus genus, TuV sequence is closely related to hNoV GII, and it is structurally more similar than MNV to hNoV (Hirneisen and Kniel 2013). Nevertheless, the binding activity between surrogate and bacteria do not completely mimic the behavior of hNoV based on current research. For instance, some of the ligands that TuV recognize overlap with hNoV but not all (Drouaz et al. 2015). Almand et al. (2017) reported the binding of GI and GII hNoV to ten selected gut microorganisms, whereas TuV bound to only five of them. With respect to AiV, it has been reported that the frequency and abundance of AiV can be higher than other enteric viruses making it potentially a good indicator of viral contamination in a given environment (Kitajima and Gerba 2015). In the present study, AiV showed the highest sensitivity to heat and the lowest to bleach which is in accordance with previous reports (Cromeans et al. 2014; Kitajima and Gerba 2015).

Acknowledgements This material is based upon work that is supported by the Arkansas Biosciences Institute with the Grant received by author Gibson (2015–2018). This work was also supported in part

by the National Institute of Food and Agriculture (NIFA), U.S. Department of Agriculture (USDA), Hatch Act Funding.

References

Ahmed, S. M., Hall, A. J., Robinson, A. E., Verhoef, L., Premkumar, P., Parashar, U. D., et al. (2014). Global prevalence of norovirus in cases of gastroenteritis: A systematic review and meta-analysis. *The Lancet Infectious Diseases, 14*(8), 725–730. https://doi.org/10.1016/S1473-3099(14)70767-4.

Almand, E. A., Moore, M. D., Outlaw, J., & Jaykus, L.-A. (2017). Human norovirus binding to select bacteria representative of the human gut microbiota. *PLoS ONE, 12*(3), e0173124. https://doi.org/10.1371/journal.pone.0173124.

Arthur, S. E., & Gibson, K. E. (2015). Physiochemical stability profile of Tulane virus: A human norovirus surrogate. *Journal of Applied Microbiology, 119*(3), 868–875. https://doi.org/10.1111/jam.12878.

Barclay, L., Park, G. W., Vega, E., Hall, A., Parashar, U., Vinjé, J., & Lopman, B. (2014). Infection control for norovirus. *Clinical Microbiology and Infection, 20*(8), 731–740. https://doi.org/10.1111/1469-0691.12674.

Bellinghausen, C., Gulraiz, F., Heinzmann, A. C. A., Dentener, M. A., Savelkoul, P. H. M., Wouters, E. F., et al. (2016). Exposure to common respiratory bacteria alters the airway epithelial response to subsequent viral infection. *Respiratory Research, 17*(1), 68. https://doi.org/10.1186/s12931-016-0382-z.

Chhabra, P., Aswath, K., Collins, N., Ahmed, T., Olórtegui, M. P., Kosek, M., et al. (2018). Near-complete genome sequences of several new norovirus genogroup II genotypes. *Genome Announcements*. https://doi.org/10.1128/genomeA.00007-18.

Cromeans, T., Park, G. W., Costantini, V., Lee, D., Wang, Q., Farkas, T., et al. (2014). Comprehensive comparison of cultivable norovirus surrogates in response to different inactivation and disinfection treatments. *Applied and Environmental Microbiology, 80*(18), 5743–5751. https://doi.org/10.1128/AEM.01532-14.

D'Souza, D. H., & Su, X. (2010). Efficacy of chemical treatments against murine norovirus, feline calicivirus, and MS2 bacteriophage. *Foodborne Pathogens and Disease, 7*(3), 319–326. https://doi.org/10.1089/fpd.2009.0426.

Diggs, R., Diallo, A., Kan, H., Glymph, C., Furness, W., B., & Chai, J., S (2008). Norovirus outbreak in an elementary school—District of Columbia, February 2007. *JAMA The Journal of the American Medical Association, 299*, 627–630.

Doultree, J. C., Druce, J. D., Birch, C. J., Bowden, D. S., & Marshall, J. A. (1999). Inactivation of feline calicivirus, a Norwalk virus surrogate. *The Journal of Hospital Infection, 41*(1), 51–57.

Drouaz, N., Schaeffer, J., Farkas, T., Pendu, J. L., & Guyader, F. S. L. (2015). Tulane virus as a potential surrogate to mimic norovirus behavior in oysters. *Applied and Environmental Microbiology, 81*(15), 5249–5256. https://doi.org/10.1128/AEM.01067-15.

Ettayebi, K., Crawford, S. E., Murakami, K., Broughman, J. R., Karandikar, U., Tenge, V. R., et al. (2016). Replication of human noroviruses in stem cell-derived human enteroids. *Science, 353*(6306), 1387–1393. https://doi.org/10.1126/science.aaf5211.

Farkas, T., Cross, R. W., Hargitt, E., Lerche, N. W., Morrow, A. L., & Sestak, K. (2010). Genetic diversity and histo-blood group antigen interactions of rhesus enteric caliciviruses. *Journal of Virology, 84*(17), 8617–8625. https://doi.org/10.1128/JVI.00630-10.

Harrington, P. R., Vinjé, J., Moe, C. L., & Baric, R. S. (2004). Norovirus capture with histo-blood group antigens reveals novel virus-ligand interactions. *Journal of Virology, 78*(6), 3035–3045.

Havelaar, A. H., Kirk, M. D., Torgerson, P. R., Gibb, H. J., Hald, T., Lake, R. J., et al. (2015). World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. *PLOS Medicine, 12*(12), e1001923. https://doi.org/10.1371/journal.pmed.1001923.

Hirneisen, K. A., & Kniel, K. E. (2013). Comparing human norovirus surrogates: Murine norovirus and Tulane virus. *Journal of Food Protection, 76*(1), 139–143. https://doi.org/10.4315/0362-028X.JFP-12-216.

Hoelzer, K., Fanaselle, W., Pouillot, R., Van Doren, J. M., & Dennis, S. (2013). Virus inactivation on hard surfaces or in suspension by chemical disinfectants: Systematic review and meta-analysis of norovirus surrogates. *Journal of Food Protection, 76*(6), 1006–1016. https://doi.org/10.4315/0362-028X.JFP-12-438.

Jones, M. K., Watanabe, M., Zhu, S., Graves, C. L., Keyes, L. R., Grau, K. R., et al. (2014). Enteric bacteria promote human and mouse norovirus infection of B cells. *Science, 346*(6210), 755–759. https://doi.org/10.1126/science.1257147.

Kingsley, D. H., Fay, J. P., Calci, K., Pouillot, R., Woods, J., Chen, H., et al. (2017). Evaluation of chlorine treatment levels for inactivation of human norovirus and MS2 bacteriophage during sewage treatment. *Applied and Environmental Microbiology, 83*(23), e01270–e01217. https://doi.org/10.1128/AEM.01270-17.

Kitajima, M., & Gerba, C. P. (2015). Aichi virus 1: Environmental occurrence and behavior. *Pathogens, 4*(2), 256–268. https://doi.org/10.3390/pathogens4020256.

Kuss, S. K., Best, G. T., Etheredge, C. A., Pruijssers, A. J., Frierson, J. M., Hooper, L. V., et al. (2011). Intestinal microbiota promote enteric virus replication and systemic pathogenesis. *Science, 334*(6053), 249–252. https://doi.org/10.1126/science.1211057.

Li, D., Breiman, A., le Pendu, J., & Uyttendaele, M. (2015). Binding to histo-blood group antigen-expressing bacteria protects human norovirus from acute heat stress. *Frontiers in Microbiology. 6*. https://doi.org/10.3389/fmicb.2015.00659.

Li, Q., Wang, D., Yang, D., Shan, L., & Tian, P. (2017). Binding of *Escherichia coli* does not protect tulane virus from heat-inactivation regardless the expression of HBGA-like molecules. *Frontiers in Microbiology, 8*, 1746. https://doi.org/10.3389/fmicb.2017.01746.

Miura, T., Sano, D., Suenaga, A., Yoshimura, T., Fuzawa, M., Nakagomi, T., et al. (2013). Histo-blood group antigen-like substances of human enteric bacteria as specific adsorbents for human noroviruses. *Journal of Virology, 87*(17), 9441–9451. https://doi.org/10.1128/JVI.01060-13.

Robinson, C. M., Jesudhasan, P. R., & Pfeiffer, J. K. (2014). Bacterial lipopolysaccharide binding enhances virion stability and promotes environmental fitness of an enteric virus. *Cell Host & Microbe, 15*(1), 36–46. https://doi.org/10.1016/j.chom.2013.12.004.

Tan, M., Wei, C., Huang, P., Fan, Q., Quigley, C., Xia, M., et al. (2015). Tulane virus recognizes sialic acids as cellular receptors. *Scientific Reports, 5*, 11784. https://doi.org/10.1038/srep11784.

Tian, P., Yang, D., Quigley, C., Chou, M., & Jiang, X. (2013). Inactivation of the Tulane virus, a novel surrogate for the human norovirus. *Journal of Food Protection, 76*(4), 712–718. https://doi.org/10.4315/0362-028X.JFP-12-361.

Wobus, C. E., Thackray, L. B., & Virgin, H. W. (2006). Murine norovirus: A model system to study norovirus biology and pathogenesis. *Journal of Virology, 80*(11), 5104–5112. https://doi.org/10.1128/JVI.02346-05.

Yamaoka, H., Nakayama-Imaohji, H., Horiuchi, I., Yamasaki, H., Nagao, T., Fujita, Y., et al. (2016). Tetramethylbenzidine method for monitoring the free available chlorine and microbicidal activity of chlorite-based sanitizers under organic-matter-rich environments. *Letters in Applied Microbiology, 62*(1), 47–54. https://doi.org/10.1111/lam.12506.

Zhang, D., Huang, P., Zou, L., Lowary, T. L., Tan, M., & Jiang, X. (2015). Tulane virus recognizes the A type 3 and B histo-blood group antigens. *Journal of Virology, 89*(2), 1419–1427. https://doi.org/10.1128/JVI.02595-14.