AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

PUBLIC AND ENVIRONMENTAL HEALTH MICROBIOLOGY

Evaluation of Chlorine Treatment Levels for Inactivation of Human Norovirus and MS2 Bacteriophage during Sewage Treatment

David H. Kingsley,[a] Johnna P. Fay,[b]* Kevin Calci,[c] Régis Pouillot,[d] Jacquelina Woods,[c] Haiqiang Chen,[b] Brendan A. Niemira,[e] Jane M. Van Doren[d]

U.S. Department of Agriculture, Agricultural Research Service, Food Safety and Intervention Technologies Research Unit, Delaware State University, Dover, Delaware, USA[a]; Department of Animal and Food Sciences, University of Delaware, Newark, Delaware, USA[b]; Food and Drug Administration, Center for Food Safety and Applied Nutrition, Gulf Coast Seafood Laboratory, Dauphin Island, Alabama, USA[c]; Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Analytics and Outreach, Division of Risk and Decision Analysis, Risk Analysis Branch, College Park, Maryland, USA[d]; U.S. Department of Agriculture, Agricultural Research Service, Food Safety and Intervention Technologies Research Unit, Eastern Regional Research Center, Wyndmoor, Pennsylvania, USA[e]

ABSTRACT This study examined the inactivation of human norovirus (HuNoV) GI.1 and GII.4 by chlorine under conditions mimicking sewage treatment. Using a porcine gastric mucin-magnetic bead (PGM-MB) assay, no statistically significant loss in HuNoV binding (inactivation) was observed for secondary effluent treatments of ≤25 ppm total chlorine; for both strains, 50 and 100 ppm treatments resulted in ≤0.8-$\log_{10}$ unit and ≥3.9-$\log_{10}$ unit reductions, respectively. Treatments of 10, 25, 50, and 100 ppm chlorine inactivated 0.31, 1.35, >5, and >5 $\log_{10}$ units, respectively, of the norovirus indicator MS2 bacteriophage. Evaluation of treatment time indicated that the vast majority of MS2 and HuNoV inactivation occurred in the first 5 min for 0.2-μm-filtered, prechlorinated secondary effluent. Free chlorine measurements of secondary effluent seeded with MS2 and HuNoV demonstrated substantial oxidative burdens. With 25, 50, and 100 ppm treatments, free chlorine levels after 5 min of exposure ranged from 0.21 to 0.58 ppm, from 0.28 to 16.7 ppm, and from 11.6 to 53 ppm, respectively. At chlorine treatment levels of >50 ppm, statistically significant differences were observed between reductions for PGM-MB-bound HuNoV (potentially infectious) particles and those for unbound (noninfectious) HuNoV particles or total norovirus particles. While results suggested that MS2 and HuNoV (measured as PGM-MB binding) behave similarly, although not identically, both have limited susceptibility to chlorine treatments of ≤25 ppm total chlorine. Since sewage treatment is performed at ≤25 ppm total chlorine, targeting free chlorine levels of 0.5 to 1.0 ppm, these results suggest that traditional chlorine-based sewage treatment does not inactivate HuNoV efficiently.

IMPORTANCE HuNoV is ubiquitous in sewage. A receptor binding assay was used to assess inactivation of HuNoV by chlorine-based sewage treatment, given that the virus cannot be routinely propagated *in vitro*. Results reported here indicate that chlorine treatment of sewage is not effective for inactivating HuNoV unless chlorine levels are above those routinely used for sewage treatment.

KEYWORDS GI.1 norovirus, GII.4 norovirus, MS2 bacteriophage, chlorine, sewage treatment

Human norovirus (HuNoV) causes a high incidence of person-to-person-, foodborne-, and waterborne-transmitted illness worldwide. Published estimates indicate that approximately 23 million illnesses per annum occur in the United States, and HuNoV is

Received 7 June 2017 **Accepted** 31 August 2017

Accepted manuscript posted online 22 September 2017

Citation Kingsley DH, Fay JP, Calci K, Pouillot R, Woods J, Chen H, Niemira BA, Van Doren JM. 2017. Evaluation of chlorine treatment levels for inactivation of human norovirus and MS2 bacteriophage during sewage treatment. Appl Environ Microbiol 83:e01270-17. https://doi.org/10.1128/AEM.01270-17.

Editor Donald W. Schaffner, Rutgers, The State University of New Jersey

Address correspondence to David H. Kingsley, david.kingsley@ars.usda.gov.

* Present address: Johnna P. Fay, Food and Drug Administration, Center for Food Safety and Applied Nutrition/OFS, Division of Seafood Safety, Shellfish, and Aquaculture Policy Branch, College Park, Maryland, USA.

the most common cause of foodborne illnesses in the United States (1). It is evident that a great deal of HuNoV enters sewage treatment facilities (2, 3), given the high frequency of HuNoV-related illnesses, with an estimated shedding period of ≥4 wk during infection and virion shedding at levels as high as 10^9 virions/g of stool (4). HuNoV is commonly found in sewage (5–9). Typical assessments of sewage treatment are based on reductions of fecal indicator coliforms rather than fecal viruses, which probably overestimates the microbiological safety of the water from a virological perspective (10, 11). Given that considerable amounts of HuNoV enter sewage treatment facilities, key questions to answer are as follows. (i) What fraction of HuNoV is inactivated by chlorine treatment? (ii) How is inactivation influenced by chlorine contact time? (iii) How is inactivation influenced by reduced chlorine treatment concentrations necessitated by high facility inflows, which are often a response to heavy precipitation events? The research described here was developed to provide insights into these questions.

HuNoVs are nonenveloped, which is a feature that makes them difficult to inactivate and environmentally resilient. Exactly how resilient these strains are has been difficult to determine, since HuNoVs cannot be reliably and reproducibly propagated *in vitro* (12–14). Some human volunteer studies have been performed, but the specific treatments that are required to chemically or thermally inactivate HuNoVs are largely unknown or uncharacterized. Estimates of HuNoV inactivation have relied on measurements of total virus (the sum of detectable infectious and noninfectious particles), propagable HuNoV research surrogates such as murine norovirus or Tulane virus, or other robust nonenveloped viruses, such as hepatitis A virus or male-specific coliphage (MSC).

Because HuNoV is currently very difficult to propagate, the principal means of detection for this virus are nucleic acid amplification techniques, such as reverse transcription (RT)-quantitative PCR (qPCR). This may result in false-positive test results, as detection of HuNoV RNA does not necessarily indicate that the virus particle was infectious, although some RT-PCR assays have been designed to assess RNA damage as an indicator of virus inactivation (15, 16). An alternative method, termed the porcine gastric mucin-magnetic bead (PGM-MB) binding assay, in combination with RT-qPCR, is a potential means of determining inactivation (17, 18). This assay estimates the virus capsid's ability to bind to a porcine gastric mucin (PGM), which is chemically similar to histo-blood group antigens in the human intestine to which the virus naturally binds during the initiation of infection (19). Recent work with Tulane virus, a rhesus macaque norovirus that is propagable and also binds histo-blood group antigens, supports the premise that the PGM-MB assay can discriminate between infectious and noninfectious virus particles (20). Research by Lou et al. (21) showed that assessments of HuNoV, after high-pressure processing, with the PGM-MB assay and with gnotobiotic swine were equivalent.

The PGM-MB assay was used to demonstrate that HuNoV is not damaged by repeated freeze-thaw cycles (22) and to evaluate HuNoV's sensitivity to chemical sanitizers. Use of the PGM-MB assay to assess chemical inactivation has suggested that HuNoV is somewhat resistant to some common sanitizing agents and food disinfectants, such as hydrogen peroxide and peroxide-acetic acid (PAA) mixtures, as well as 350 ppm chlorine dioxide (23). When used at relatively high concentrations, chlorine bleach was found to inactivate HuNoVs, as judged by the PGM-MB assay. For example, 1-min treatments of a 10% human stool filtrate with 33 and 189 ppm free chlorine reduced PGM-MB binding by 1.5 and 4.1 $\log_{10}$ units, respectively (23). Notably, chlorine-based sewage treatment is typically performed using free chlorine levels of ≤1 ppm (24, 25).

In this study, we mimic chlorine treatment of sewage, analyze the effects of total (input) and free (residual) chlorination levels and contact times on HuNoV, and compare the results with those for MS2 bacteriophage, which is commonly found in sewage and is frequently used as a municipal sewage indicator (8). This work was performed by

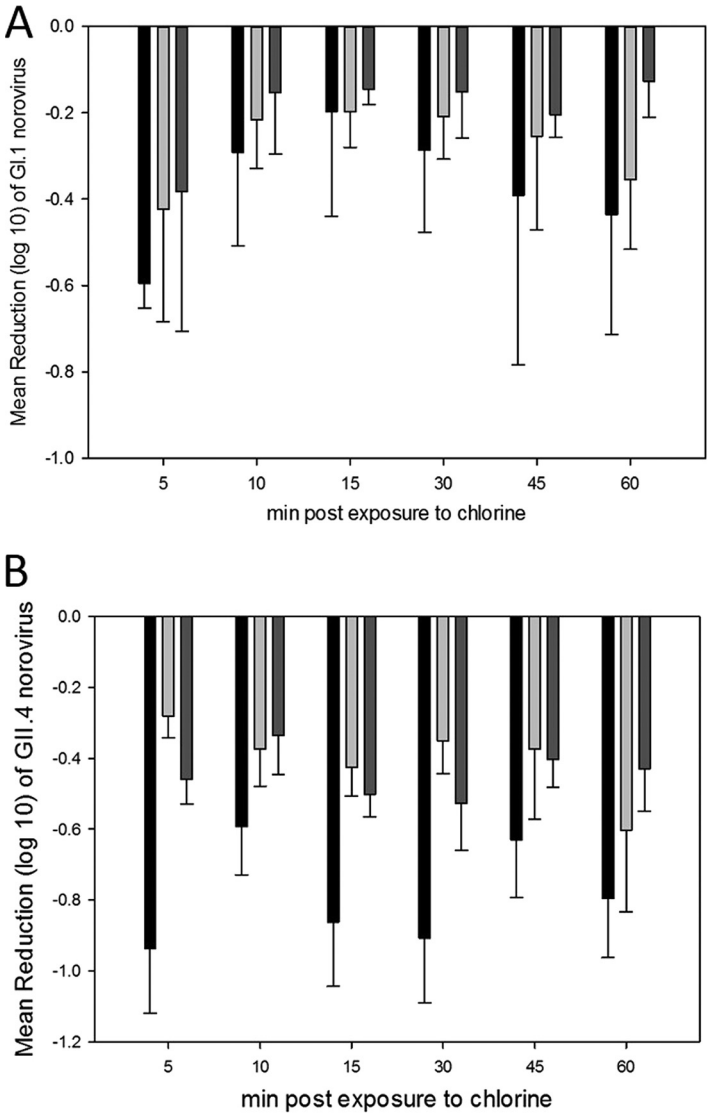

FIG 1 Comparison of reductions of bound, unbound, and total HuNoV particles after treatment with 50 ppm initial total chlorine. (A) GI.1 HuNoV. (B) GII.4 HuNoV. Amounts of PGM-MB-bound HuNoV (black bars), unbound HuNoV (light gray bars), and total HuNoV (sampled before the PGM-MB assay) (dark gray bars) are shown. Error bars represent standard deviations (SDs).

treating secondary effluents seeded with GI.1 and GII.4 HuNoV strains or MS2 with low levels of chlorine bleach and characterizing changes in PGM-MB binding by RT-qPCR.

RESULTS

HuNoV and MS2 reductions. The ability of chlorine to inactivate HuNoV and MS2 bacteriophage in wastewater after secondary treatment was assessed in order to mimic chlorine sewage treatment. Initial experiments evaluated the effects of 1, 5, 10, 25, 50, and 100 ppm total chlorine treatments for up to 60 min on GI.1 and GII.4 HuNoVs. Results indicated that 1, 5, and 10 ppm total chlorine treatments for $\leq$60 min did not lead to losses of the GI.1 or GII.4 HuNoV strains tested, as judged by the PGM-MB binding-RT-qPCR assay (data not shown). Instead, we observed a slight enhancement of PGM-MB binding at these levels of chlorine exposure, which was not significant; the reasons for this are unknown. For 25 ppm total chlorine treatments of GI.1 and GII.4, reduction of PGM-MB binding was not statistically significant (data not shown). For 50 ppm treatments, HuNoV binding reduction across all contact times averaged 0.4 $\pm$ 0.1 and 0.8 $\pm$ 0.1 $\log_{10}$ units for GI.1 and GII.4, respectively (Fig. 1A and B). For 100 ppm

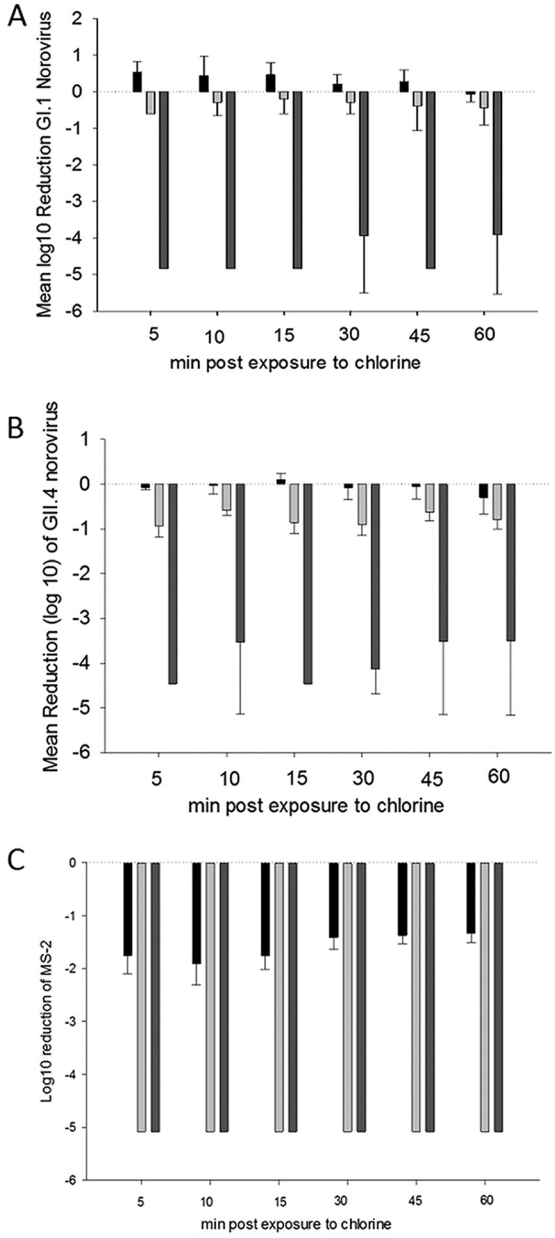

FIG 2 Effects of chlorine treatments on GI.1 HuNoV (A), GII.4 HuNoV (B), and MS2 bacteriophage (C). HuNoV or MS2 seeded in 0.2-μm-filtered secondary effluent was exposed to initial total chlorine levels of 25 (black bars), 50 (light gray bars), or 100 ppm (dark gray bars) for periods ranging from 0 to 60 min. Reductions of HuNoV were based on loss of PGM-MB-binding ability, and MS2 was assessed by plaque formation. Error bars represent SDs.

treatments, average binding reductions of 4.5 $\pm$ 0.5 and 3.9 $\pm$ 0.5 $\log_{10}$ units were observed for GI.1 and GII.4, respectively. The data for 25, 50, and 100 ppm treatments as a function of contact time are illustrated in Fig. 2A and B.

For bacteriophage seeded into secondary effluent (Prechlor), the inactivation of MS2 by chlorine treatment was assessed by plaque assay. Treatment with 1 and 5 ppm total chlorine levels had no discernible inactivation effect on MS2 (data not shown). However, plaque reductions of 0.3 $\pm$ 0.1 and 1.4 $\pm$ 0.2 $\log_{10}$ units were observed for 60-min treatments with 10 and 25 ppm chlorine, respectively, and 50 and 100 ppm total chlorine treatments reduced MS2 to undetectable levels ($>$5 $\log_{10}$ units) (Fig. 2C).

Influence of contact time. Results from the PGM-MB binding assays with GI.1 and GII.4 indicated that the greatest reduction of PGM binding occurred upon initial contact

with chlorine, rather than gradually over the 60-min time course, specifically since the 5-min reductions were similar to the 60-min reductions observed for total chlorine treatments of 25, 50, and 100 ppm (Fig. 2A and B). Mimicking the results observed for HuNoV, the MS2 plaque assay results indicated that most plaque reduction with 25, 50, and 100 ppm total chlorine treatments occurred within the first 5 min of treatment (Fig. 2C).

Chlorine rapidly reacts with available organic compounds, ammonia, and sulfur ions in solution (25). Thus, there is an oxidative chlorine burden that can substantially reduce effectiveness in different solutions and substrates. After the chlorine demand has been satisfied, the remaining effective chlorine is free chlorine. In order to understand how free chlorine levels were affected by the inherent organic content of prechlorinated secondary effluent (Prechlor) and further seeding with HuNoV stool and MS2, free chlorine levels were evaluated before and after filtration and after mixing with GI.1 and GII.4 norovirus stool stocks or MS2 stocks. Free chlorine levels for total chlorine treatments are shown in Fig. 3A to C. For the 25 ppm total chlorine treatment, free chlorine levels were generally minimal, measuring 0.21 to 0.58 ppm after 60 min (Fig. 3A). Samples treated with 50 ppm total chlorine yielded higher free chlorine levels after 60 min of contact with Prechlor, ranging from 0.28 to 16.7 ppm (Fig. 3B). Additionally, the amount of free chlorine with 60-min 100 ppm treatments increased substantially, compared to the 50 ppm treatments, ranging from 11.6 to 53 ppm (Fig. 3C). Thus, measurements of free chlorine levels suggested that, until the initial treatments exceeded 25 ppm, most of the initial chlorine was rapidly lost due to the high chlorine demand and organic content of Prechlor sewage. It is noteworthy that free chlorine levels of spiked Prechlor samples were substantially different; measured free chlorine levels indicated that different stool sources from the GI.1 and GII.4 stocks influenced the oxidative chlorine burden somewhat differently. Interestingly, Prechlor samples seeded with the GII.4 stool reduced free chlorine levels substantially more than those seeded with the GI.1 stool.

To evaluate the effects of filtration and seeding on the chlorine demand, free chlorine levels were evaluated in unfiltered Prechlor, 0.2-μm-filtered Prechlor, and GI.1-, GII.4-, and MS2-seeded Prechlor samples. As noted previously, analysis of chlorine concentrations indicated that most chlorine was rapidly lost during initial contact with Prechlor and residual free chlorine levels generally remained similar throughout the 60-min period (Fig. 3A to C). One exception was unfiltered Prechlor, which presumably had larger fecal particles present; when samples were treated at 25 ppm, the free chlorine level was initially 1.6 ppm but gradually declined to 0.24 ppm over 60 min (Fig. 3A).

HuNoV reductions assessed by detection of RNA alone (without PGM-MB binding). We examined the impact of chlorine treatments, as measured with RT-qPCR, on all HuNoV particles before the PGM-MB assay and on the population of HuNoV particles that did not bind during the PGM-MB assay, and we compared these data with the data obtained for PGM-MB-bound HuNoV. These data can potentially provide insights into the mechanism of chlorine disinfection, as well as information regarding whether RT-qPCR data alone can provide an accurate description of the impact of chlorine treatment.

For HuNoV GI.1 after chlorine treatment but prior to interaction with PGM-MBs, the average log reductions across all contact times were 0.02 $\pm$ 0.08, 0.2 $\pm$ 0.2, and 4.2 $\pm$ 0.84 $\log_{10}$ units after 25, 50, and 100 ppm total chlorine treatments, respectively. For GII.4 after chlorine treatment but prior to interaction with PGM-MBs, the mean log reductions across all contact times were 0.3 $\pm$ 0.3, 0.4 $\pm$ 0.1, and 4.4 $\pm$ 0.3 $\log_{10}$ units after treatments of 25, 50, and 100 ppm total chlorine, respectively. Overall log reductions of unbound HuNoV particles were not statistically different from those of total HuNoV particles, since the amount bound was a limited fraction of the total HuNoV present. However, the log reductions of bound HuNoV after 50 ppm total chlorine treatment were significantly larger (P values of $\leq$0.05 for all times) than those of total HuNoV prior to the binding assay. Statistical differences between bound HuNoV

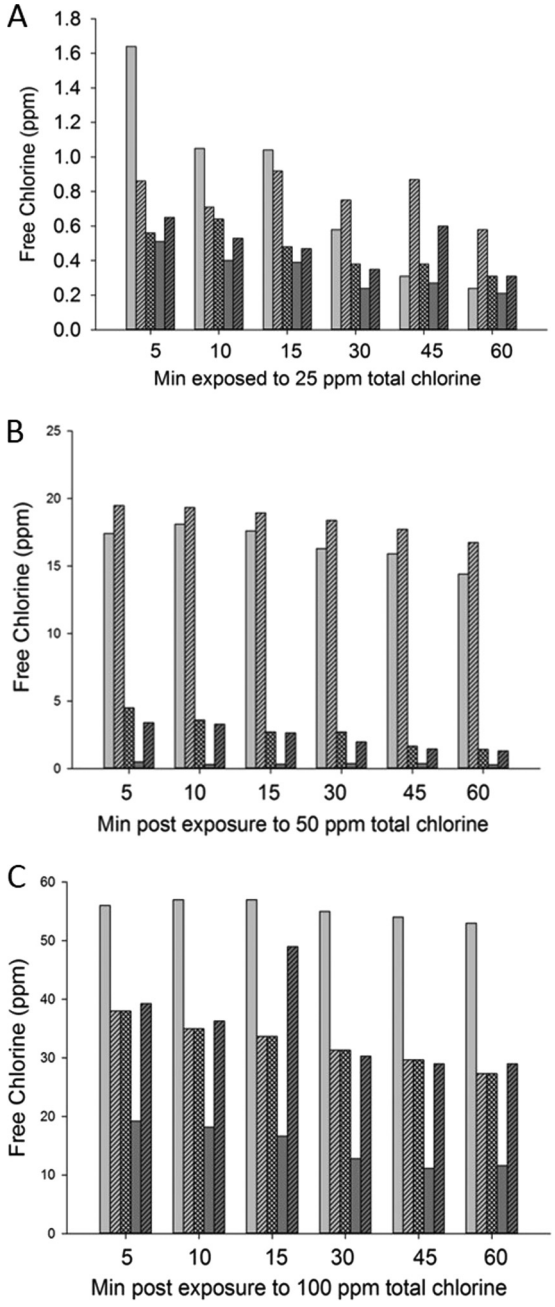

FIG 3 Evolution of free chlorine levels after initial chlorine treatment. Free chlorine levels observed after total chlorine treatments of 25 ppm (A), 50 ppm (B), and 100 ppm (C) for raw secondary sewage effluent (light gray bars), 0.2-μm-filtered secondary effluent (light gray hatched bars), 0.2-μm-filtered secondary effluent seeded with GI.1 HuNoV (crosshatched bars), 0.2-μm-filtered secondary effluent seeded with GII.4 HuNoV (dark gray bars), and 0.2-μm-filtered secondary effluent seeded with MS2 (dark gray hatched bars) were assessed as a function of time (0 to 60 min).

and total HuNoV were not observed for the 100 ppm total chlorine treatment, with which all levels were substantially reduced, or for $\leq$25 ppm total chlorine treatments, for which only small log reductions were observed.

While MS2 viability was reduced by 0.31 and 1.35 $\log_{10}$ units after treatment with 10 and 25 ppm total chlorine, respectively, an effect of chlorine on RT-qPCR detection of MS2 bacteriophage RNA was also noted. Compared to untreated samples, 10 and 25 ppm total chlorine treatments somewhat reduced the amounts of amplifiable RNA; the RT-qPCR threshold cycle (C_T) values increased by 1.0 and 2.3 cycles, respectively. Log_{10}

unit reduction values for MS2 were not determined, due to a lack of suitable MS2 RNA for standard curve determinations. Treatments with 50 and 100 ppm total chlorine were observed to render MS2 RNA undetectable by RT-qPCR.

DISCUSSION

The principal goal of this study was to determine and to characterize the chlorine inactivation of HuNoV associated with the sewage treatment process. Here, wastewater after secondary treatment, prior to disinfection, was used to imitate actual sanitization conditions. While treatments are reported as total chlorine levels, it is important to note that actual or free chlorine levels are dependent on the chlorine demand of the solution matrix, which is a function of the organic content and suspended solids, as well as ammonia and sulfide levels. For this reason, free chlorine levels after treatments were measured by colorimetry. For the two strains tested, GI.1 and GII.4, total chlorine levels of ≤25 ppm did not appear to be effective against HuNoV. We found that, as judged by PGM-MB binding and RT-qPCR assays, HuNoV was not substantially inactivated, suggesting that typical chlorine levels and contact times used for sewage treatment are ineffective.

The use of chlorine by sewage treatment plants is closely managed, to reduce costs. Typical total chlorine levels used by a traditional sewage treatment plant are ≤25 ppm, with targeted free chlorine levels of approximately 0.5 to 1 ppm (24, 25). Thus, it appears that substantial inactivation of HuNoV during traditional chlorine-based sewage treatment should not be expected. The results of this study suggest that a substantial fraction of HuNoV present in wastewater treatment plant influent may pass through the plant without inactivation.

There are a number of caveats that must be noted regarding use of the PGM-MB assay to assess HuNoV inactivation. Without use of an animal model, human volunteers, or an *in vitro* replication system, it cannot be stated for certain that HuNoV is not inactivated at chlorine levels of ≤25 ppm and contact times of ≤60 min, which are typically used for sewage treatment. The PGM-MB assay is principally a measure of the loss of the virus' ability to interact with its receptor. Loss of binding thus reflects inactivation, since a virus that cannot bind to its receptor cannot initiate infection. However, it is possible that chlorine could cause subtle damage to capsid peptides not involved in attachment (or to other virus proteins) that does not prevent PGM-MB binding, possibly resulting in the PGM-MB assay underrepresenting actual inactivation.

Results indicated that, as chlorine treatment levels were increased from 25 to 50 ppm, greater loss of HuNoV binding to PGM-MB occurred, compared with the overall reduction of total RT-qPCR counts. This suggests that, while chlorine-associated damage to the virus RNA undoubtedly occurs, virion damage is substantial enough to inactivate HuNoV (eliminate binding) but not substantial enough to have a discernible effect on amplification of the genome by RT-qPCR. A caveat would be that this assumes that damage would not be targeted to a specific location within HuNoV genomic RNA. It is noteworthy that, for hepatitis A virus, a virus with some characteristics in common with HuNoV, chlorine was shown to predominately damage the 5′ end of the virus genome (26). Since the HuNoV RT-qPCR primers used in this study do not amplify this region of HuNoV, it is conceivable that chlorine damage to the HuNoV genome may not be reflected in the qRT-PCR results.

Other considerations might also have influenced the results reported here. It is likely that viruses naturally present within sewage treatment effluent are largely associated with or sequestered within suspended fecal solids. First, since levels of HuNoV higher than those ordinarily found in sewage after secondary treatment were desirable for testing purposes, Prechlor samples were seeded with GI.1 and GII.4 virus. HuNoV particles are on the order of 30 nm in size. The virus used to seed the Prechlor was filtered using a filter with an average pore size of 200 nm (0.2 μm), making it conceivable that some HuNoV was sequestered within the fecal matrix, which perhaps shielded the virus from chlorine exposure. In nonfiltered effluent, the particles are highly variable, being as large as 100 μm, with a median particle size of approximately 5 to 50 μm (27).

Thus, filtered and nonfiltered Prechlor samples may have different proportions of virus sequestered within fecal solids and different responses to chlorine treatment. Indeed, for unfiltered Prechlor, a more gradual decline of free chlorine levels was observed, presumably because organic compounds were not immediately accessible to chlorine. This will be an important area of further research in quantifying the efficacy of treatments.

A comparison of the free chlorine values observed when MS2 plaque reductions and PGM-MB binding reductions become apparent suggests that MS2 bacteriophage and GI.1 and GII.4 HuNoVs behaved similarly, although not identically, when exposed to similar levels of free chlorine. For example, in this study, 60-min free chlorine levels were associated with observed virus reduction. A free chlorine level of 1.4 ppm (from 50 ppm initial treatment) yielded a reduction of 0.4 $\pm$ 0.3 $\log_{10}$ units for GI.1 HuNoV. For the GII.4 samples treated with 25 and 50 ppm initial chlorine for 60 min, 0.21 and 0.28 ppm free chlorine were observed, respectively, and yielded PGM-MB binding reductions of 0.3 $\pm$ 0.37 $\log_{10}$ units and 0.8 $\pm$ 0.2 $\log_{10}$ units, respectively. For MS2, a 60-min total chlorine treatment of 10 ppm yielded a free chlorine level of 0.27 ppm and a corresponding plaque reduction of 0.31 $\pm$ 0.12 $\log_{10}$ units.

Previous studies using RT-qPCR have shown that, while sewage treatment can reduce enteric virus levels, a substantial amount of virus RNA is commonly detected in treatment plant effluent (6, 8, 10, 28). The degree to which this is representative of infectious viruses is largely unknown, although one study on virus viability for some propagable enteroviruses suggested that viruses in final effluents and biosolids of wastewater treatment plants are infectious (29). Secondary wastewater treatment by itself reduces viral loads, presumably via flocculation and sedimentation of virus particles. For example, Flannery et al. (5) reported reductions of GI and GII HuNoV levels of $<$1 $\log_{10}$ unit after secondary treatment, based on total RT-qPCR, while Kitajima et al. (6) reported reductions of 1.7 to 2.6 $\log_{10}$ units for GI.1 HuNoV and 2.1 to 2.9 $\log_{10}$ units for GII.4 HuNoV, as assessed by RT-qPCR. A recent meta-analysis estimated that chlorine-based sewage treatment resulted in average reductions of 2.4 and 2.7 $\log_{10}$ genomic copies/liter for GI HuNoV and GII HuNoV, respectively, and 2.9 $\log_{10}$ PFU/liter for MSC, although substantial variation in the performance of wastewater treatment plants was noted (8). This meta-analysis evaluated the disinfection effects for GI and GII HuNoVs in these wastewater treatment plants and found no statistically significant effects on the levels of GI and GII HuNoVs, as measured with RT-qPCR alone, and a small effect for MSC, as measured with a plaque assay. These data are consistent with the results of this study. Presumably, while the MS2 plaque assay is a direct and sensitive method for assessing reductions in response to chlorine treatment, the results reported here suggest that the RT-qPCR assay could potentially be associated with MS2 (or MSC) plaque reductions and with HuNoV RT-qPCR results, to provide some assessment of virus inactivation during sewage treatment.

The results presented here indicate that only limited amounts of virus are inactivated by current sewage treatment protocols. Increasing chlorine levels would ostensibly reduce HuNoV levels in wastewater effluent. However, chlorine can combine with organic compounds to generate chlorinated organic compounds, which may be a source of environmental carcinogens (30). Also, all additional chlorine needs to be neutralized before release into the sewage plant effluent, to prevent harm to aquatic fauna. For these reasons, wastewater chlorine treatment levels are closely managed to minimize expenses and to comply with environmental regulations.

An early study by Keswick et al. (31), utilizing human volunteers, suggested that 1 ppm free chlorine treatment of drinking water for 30 min would not be sufficient to render HuNoV noninfectious. However, subsequent work suggested that this result was due to the use of nonpurified, unfiltered virus samples (15). In our study, filtered effluent was spiked with frozen stocks of 0.22-μm-filtered HuNoV stool. Thus, our results are consistent with those of Keswick et al. (31), since we also found that $\leq$25 ppm total chlorine was not sufficient to substantially inactivate HuNoV and a measured free chlorine level of 1.4 ppm gave reductions of GI.1 HuNoV levels of $<$0.5 $\log_{10}$ units.

In this study, chlorine treatments were performed at room temperature. Real-world chlorine treatments are performed at different temperatures. Typically, oyster-associated norovirus outbreaks occur in cooler seasons, since the virus is known to be more prevalent in winter (i.e., causing winter vomiting disease). How temperature might influence inactivation by chlorine-based sewage treatment is currently unknown. Pouillot et al. (8) found no significant impact by month for HuNoV reductions, while the MSC reductions were significantly smaller than average from February to June and significantly larger from August to December.

With regard to MS2 inactivation, results indicate that the bacteriophage is not substantially inactivated by traditional chlorine treatment of sewage. MSCs have been proposed as HuNoV surrogates by food safety researchers and regulators, on the basis of their ubiquity in community sewage effluent and their similarities to HuNoVs, such as being nonenveloped, being environmentally stable, and containing a similar-sized RNA genome (32, 33). The results presented here suggest that MSC may be a viable surrogate with regard to the effects of sewage and may act as an indicator of the potential environmental presence of infectious HuNoV in estuaries.

MATERIALS AND METHODS

MS2 and HuNoV stocks. HuNoV stock 8FIIa was prepared from stool containing the GI.1 Norwalk strain of HuNoV 8FIIa, from a volunteer study (34). GI.1 HuNoV-positive stool was suspended and diluted 1:10 in Dulbecco's modified Eagle's medium (DMEM) (Gibco-Thermo Fisher Scientific, Wilmington, DE), followed by centrifugation at 12,000 × *g* for 20 min at 4°C. GII.4 HuNoV-positive stool was obtained from the New Hampshire Health Department. This stool stock was diluted 1:10 in distilled water, followed by centrifugation at 3,200 × *g* for 15 min at 4°C. Diluted GI.1 and GII.4 supernatant HuNoV stocks were passed through a polyethersulfone 0.22-μm filter (Nalgene, Rochester, NY), and 1-ml aliquots were stored at −80°C. *Escherichia coli* bacteriophage MS2 15597-B1 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and propagated by inoculation of *E. coli* HS(pFamp)R (ATCC 700891) in growth medium for 24 to 48 h at 37°C. Growth broth consisted of 10.0 g of tryptone (Becton, Dickinson and Co., Sparks, MD), 1.0 g of dextrose, 5.0 g of NaCl, and 1 liter of distilled water. Contents were dissolved after mixing, autoclaved at 121°C for 15 min, and stored at 4°C until use. Bacterial cells and debris were pelleted at 3,000 × *g* for 15 min, and supernatant containing MS2 bacteriophage was filtered through a 0.2-μm filter and stored at 4°C prior to use.

Chlorine treatments. Chlorine (8.25% sodium hypochlorite) was obtained at a local market. Prechlor samples were obtained from a sewage treatment plant in Mobile, Alabama, and filtered through a 0.22-μm filter. These were grab samples taken from the final secondary effluent just prior to disinfection. Samples were shipped with refrigeration packs and stored at −80°C prior to use. Prior to the laboratory chlorine treatments, HuNoV and MS2 were equilibrated to room temperature.

For initial experiments, separate sewage aliquots of 1 ml for bioassays and 20 ml for chlorine analysis were prepared; the Prechlor filtrate was seeded with GI.1 or GII.4 HuNoV-infected stool stock (10^5 genomic copies/ml) or MS2 (10^6 PFU/ml) and subsequently treated for 60 min with initial chlorine concentrations of 1, 5, 10, 25, 50, or 100 ppm, at 25°C. To address the effects of 25, 50, and 100 ppm chlorine treatments over time, separate 1-ml bioassay samples and 20-ml chlorine analysis aliquots of Prechlor were initially incubated for 60 min at room temperature with 10^5 genomic copies/ml of either GI.1 or GII.4 or 10^6 PFU/ml of MS2. After equilibration to room temperature, aliquots were treated with different chlorine concentrations and incubated at 25°C for 5, 10, 15, 30, 45, or 60 min. After chlorine treatment, 1 M sodium thiosulfate was used to inactivate residual chlorine in the bioassay samples. All bioassay experiments were performed in triplicate.

In separate experiments that did not assess PGM binding or HuNoV inactivation, free chlorine concentrations of Prechlor, with or without HuNoV or MS2, were evaluated before and after 0.2-μm filtration. For safety purposes and prevention of equipment contamination, HuNoV stocks were heated to 99°C for 15 min, to inactivate the virus, and then were cooled to room temperature prior to chlorine treatments and free chlorine measurements. Assessment of free chlorine was performed using a Hach DR900 colorimeter (Hach Inc., Loveland, CO) and U.S. EPA DPD method 8021.

HuNoV assay. PGM-MBs were prepared as described by Tian et al. (35) and Kingsley et al. (23). A magnetic bead attractor was used to separate the PGM-MBs from the treated virus-laden sewage and the beads were washed three times, as described previously (23). After HuNoV was bound to the PGM-MBs, viral RNA was extracted from viruses bound to PGM-MBs by using the Qiagen viral RNA minikit (Qiagen Inc., Germantown, MD). Samples were immediately stored on ice pending RT-qPCR assay. The HuNoV GI and GII primer sets and TaqMan probes used for the RT-qPCR assay were originally described by Stals et al. (36). Preparation and performance of RT-qPCRs were also as described previously (17, 23). Inactivation was assessed on the basis of the reduction of virions bound after treatment, compared to untreated bound samples.

MS2 bacteriophage assay. MS2 was enumerated using a plaque assay based on the method described by Debartolomeis and Cabelli (37). The modified double-agar overlay method assayed 100 μl of sewage in 5 ml of soft agar, seeded with 200 μl of log-phase *E. coli* F_{amp} host and plated in a 100-mm petri dish containing 15 ml of bottom agar. Bottom agar contained 10 g tryptone, 1 g dextrose, 5 g NaCl,

and 15 g agar (Becton, Dickinson and Co.) in 1 liter of distilled water. After autoclaving of the medium for 15 min at 121°C, the bottom agar was supplemented with 50 mg each of streptomycin sulfate (Corning, Mediatech, Inc., Manassas, VA) and ampicillin (Sigma Chemical Co., St. Louis, MO), from filter-sterilized solutions. The top soft agar consisted of 10.0 g tryptone, 1.0 g dextrose, 5.0 g NaCl, 7.0 g agar, 500 μl of previously prepared 1 M $CaCl_2$ (Sigma Chemical Co.) solution, and 500 ml of distilled water.

MS2 RNA was also extracted using the Qiagen viral RNA minikit. RT-qPCR of MS2 was performed as described by Gendron et al. (38), using the forward primer 5′-GTCCATACCTTAGATGCGTTAGC-3′, the reverse primer 5′-CCGTTAGCGAAGTTGCTTGG-3′, and the dually labeled probe 5′-6-carboxyfluorescein (FAM)-ACGTCGCCAGTTCCGCCATTGTCG-black hole quencher (BHQ)-3′ (Integrated DNA Technologies, San Jose, CA). The final concentration of the forward and reverse primers was 1 μM and that of the probe was 150 nM, utilizing a one-step RT-PCR kit according to the manufacturer's instructions (Qiagen). RT was performed for 30 min at 50°C, with a polymerase activation step for 3 min at 94°C, followed by 50 cycles of amplification using 15 s of denaturation at 94°C and 1 min of annealing/extension at 60°C.

Data and statistical analyses. Serially diluted HuNoV RNA, in triplicate, was used to generate a standard curve of RT-qPCR C_T values; RT-qPCR C_T values were then converted to RT-qPCR RNA units. To quantitate HuNoV reductions after chlorine treatments, untreated RT-qPCR units were compared with chlorine-treated RT-qPCR units. Log_{10} reductions were determined using the equation $y = -3.2475x + 38.156$ ($R^2 = 0.99$) for GI.1 and the equation $y = -3.4599x + 39.953$ ($R^2 = 0.99$) for GII.4, as generated from the standard curve. Significant differences in detected HuNoV RNA concentrations were calculated with Student's *t* tests. Results were considered significantly different when *P* values were ≤ 0.05. MS2 reductions were determined by plaque assay.

ACKNOWLEDGMENTS

We thank Gary P. Richards (USDA, Dover, DE), William Burkhardt (USFDA, Dauphin Island, AL), and Jessica Jones (USFDA, Dauphin Island, AL) for critical reading of the manuscript.

Funding for J.P.F. was provided via interagency agreement 8072-42000-081-01L between the USDA Agricultural Research Service and the FDA Center for Food Safety and Applied Nutrition.

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture or Food and Drug Administration.

REFERENCES

1. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States: major pathogens. Emerg Infect Dis 17:7–15. https://doi.org/10.3201/eid1701.P11101.
2. Eftim SE, Hong T, Soller J, Boehm A, Warren I, Ichida A, Nappier SP. 2017. Occurrence of norovirus in raw sewage: a systematic literature review and meta-analysis. Water Res 111:366–374. https://doi.org/10.1016/j.watres.2017.01.017.
3. Montazeri N, Goettert D, Achberger EC, Johnson CN, Prinyawiwatkul W, Janes ME. 2015. Pathogenic enteric viruses and microbial indicators during secondary treatment of municipal wastewater. Appl Environ Microbiol 81:6436–6445. https://doi.org/10.1128/AEM.01218-15.
4. Atmar RL, Opekun AR, Gilger MA, Estes MK, Crawford SE, Neill FH, Graham DY. 2008. Norwalk virus shedding after experimental human infection. Emerg Infect Dis 14:1553–1557. https://doi.org/10.3201/eid1410.080117.
5. Flannery J, Keaveney S, Rajko-Nenow P, O'Flaherty V, Doré W. 2012. Concentration of norovirus during wastewater treatment and its impact on oyster contamination. Appl Environ Microbiol 78:3400–3406. https://doi.org/10.1128/AEM.07569-11.
6. Kitajima M, Iker BC, Pepper IL, Gerba CP. 2014. Relative abundance and treatment reduction of viruses during wastewater treatment processes: identification of potential viral indicators. Sci Total Environ 488-489:290–296. https://doi.org/10.1016/j.scitotenv.2014.04.087.
7. Maalouf H, Pommepuy M, Le Guyander FS. 2010. Environmental conditions leading to shellfish contamination and related outbreaks. Food Environ Virol 2:136–145. https://doi.org/10.1007/s12560-010-9043-4.
8. Pouillot R, Van Doren JM, Woods J, Plante D, Smith M, Goblick G, Roberts C, Locas A, Hajen W, Stobo J, White J, Holtzman J, Buenaventura E, Burkhardt W, III, Catford A, Edwards R, DePaola A, Calci K. 2015. Meta-analysis of the reduction of norovirus and male-specific coliphage concentrations in wastewater treatment plants. Appl Environ Microbiol 81:4669–4681. https://doi.org/10.1128/AEM.00509-15.
9. Sima LC, Schaeffer J, Le Saux JC, Parnaudeau S, Elimech M, Le Guyader FS. 2011. Calicivirus removal in a membrane bioreactor wastewater treatment plant. Appl Environ Microbiol 77:5170–5177. https://doi.org/10.1128/AEM.00583-11.
10. Campos CJA, Lees DN. 2014. Environmental transmission of human norovirus in shellfish waters. Appl Environ Microbiol 80:3552–3561. https://doi.org/10.1128/AEM.04188-13.
11. Tyrrell SA, Rippey SR, Watkins WR. 1995. Inactivation of bacterial and viral indicators in secondary sewage effluents using chlorine and ozone. Water Res 29:2483–2490. https://doi.org/10.1016/0043-1354(95)00103-R.
12. Duizer E, Schwab KJ, Neill FH, Atmar RL, Koopmans MP, Estes MK. 2004. Laboratory efforts to cultivate noroviruses. J Gen Virol 85:79–87. https://doi.org/10.1099/vir.0.19478-0.
13. Herbst-Kralovetz MM, Radtke AL, Lay MK, Hjelm BE, Bolick AN, Sarker SS, Atmar RL, Kingsley DH, Arntzen CJ, Estes MK, Nickerson CA. 2013. Lack of norovirus replication and histo-blood group antigen expression in 3-D intestinal epithelial cell cultures. Emerg Infect Dis 19:431–438. https://doi.org/10.3201/eid1903.121029.
14. Papafragkou E, Hewitt J, Park GW, Greening G, Vinjé J. 2014. Challenges of culturing human norovirus in three-dimensional organoid intestinal cell culture models. PLoS One 8:e63485. https://doi.org/10.1371/journal.pone.0063485.
15. Shin GA, Sobsey MD. 2008. Inactivation of norovirus by chlorine disinfection of water. Water Res 42:4562–4568. https://doi.org/10.1016/j.watres.2008.08.001.
16. Tung G, Macinga D, Arbogast J, Jaykus LA. 2013. Efficacy of commonly used disinfectants for inactivation of human noroviruses and their surrogates. J Food Prot 76:1210–1217. https://doi.org/10.4315/0362-028X.JFP-12-532.
17. Dancho BA, Chen H, Kingsley DH. 2012. A method to discriminate between infectious and inactive human noroviruses. Int J Food Microbiol 155:222–226. https://doi.org/10.1016/j.ijfoodmicro.2012.02.010.
18. Wang D, Tian P. 2014. Inactivation conditions for human norovirus

measured by an in situ capture-qRT-PCR method. Int J Food Microbiol 172:76–82. https://doi.org/10.1016/j.ijfoodmicro.2013.11.027.
19. Tian P, Jiang X, Zhong W, Jensen HM, Brandl M, Bates AH, Engelbrektson AL, Mandrell R. 2007. Binding of recombinant norovirus like particle to histo-blood group antigen on cells in the lumen of pig duodenum. Res Vet Sci 83:410–418. https://doi.org/10.1016/j.rvsc.2007.01.017.
20. Li X, Chen H. 2015. Evaluation of the porcine gastric mucin binding assay for high-pressure-inactivation studies using murine norovirus and Tulane virus. Appl Environ Microbiol 81:515–521. https://doi.org/10.1128/AEM.02971-14.
21. Lou F, Ye M, Ma Y, Li X, Chen H, Krakowka S, Hughes J, Kingsley DH, Li J. 2015. A gnotobiotic pig model for determining human norovirus inactivation by high-pressure. Appl Environ Microbiol 81:6679–6687. https://doi.org/10.1128/AEM.01566-15.
22. Richards GP, Watson MA, Kingsley DH. 2004. A SYBR green, real-time RT-PCR method to detect and quantitate Norwalk virus in stools. J Virol Methods 116:63–70. https://doi.org/10.1016/j.jviromet.2003.10.011.
23. Kingsley DH, Vincent E, Meade GK, Watson C, Fan X. 2014. Inactivation of human norovirus using chemical sanitizers. Int J Food Microbiol 171:94–99. https://doi.org/10.1016/j.ijfoodmicro.2013.11.018.
24. Tchobanoglous G, Stensel HD, Tsuchihashi R, Burton F. 2014. Wastewater engineering: treatment and resource recovery, 5th ed. McGraw Hill Education, New York, NY.
25. Salvato JA, Neerow NL, Agardy FJ (ed). 2003. Environmental engineering, 5th ed. J. Wiley and Sons, Hoboken, NJ.
26. Li JW, Xin ZT, Wang XW, Zheng JL, Chao FH. 2002. Mechanisms of inactivation of hepatitis A virus. Appl Environ Microbiol 68:4951–4955. https://doi.org/10.1128/AEM.68.10.4951-4955.2002.
27. Tiehm A, Herwig V, Neis U. 1999. Particle size analysis for improved sedimentation and filtration in waste water treatment. Water Sci Technol 39:99–106.
28. Tian P, Yang D, Pan L, Mandrell R. 2012. Application of a receptor-binding capture quantitative reverse transcription-PCR assay to concentrate human norovirus from sewage and to study the distribution and stability of the virus. Appl Environ Microbiol 78:429–436. https://doi.org/10.1128/AEM.06875-11.
29. Simmons FJ, Xagoraraki I. 2011. Release of infectious human enteric viruses by full scale wastewater utilities. Water Res 45:3590–3598. https://doi.org/10.1016/j.watres.2011.04.001.
30. Di Cristo C, Esposito G, Leopardi A. 2013. Modelling trihalomethanes formation in water supply systems. Environ Technol 34:61–70. https://doi.org/10.1080/09593330.2012.679702.
31. Keswick BH, Satterwhite TK, Johnson PC, DuPont HL, Secor SL, Bitsura J, Gary AGW, Hoff JC. 1985. Inactivation of Norwalk virus in drinking water by chlorine. Appl Environ Microbiol 50:261–264.
32. U.S. Food and Drug Administration. 2015. National Shellfish Sanitation Program (NSSP) guide for the control of molluscan shellfish, 2015 revision. U.S. Food and Drug Administration, Silver Spring, MD. https://www.fda.gov/downloads/Food/GuidanceRegulation/FederalStateFoodPrograms/UCM505093.pdf.
33. Flannery J, Keaveney S, Doré W. 2009. Use of FRNA bacteriophages to indicate the risk of norovirus contamination in Irish oysters. J Food Prot 72:2358–2362. https://doi.org/10.4315/0362-028X-72.11.2358.
34. Richards GP, Watson MA, Meade GK, Hovan GL, Kingsley DH. 2012. Resilience of norovirus GII.4 to freezing and thawing: implications for virus infectivity. Food Environ Virol 4:192–197. https://doi.org/10.1007/s12560-012-9089-6.
35. Tian P, Engelbrektson A, Mandrell R. 2008. Two-log increase in sensitivity for detection of norovirus in complex samples by concentration with porcine gastric mucin conjugated to magnetic beads. Appl Environ Microbiol 74:4271–4276. https://doi.org/10.1128/AEM.00539-08.
36. Stals A, Baert L, Botteldoorn N, Werbrouck H, Herman L, Uyttendaele M, Van Coillie E. 2009. Multiplex real-time RT-PCR for simultaneous detection of GI/GII noroviruses and murine norovirus 1. J Virol Methods 161:247–253. https://doi.org/10.1016/j.jviromet.2009.06.019.
37. Debartolomeis J, Cabelli VJ. 1991. Evaluation of an *Escherichia coli* host strain for enumeration of F male-specific bacteriophages. Appl Environ Microbiol 57:1301–1305.
38. Gendron L, Verreault D, Veilette M, Moineau S, Duchaine C. 2010. Evaluation of filters for the sampling and quantification of RNA phage aerosols. Aerosol Sci Technol 44:893–901. https://doi.org/10.1080/02786826.2010.501351.