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Vesicle-Cloaked Rotavirus Clusters are Environmentally Persistent and Resistant to Free Chlorine Disinfection

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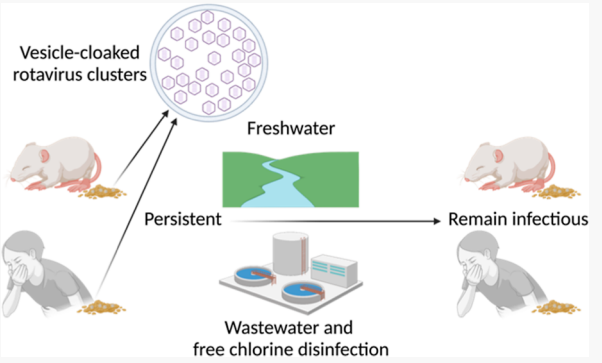
ABSTRACT: Recent discovery of vesicle-cloaked virus clusters (i.e., viral vesicles) has greatly challenged the central paradigm of viral transmission and infection as a single virion. To understand the environmental transmission of viral vesicles, we used an in vivo model to investigate their environmental persistence and engineering control by disinfection. Murine rotavirus vesicles maintained both their integrity and infectivity after incubation in filtered freshwater and wastewater for at least 7 days, with 24.5-27.5% of the vesicles still intact at 16 weeks after exposure to both waters. Free chlorine disinfection at a dosage of 13.3 mg min L^{-1} did not decompose murine rotavirus vesicles, and it was much less effective in inactivating rotaviruses inside vesicles than free rotaviruses based on the quantification of rotavirus shedding in mouse stool and rotavirus replication in small intestines. Rotavirus vesicles may be more environmentally transmissible than free rotaviruses regardless of disinfection. Vesicle-mediated *en bloc* transmission could be responsible for vesicles' resistance to disinfection due to an increased multiplicity of infection and/or genetic recombination or reassortment to promote infection. Our work highlights the environmental, biological, and public health significance of viral vesicles, and the findings call for urgent action in advancing disinfection for pathogen control.



KEYWORDS: *vesicle-cloaked virus clusters, rotavirus, environmental persistence, free chlorine disinfection*

INTRODUCTION

Vesicle-cloaked virus clusters (here referred to as viral vesicles) are emerging pathogenic units distinct from both enveloped and non-enveloped viruses, and they are phospholipid-bilayer-encapsulated extracellular vesicles (EVs) containing multiple virions or multiple copies of naked viral genomes (Figure S1).[1,2] The discovery of viral vesicles greatly challenges the central paradigm of viral transmission and infection as a single virion. A broad spectrum of enveloped and non-enveloped viruses, especially enteric viruses like poliovirus, coxsackievirus, norovirus, and rotavirus, have been found to be present as viral vesicles in their lifecycle from egressing from host cells to spreading and then infecting the next host.[2,3] Viral vesicles can deliver from 1 to 5 to more than 25 virions to a host cell at the same time, and the vesicle size ranges from 50 to 100 nm to several hundred nanometers, depending on the virus type and the cellular organelles viral vesicles originate from, including plasma membranes, autophagosomes, and multivesicular bodies.[2,3] For example, rotavirus vesicles can contain more than 25 virions, and they directly bud from the plasma membranes as microvesicles.[4]

Viral vesicles are environmentally and biologically important because they are ubiquitously present in feces (contributing up to ~50% of the total virus population) that can contaminate the environment, they are highly persistent under diverse environmental stressors, and they are more infectious and more resistant to ultraviolet (UV_{254}) disinfection compared to their counterparts of free viruses.[1,2] Viral vesicles not only increase clinical burden but also promote environmental transmission of viral pathogens and eventually pose a higher risk to public health when compared to free viruses. Vesicle-mediated *en bloc* transmission of virions enhances the multiplicity of infection (MOI), enables multiple viral genomes to enter into one host cell simultaneously, overcomes the replication barrier, and initiates successful infection.[2,3] The delivery of multiple viral genomes to the host cell also promotes genetic recombination or reassortment and diminishes the lethal effect of genome mutation and/or damage, resulting in enhanced virus infectivity and resistance to disinfection like UV_{254} irradiation, which mainly destroys the viral genome.[1] In addition, EVs also promote the inter-organismal viral transmission by shielding viral cargos from host antibodies and suppressing host immune defenses.[2] Our

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recent study has shown that murine norovirus vesicles have 1.89−3.17-times higher infectivity, and they are ca. 2.16-times more resistant to UV_{254} disinfection when compared with free murine noroviruses in vitro.[1] Besides norovirus, poliovirus,[5] coxsackievirus,[6] rhinovirus,[5] marseillevirus,[7] polyomavirus,[8] and rotavirus[4] have all been reported with an enhanced infectivity in vesicles than as individual viral particles. For example, the infectivity of murine rotavirus vesicles was equivalent to that of five times-concentrated free murine rotaviruses based on an in vivo study.[4] Nevertheless, our recent study is the first and the only one that reports the resistance of viral vesicles to disinfection to date. Moreover, environmental persistence of viral vesicles further increases the health risk of virus transmission and infection. Murine rotavirus vesicles are highly resistant to digestive enzymes and low stomach pH since they remain intact after passing through the gastrointestinal tract of mice.[4] Murine norovirus vesicles harvested from cell culture remained intact under temperature variation (i.e., 20 cycles of freeze-thaw) and only partially decomposed after detergent treatment.[2] Particularly, EVs harvested in vivo have a higher loading of cholesterol and sphingomyelin than those isolated in vitro,[9] which modulates the fluidity and stability of EVs and in turn promotes their environmental persistence.[10−13]

Our study aims to understand the environmental persistence of vesicle-cloaked rotavirus vesicles in freshwater and wastewater and to elucidate how resistant the viral vesicles are to free chlorine disinfection. Research findings will shed light on the broad environmental impact of viral vesicles, from fate and transport to engineering control of these emerging yet largely unknown pathogenic units. Our research will also answer the question of whether the current disinfection paradigm can sufficiently inactivate waterborne viruses, and the outcome will provide guidelines for advancing disinfection, sanitation, and hygiene practices. Rotavirus was selected because of its significant public health concern: it is the leading cause of severe acute gastroenteritis and diarrhea-associated mortality worldwide, resulting in more than 500,000 deaths of infants and young children every year.[14,15] Rotavirus is a nonenveloped, segmented, and double-stranded RNA virus from the family of *Reoviridae*,[14] and it can be transmitted by the fecal-oral route through contaminated water, food, contact surfaces, and others. Rotavirus is notorious for spreading infectious diseases because of its low infectious dose, high shedding concentration in host excreta, and high prevalence and persistence in the environment.[16−20]

In our study, the persistence of murine rotavirus vesicles from the stool of rotavirus-infected mouse pups was investigated in filtered freshwater (i.e., Potomac River water) and wastewater for up to 16 weeks. The resistance of murine rotavirus vesicles to free chlorine disinfection was also investigated and compared with that of free murine rotaviruses using a mouse model. Our work is innovative because it is the first study to quantify the persistence of viral vesicles in environmentally relevant conditions and evaluate viral vesicle disinfection by free chlorine, one of the most widely used disinfectants. Our unique study also used the mouse model instead of cell culture to isolate rotaviruses and evaluate virus infectivity (in vivo vs in vitro), and it best mimics the environmental and biological behavior of rotaviruses that are shed in human excreta and are present in the environment.

EXPERIMENTAL METHODS

Mouse Model. The infectivity of rotavirus was tested using a mouse model as previously described by Santiana et al.[4] Pathogen-free BALB/c mice (including female adults and litters) were purchased from Taconic Farms, maintained at the National Heart, Lung, and Blood Institute (NHLBI) Animal Care facility, housed, and bred following the Guide for the Care and Use of Laboratory Animals under an animal study proposal approved by the NHLBI Animal Care and Use Committee. Briefly, the mice were housed in cages supplied with hardwood bedding and nest packs in ventilated racks and bred with the National Institutes of Health (NIH) 31 feed and autoclaved water. In the animal facility, the temperature was maintained at 22 °C, and a 6 a.m. to 6 p.m. light cycle was followed. All animal experiments were performed at an American Association for the Accreditation of Laboratory Animal Care (AAALAC) accredited animal facility. All experimented mice were 10 day old pups including both females and males since no gender bias was reported for rotavirus infection in mice. Animal euthanization was also carried out by following the AAALAC guidelines, where mice were first euthanized by 2−3 min of CO_2 exposure (USP Grade A) at 3 L min^{-1} and then followed by physical euthanization (decapitation) until the lack of respiration and a faded eye color were observed.

Rotavirus Stock Preparation. Murine rotavirus of the epizootic diarrhea of infant mice (EDIM) strain was kindly provided by Dr. H. Greenberg (Stanford University, Palo Alto, CA) and was propagated in BALB/c mice. 6 day old mouse pups were orally gavaged with 100-fold diluted rotavirus stock with 1× phosphate-buffered saline (PBS). Small intestines were surgically extracted at 3 days post infection (dpi) after the pups were euthanized when the pups showed serious diarrhea. The tissues were homogenized in M199 medium and centrifuged at 5000×*g* for 10 min at 4 °C. The clear supernatant was collected as the rotavirus stock, and it was aliquoted and stored at −80 °C for further use.

Rotavirus Vesicle Preparation. After 6 day old BALB/c mouse pups were orally inoculated with 100-fold diluted rotavirus stock, the stool was harvested twice a day from the diarrhea symptom onset to 5 dpi. Mouse pups' bellies were gently pressed for stool collection at anuses until no stool was released. The intestine content was also collected after pups were euthanized, and the intestines were surgically extracted. All rotavirus-positive stools and the intestine content were suspended with 1× PBS buffer to obtain a 10−20% (w/v) fecal suspension before proceeding to a series of sequential centrifugations for preparing a clarified fecal solution. The sequential centrifugations were conducted for 10 min each with the speed of 500, 1000, 2000, 4000, and 5000×*g* at 10 °C. The fecal supernatant was transferred to a new tube after the centrifugation at 1000, 4000, and 5000×*g*, and multiple rounds of centrifugation at 5000×*g* were conducted when needed. The clarified fecal solution was then subjected to vesicle purification using the MagCapture Exosome Isolation Kit PS (Fujifilm Wako Pure Chemical, 293-77601), which uses TIM4-coated magnetic beads to selectively bind viral vesicles but not free viruses.[1] Rotavirus vesicles were stored in the elution buffer of the kit at 4 °C for further use.

Free Rotavirus Preparation. A non-detergent lysis buffer (Detergent-free Exosomal Protein Extraction Kit, 101Bio, P201) was applied for releasing free rotaviruses from vesicles,

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which effectively lyses EVs liberating viruses but does not negatively impact virus infectivity in both in vitro and in vivo studies.[1,4] Therefore, the non-detergent lysis buffer prepares a mixture of free rotaviruses and rotavirus vesicles, with free rotaviruses dominating the viral population. Rotavirus vesicles were first ultracentrifuged at 100,000×*g* at 10 °C for 30 min. The supernatant that mainly contained free rotaviruses was collected separately, and pellets were lysed with 20 μL of lysis buffer A and neutralized with lysis buffer B from the Detergent-free Exosomal Protein Extraction Kit sequentially. A vortex of 15 s was applied between the lysis and neutralization. The collected supernatant was then added back to the lysed pellets to maximize the collection of free rotaviruses. Free rotaviruses were stored in the mixture of the elution buffer (MagCapture Exosome Isolation Kit PS) and the lysis buffer (Detergent-free Exosomal Protein Extraction Kit) at 4 °C for further use.

Incubation of Rotavirus Vesicles in Freshwater and Wastewater. The persistence of free rotaviruses in various water matrices has been intensively investigated in previous studies, which showed that rotaviruses could persist in water for up to months.[18,19,21] Our objective is to investigate the persistence of rotavirus vesicles in freshwater and wastewater, and we did not intend to compare the persistence of rotavirus vesicles with free rotaviruses. Freshwater was collected from the Potomac River in VA, and raw wastewater was collected from a local wastewater reclamation facility (VA) after screening. All water samples were filtered with 0.22 μm membranes to remove large particles and bacteria, protists, protozoa (like amoebas), zooplanktons, and other higher organisms immediately after sample collection and then stored at −80 °C for further experiments. We focus on the physical and chemical stability of rotavirus vesicles under different water chemistries and potential exposure to enzymes (e.g., proteolytic enzymes secreted by bacteria[22]) by excluding the impact of microbial grazing.[23] Water quality parameters are listed in Table S1. To evaluate the short- and long-term stability of rotavirus vesicles, 10 μL of rotavirus vesicles were seeded to 90 μL of filtered freshwater and wastewater and settled at room temperature in the dark for up to 7 days and 16 weeks without mixing. After incubation, 1× PBS buffer was added until the solution volume reached 1 mL, and then the vesicles were re-pulled down using the MagCapture Exosome Isolation Kit PS. The harvested vesicles were next subjected to RNA isolation using the Quick-RNA MicroPrep Kit (Zymo Research, CA, USA, R1050) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) quantification. For short-term stability evaluation, rotavirus vesicles were mixed with filtered freshwater and wastewater and immediately re-pulled down by the MagCapture Exosome Isolation Kit PS, respectively, and these samples were defined as the 0 day control. For long-term stability evaluation, rotavirus vesicles were first maintained in the elution buffer provided by the MagCapture Exosome Isolation Kit PS at 4 °C for 16 weeks and re-pulled down using the same kit immediately after being mixed with filtered freshwater and wastewater, respectively, which was defined as the 0 day$^{\#}$ control. Ethylenediaminetetraacetic acid (EDTA) in the elution buffer and low-temperature preservation were proved to largely maintain the integrity of the viral vesicles.[24]

We have demonstrated the validity of preparing rotavirus vesicles and norovirus vesicles by using TIM4-coated magnetic beads because the beads selectively and strongly bind to phosphatidylserine (PS) on the vesicle membrane but not naked viruses.[1,4] Transmission electron microscopy (TEM) characterizes the morphology of viral vesicles, and immuno-electron microscopy confirms the presence of viruses in the vesicles.[1,4] Nanoparticle tracking analysis (NTA) also characterizes the integrity of vesicles before and after the treatment by detergents, the non-detergent lysis buffer, and UV_{254} disinfection. However, both TEM and NTA could bias the results because (i) viruses cannot be observed under TEM when cloaked in intact vesicles, (ii) broken viral vesicles and the loss of viruses from the broken vesicles could result from the artifacts of TEM sample preparation, and (iii) a large number of exosomes in viral vesicle samples purified from the mouse stool may interfere with NTA characterizations. Notably, separation by TIM4-coated magnetic beads and subsequent RT-qPCR provides reliable quantitative analyses of intact viral vesicles, in contrast to TEM and NTA.[1]

The infectivity of rotavirus vesicles after wastewater incubation was also evaluated. 8 μL of rotavirus vesicles were first incubated in 72 μL of filtered wastewater for 7 days at room temperature in the dark. The mixture post incubation was diluted with 1× PBS buffer to a total volume of 100 μL and orally gavaged to each 10 day old BALB/c mouse pup. Rotavirus vesicles of the same viral load but without exposure to the wastewater were fed to the mouse pup as a positive control. Vesicles free of viruses were isolated from the stool of uninfected healthy mouse pups, incubated with filtered wastewater at room temperature in the dark for 7 days, and then fed to mouse pups as a negative control. Six mouse pups were used as replicates for each experimental condition.

Free Chlorine Disinfection. Free chlorine disinfection was conducted with chlorine water (saturated aqueous solution, ca. 0.3% w/v, Ricca Chemical) in a 10 mM phosphate buffer at pH 6, and the final free chlorine concentration was diluted to 6.2 mg L^{-1}. A pH of 6 was used to maximize disinfection performance because hypochlorous acid was the dominant species, and it outperformed hypochlorite. All working solutions and buffers were prepared with chlorine demand-free water. The elution buffer and non-detergent lysis buffer were diluted 20- and 100-fold by 10 mM phosphate buffer (pH 6), respectively, for free chlorine disinfection to eliminate chlorine consumption by the constituents in these buffers. The contact time of free chlorine with rotaviruses was 128 s to obtain a final disinfectant concentration × contact time (CT) value of 13.3 mg min L^{-1}, which should achieve more than 5-$\log_{10}$s inactivation of rotaviruses based on published literature.[25−27] Residual free chlorine after disinfection was measured by the *N*,*N*-diethyl-*p*-phenylenediamine colorimetric method (see Supporting Information (SI) for details),[28] and this method does not quantify the combined chlorine. No combined chlorine existed in our system. The free chlorine standard curve was calibrated with the presence of 20-fold diluted elution buffer or the mixture of 100-fold diluted lysis buffer and 20-fold diluted elution buffer to exclude the impact of buffer constituents on free chlorine measurements. To end disinfection, residual free chlorine was immediately quenched by an excessive amount of ascorbic acid at the end of the reaction (i.e., four times higher dosage of ascorbic acid compared to initial free chlorine). Rotavirus vesicles and free rotaviruses without chlorination but with the same composition and loading of buffers (e.g., elution buffer, lysis buffer, and phosphate buffer) and ascorbic acid were used as positive controls. 100 μL of rotavirus vesicles or free rotaviruses with or without chlorination were orally gavaged to each 10 day old

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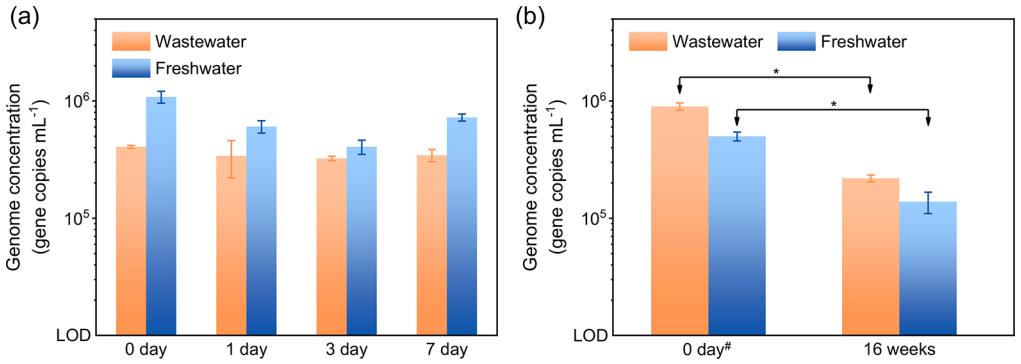

Figure 1. Rotavirus vesicles are persistent in filtered wastewater and freshwater in a (a) short-term and (b) long-term study. 0 day represents that rotavirus vesicles were immediately re-pulled down using TIM4-coated magnetic beads after being mixed with filtered freshwater and wastewater; 0 day# represents that rotavirus vesicles were first stored in an EDTA-containing elution buffer at 4 °C for 16 weeks and then mixed with filtered freshwater and wastewater and immediately re-pulled down using TIM4-coated magnetic beads. All data were measured by RT-qPCR. The LOD was 1.00×10^4 gene copies mL^{-1}. All data below LOD were substituted by the LOD. n = 4−6. *represents $p < 0.05$.

BALB/c mouse pup, and stools and small intestines at different time intervals post infection were collected for RT-qPCR quantification to evaluate rotavirus infectivity in mice. To understand the impact of disinfection on vesicle integrity, chlorinated rotavirus vesicles were re-pulled down using the MagCapture Exosome Isolation Kit PS, isolated for RNA using the Quick-RNA MicroPrep Kit, and quantified by RT-qPCR. 8−12 mouse pups were used as replicates for each experimental condition.

The total rotavirus load in the stool, defined as the cumulative rotavirus amount in the mouse stool per pup from 1 to 7 dpi, was calculated by

$$\text{Total rotavirus load in stool} = \sum_{j=1}^{7} \left(\frac{C_{\text{rotavirus}} \times W_{\text{stool}}}{N} \right)_{\text{at } j \text{ dpi}}$$

where $C_{\text{rotavirus}}$ is the rotavirus concentration in the stool (gene copies per gram of stool), W_{stool} is the stool weight collected from all mouse pups (g), and N is the number of mouse pups. Special attention should be paid to the assumption in estimating the total rotavirus load in the stool: collected rotaviruses in the stool to overall defecated rotaviruses remained constant for any mouse pup infected with rotaviruses after any treatment. It was impossible to collect all the stool as the pups defecated all day long, but we collected the stool at the exact same time each dpi and harvested all the available stool in each collection.

Pre-treatment of Stool and Small Intestines for the Reverse Transcription-Quantitative Polymerase Chain Reaction Assay. Rotavirus shedding in the stool and replication in small intestines were monitored as the indicators of rotavirus infectivity in vivo. Zero dpi samples included the stool collected before rotavirus inoculation to mouse pups and the small intestines collected at 6 h post infection (hpi). Stool was collected at pups' anuses every 24 hpi (equivalent to 1 dpi) by gently pressing pups' bellies until no stool was released. Small intestines were surgically extracted at different dpi after the pups were euthanized. The stool and small intestines were stored at 4 and −80 °C until RNA isolation.

The stool was suspended in 1× PBS buffer to obtain a 10% (w/v) stool suspension, and the clarified supernatant was collected after centrifugation at 1000×g for 5 min at 10 °C. Then, 5 μL of the supernatant was added to 100 μL of RNA lysis buffer and then proceeded to the column purification of RNA using the Quick-RNA MicroPrep Kit. RNA was eluted in 15 μL of RNase-DNase free water and stored at −20 °C until 5 μL of RNA was further proceeded to the reverse transcription to cDNA.

Only the upper small intestines were proceeded for quantifying rotavirus replication. Around 50 mg of the tissue was first homogenized in 300 μL of DNA/RNA shield buffer provided using the Quick-RNA MiniPrep Kit (Zymo Research, CA, USA, R1055) supplemented with proteinase K, followed by further digestion for 5 h at 55 °C. An equal volume of RNA lysis buffer was added to the digested tissue and frozen at −20 °C overnight. Frozen samples were thawed at room temperature and centrifuged at 15,000×g for 2 min. The clear supernatant was then subjected to the column purification of RNA using the Quick-RNA MiniPrep Kit. RNA was eluted in 50 μL of RNase-DNase free water, quantified using NanoDrop (Thermo Fisher Scientific, NanoDrop 2000c spectrophotometer) for concentration, and stored at −20 °C. 2 μg of total RNA was further subjected to the reverse transcription to cDNA.

Reverse Transcription-Quantitative Polymerase Chain Reaction. 10 μL of cDNA was prepared with the RNA template, 2 μL of 5× Reaction Mix, 1 μL of Maxima Enzyme Mix, and nuclease-free water provided by the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA, K1672). Reverse transcription was performed according to the following temperature program: 25 °C for 10 min, 50 °C for 60 min, 85 °C for 5 min, and finally cooling down to 4 °C. cDNA was stored at −20 °C until further RT-qPCR quantification.

RT-qPCR was conducted using the iTaq Universal SYBR Green Supermix kit (Bio-Rad, 1725124). Each 10 μL of reaction mix contained 3 μL of four/16-fold diluted cDNA template, 5 μL of SYBR Green Supermix, 0.67 μL of forward and reverse primer (10 μM), and 1.33 μL of nuclease-free water. The absolute genome copy numbers were standardized using a series of 10-fold dilutions (10^3 to 10^{10} copies mL^{-1}) of synthetic cDNA oligos from Integrated DNA Technologies (IDT) (Coralville, Iowa). Detailed sequence information for primer pairs and the standard is listed in Table S2. The RT-qPCR program was run with the real-time PCR system of the LightCycler 96 Instrument (Roche) or Applied Biosystems QuantStudio 7 Pro (Thermo Fisher Scientific): preincubation (95 °C for 90 s), 40 cycles of two-step amplification (95 °C for

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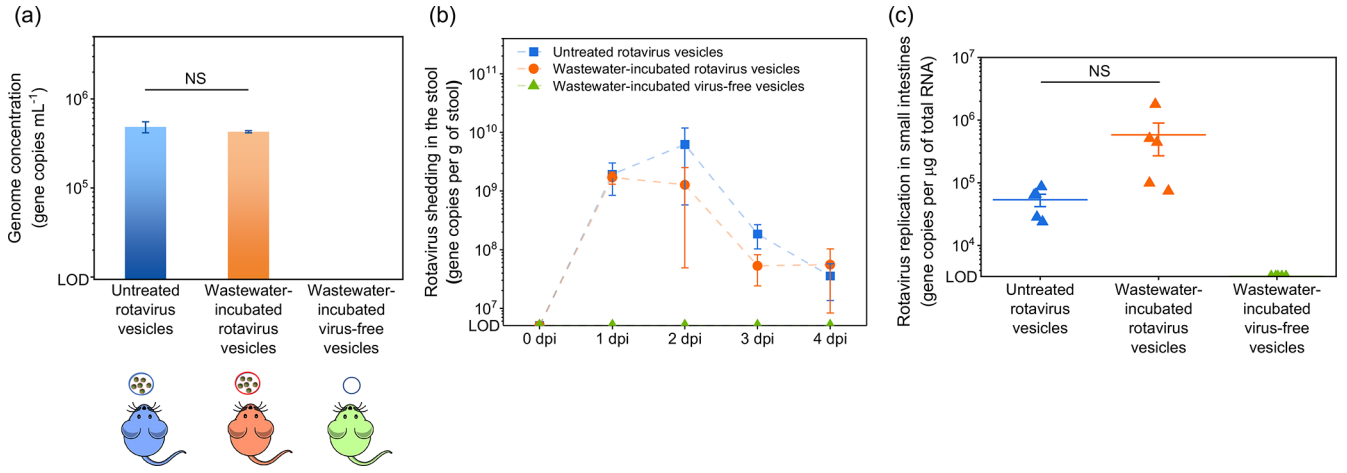

Figure 2. Wastewater-incubated rotavirus vesicles remain infectious to mouse pups. (a) Rotavirus concentrations in gavage inoculum for mouse pups; (b) rotavirus shedding in the mouse stool at 0−4 days post infection (dpi); and (c) rotavirus replication in small intestines at 4 dpi. Virus-free vesicles were harvested from the stool of healthy mouse pups. For wastewater-incubated rotavirus vesicles and virus-free vesicles, rotavirus vesicles and virus-free vesicles were incubated in filtered raw wastewater for 7 days at room temperature in the dark before being inoculated to 10 day old BALB/c mouse pups. All data were measured by RT-qPCR. The LOD was 9.40×10^3 gene copies mL^{-1} for (a), 5.05×10^6 gene copies per g of stool for (b), and 6.73×10^3 gene copies per μg of total RNA for (c). All data below LOD were substituted by the LOD. Six mouse pups were involved in each experiment. NS represents $p > 0.05$.

10 s, 52 °C for 10 s, and 72 °C for 10 s), melting (95 °C for 10 s, 65 °C for 60 s, and 97 °C for 1 s), and cooling at 37 °C for 30 s. RNA-free water was used as the negative control. qPCR efficiency for rotavirus was 88.0−102.9%, and its coefficient of determination was 0.9931−0.9994 (Table S3). The cDNA template was diluted four/16-fold to avoid inhibition for qPCR. All cycle threshold values above 30 were considered under the limit of detection (LOD), where deviation from the linear relationship was found in the standard curve of $\log_{10}$ cDNA concentrations versus the cycle threshold values. All the RT-qPCR data were reported following MIQE guidelines.[29]

Statistical Analysis. Unpaired Student's *t*-test was applied for the statistical comparison, which returned to a *p* value. All *p* values < 0.05 were considered statistically significant.

RESULTS

Rotavirus Vesicles are Persistent in Freshwater and Wastewater. After rotavirus vesicles were incubated with filtered Potomac River water and wastewater at room temperature for a certain duration, they were recovered with TIM4-coated magnetic beads and quantified by RT-qPCR. TIM4 is known to specifically bind to PS on the EV membrane but not naked viruses; therefore, RT-qPCR only quantified the viruses associated with vesicles, like intact viral vesicles. Figure 1a indicates that rotavirus vesicles remained intact upon exposure to filtered freshwater and wastewater for at least 7 days, and Figure 1b highlights that 27.5 ± 4.4 and 24.5 ± 2.2% of the vesicles still remain intact in the filtered freshwater and wastewater at 16 weeks, respectively ($p < 0.05$). The results clearly demonstrate that rotavirus vesicles isolated from animal excreta are highly persistent in aquatic environments. Special attention should be paid to the recovery of viral vesicles using the TIM4-based approach because viral vesicles with a membrane opening smaller than the size of an individual virion or broken vesicles with virions attached could still be harvested and the viruses could be quantified. This might overestimate the contribution of intact viral vesicles in the whole viral population, and damaged vesicles might bring multiple virions into the host cell and increase MOI for successful infection.

Rotavirus Vesicles Maintain Their Infectivity after Incubation in Wastewater. Beyond the integrity of rotavirus vesicles, we also evaluated the infectivity of rotavirus vesicles after exposure to filtered wastewater at room temperature for 7 days. Rotavirus vesicles with or without 7 day wastewater incubation were orally fed to 10 day old BALB/c mouse pups with the same dosage ($4.85 \pm 0.68 \times 10^4$ and $4.28 \pm 0.12 \times 10^4$ gene copies per mouse pup for untreated and wastewater-incubated rotavirus vesicles, respectively, $p > 0.05$, Figure 2a) to understand the impact of wastewater incubation on virus infectivity. Both groups of the mouse pups started showing diarrhea symptoms from 1 dpi (Figure S2) and shed a similar level of rotaviruses in the stool from 1 to 4 dpi (Figure 2b). Moreover, rotavirus replication in small intestines at 4 dpi was statistically the same between the two groups ($p > 0.05$, Figure 2c). In contrast, the negative control was conducted by feeding mouse pups with virus-free vesicles (isolated from healthy mouse pups) after 7 days of wastewater incubation, and neither diarrhea symptoms, rotavirus shedding in the stool, nor rotavirus replication in small intestines was observed (Figures S2 and 2b,c). These results suggest that the viruses inside the vesicles but not the vesicle lumen contents nor wastewater lead to rotavirus infection and that rotavirus vesicles isolated from the animal stool are highly persistent and remain infectious in wastewater environments.

Rotavirus Vesicles are Resistant to Free Chlorine Disinfection. We next evaluated the resistance of rotavirus vesicles to free chlorine disinfection compared to that of free rotaviruses. Free rotaviruses were released from the same batch and amount of rotavirus vesicles by a non-detergent lysis buffer and they had the same viral load as rotavirus vesicles for infecting 10 day old BALB/c mouse pups ($2.14 \pm 0.18 \times 10^5$ and $1.97 \pm 0.07 \times 10^5$ gene copies per mouse pup for rotavirus vesicles and free rotaviruses, respectively, $p > 0.05$, Figure 3a). The non-detergent lysis buffer was selected because it effectively lyses EVs liberating viruses but does not negatively impact virus infectivity in animal studies.[1,4] Next, rotavirus

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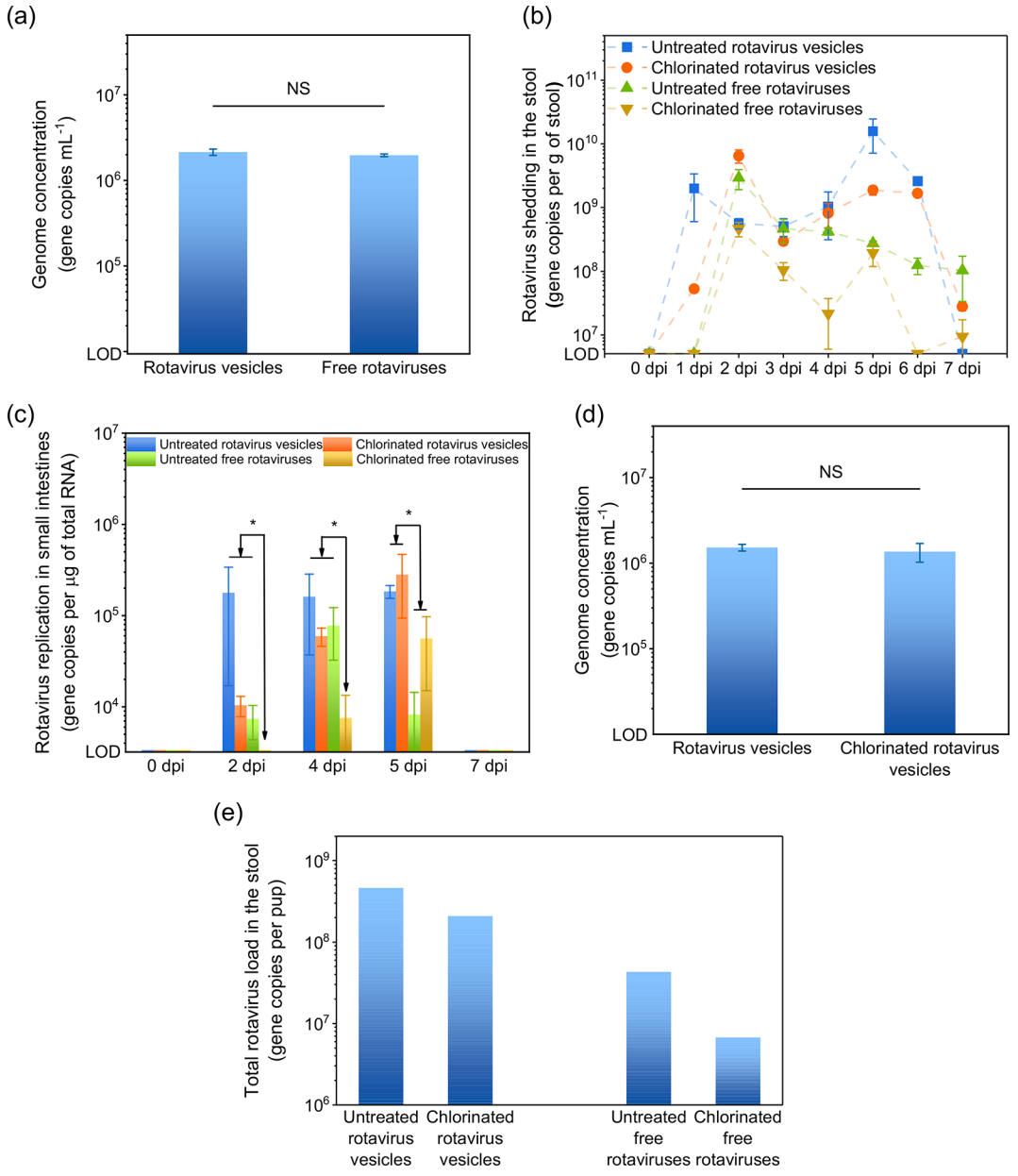

Figure 3. Rotavirus vesicles are resistant to free chlorine disinfection. (a) Rotavirus concentrations in the gavage inoculum for mouse pups; (b) rotavirus shedding in the mouse stool at 0−7 days post infection (dpi); (c) rotavirus replication in small intestines at 0, 2, 4, 5, and 7 dpi; (d) rotavirus vesicles recovered using TIM4-coated magnetic beads before and after chlorination; and (e) cumulative rotavirus loads in the mouse stool from 1−7 dpi. For chlorinated rotavirus vesicles and free rotaviruses, the viruses were treated with 13.3 mg min L^{-1} of free chlorine and then orally inoculated to 10 day old BALB/c mouse pups. All data were measured by RT-qPCR. The LOD was 9.40×10^3 gene copies mL^{-1} for (a), 5.05×10^6 gene copies per g of stool for (b), 6.73×10^3 gene copies per μg of total RNA for (c), and 1.00×10^4 gene copies mL^{-1} for (d). All data below LOD were substituted by the LOD. 8−12 pups were involved in each experiment. NS and * represent $p > 0.05$ and $p < 0.05$, respectively.

vesicles and free rotaviruses were exposed to the same dosage of free chlorine at 13.3 mg min L^{-1}, and their infectivity, along with the infectivity of rotavirus vesicles and free rotaviruses without disinfection, was evaluated. Consistent with our previous findings,[4] mouse pups infected with free rotaviruses when compared to rotavirus vesicles, regardless of disinfection, showed delayed diarrhea symptom onsets (2 vs 1 dpi, Figures 3b and S3). Free rotavirus-inoculated mouse pups exhibited much less rotavirus shedding in the stool and replication in small intestines at 1 and 5−7 dpi (Figure 3b,c). More importantly, mouse pups infected with chlorinated free rotaviruses, when compared to untreated free rotaviruses, showed a consistently lower level of rotavirus shedding in the stool across the whole infection period (Figure 3b) and less rotavirus replication in small intestines before virus clearance at 5 dpi ($p < 0.05$, Figure 3c). In contrast, rotavirus shedding in the stool from mouse pups either inoculated with chlorinated or untreated rotavirus vesicles was similar from 3 to 7 dpi (Figure 3b), and rotavirus replication in small intestines was also statistically the same at 2, 4, and 5 dpi ($p > 0.05$, Figure 3c). Note that using TIM4 bead-based vesicle-pull down, we did not observe a significant change in recovered rotavirus vesicles after free chlorine treatment ($1.52 \pm 0.14 \times 10^6$ and $1.36 \pm 0.34 \times 10^6$ gene copies mL^{-1} for rotavirus vesicles before and after chlorination, respectively, $p > 0.05$, Figure 3d), implying that vesicles may remain intact and lipid molecules

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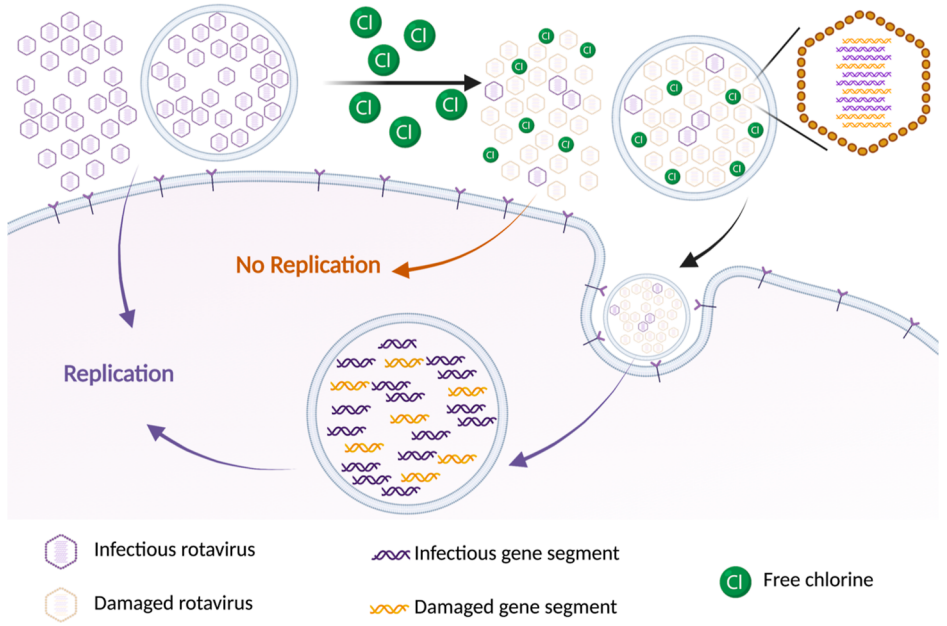

Figure 4. Rotavirus vesicle infection after chlorination. Each rotavirus vesicle might contain >25 virions.

like PS might not react with free chlorine effectively.[30] Special attention should be paid to the fact that damaged yet virus-associated vesicles could exist.

We propose the total rotavirus load in the stool, that is, cumulative rotaviruses in the stool per mouse pup over the infection period of 7 days, as an indicator for qualitatively evaluating the environmental transmissibility of rotaviruses by the fecal-oral route. The clinical symptoms of mouse pups (e.g., watery stool and diarrhea) were positively correlated to virus shedding in stool and virus replication in small intestines (Figures S2 and S3), which is also consistent with our previous study.[4] Mouse pups inoculated with untreated rotavirus vesicles shed at least 10-times more viruses in their stool when compared to mouse pups infected by untreated free rotaviruses (Figure 3e). The reduction of the total rotavirus load in the stool resulting from chlorination was much more significant for free rotavirus-inoculated mouse groups than for rotavirus vesicle-inoculated mouse groups. Notably, mouse pups infected with chlorinated rotavirus vesicles still shed significantly more stool viruses than pups inoculated with untreated free rotaviruses. The concentration of rotavirus shedding in the stool, the rotavirus replication level in small intestines, and the total rotavirus load in the stool were applied together as indicators from multi-dimensions to reflect the rotavirus infectivity before and after free chlorine disinfection. Collectively, these data indicate that, in striking contrast to free rotaviruses, the in vivo infectivity of rotavirus vesicles is largely maintained even after free chlorine disinfection and that rotavirus vesicles may be more environmentally transmissible than free rotaviruses regardless of disinfection. Special attention should be paid to interpreting the data because the total rotavirus load in the stool could reach a plateau with a continuous increase in the amount of infectious rotaviruses for feeding mouse pups, which may lead to an inaccurate quantitative comparison of the infectivity. Therefore, we use the total rotavirus load in the stool as a metric for qualitative comparison of the environmental transmissibility of rotaviruses before and after disinfection. We also acknowledge that the conclusion is valid under the current experimental conditions (e.g., virus strain, host type, disinfection conditions, and viral load in the inoculum for feeding pups). Future work needs to validate the total virus load in the stool for quantitative analyses and develop new metrics to accurately reflect virus infectivity in vivo.

DISCUSSION

Viral vesicles have significant environmental impacts because they can be highly persistent in freshwater and wastewater up to months (Figure 1). The persistence of viral vesicles could be attributed to the unique chemical composition of vesicle membranes because a threefold enrichment of cholesterol and sphingomyelin was found in viral vesicle membranes when compared to plasma membranes, and these chemicals are well-known for facilitating membrane integrity.[4,10,12,31] Water is a key environmental medium for virus transmission, especially enteric viruses. Untreated or inadequately treated wastewater can carry viral vesicles to natural aquatic environments and to reused water for agricultural irrigation, recreation, and drinking. Non-enveloped viruses, like enteric viruses, pose significant health concerns because they can persist as free viruses in aquatic environments for days to months under ambient conditions with limited decay of the infectivity.[18,19,21] Even worse, many non-enveloped viruses have been found in vesicles to date, including rotavirus, norovirus, coxsackievirus, poliovirus, and hepatitis A and E viruses,[2,3] and the excellent stability of viral vesicles further exacerbates public health concerns about these pathogens because of the significantly increased infectivity of viral vesicles in comparison to their free virus counterparts.[2,3] Even if the vesicle membrane is broken, free viruses are released and they can still infect susceptible hosts. This behavior is in sharp contrast to that of enveloped viruses that also possess a lipid membrane. Enveloped viruses are generally less stable in the environment than non-enveloped viruses,[32,33] and they lose their fusion capacity and infectivity once the envelope is broken. Therefore, viral vesicles are unique pathogenic units; they blur the distinction between non-enveloped and enveloped viruses but behave like neither of them.

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Viral vesicles have significant public health impacts because they are resistant to disinfection. Free chlorine is known to react with viral proteins and genomes instead of lipids,[30,34,35] and we also did not observe a significant change in vesicle integrity after free chlorine exposure. Therefore, it is reasonable to speculate that hypochlorous acid (the dominant free chlorine species at pH 6) could penetrate through the vesicle membrane and oxidize the viral capsid proteins and genomes, whereas the vesicle membrane structure remains intact (schematic model, Figure 4). Although viral proteins and genomes are compromised after disinfection, viral vesicles could benefit from the *en bloc* transmission of multiple virions for successful infection through increased MOI and/or genetic recombination or reassortment (Figure 4). *En bloc* transmission through vesicles brings multiple viral genomes into a host cell, which produces enough viral proteins at the beginning of the infection to overcome host defenses. The lesion of an individual genome after disinfection could also be remediated through recombination or reassortment since multiple viral genomes are present in the cytoplasm.[36,37] Considering that each rotavirus vesicle might contain more than 25 virions, *en bloc* transmission would be highly beneficial for rotavirus replication in host cells even if a large portion of virions are damaged in disinfection.

Discrepancy of rotavirus resistance to disinfection was observed in in vivo versus in vitro studies. Free chlorine at a high dosage of 13.3 mg min L^{-1} is expected to inactivate more than 5-log_{10}s of rotaviruses based on published in vitro studies.[25−27] Considering ~10^5 gene copies of rotaviruses were fed to mouse pups and assuming all rotaviruses were infectious before disinfection, none or only few infectious virions should be left after disinfection. Surprisingly, both rotavirus vesicles and free rotaviruses after disinfection were still infectious to mouse pups. This result calls for careful inspection and comparison of disinfection performance among different studies because the results could be highly dependent on the virus strain, host type, and in vitro and in vivo model systems.[25−27,38] Particularly, the minimum number of virions for successful infection, which is critical to determine disinfection efficacy, may vary in different systems. A virus strain that could be tested both in vitro and in vivo might be a good choice to investigate virus persistence and resistance to disinfectants.

Environmental Implications. Our work highlights the environmental, biological, and public health impacts of viral vesicles as emerging pathogenic units. Viral vesicles are highly prevalent in human and animal excreta that can contaminate the environment like water;[4] they are extremely persistent in freshwater and wastewater, and they are resistant to disinfection by free chlorine and UV_{254}.[1] With an ever-increasing demand of freshwater supply, water reuse has emerged as a promising practice for providing sufficient and safe water for agricultural, recreational, industrial, and drinking purposes. However, the presence of viral vesicles could significantly challenge the microbial safety of reused water. We need to conduct surveillance to understand the presence, removal, and inactivation of viral vesicles in wastewater treatment and water reuse chains and scrutinize current treatment processes for effective control of the viral vesicles. Moreover, since viruses can also transmit through other environmental media like food, air, and fomites, there is also a pressing need to understand the prevalence and persistence of viral vesicles in these environments.

Our study also calls for attention on selecting appropriate human pathogen surrogates and model systems for evaluating virus infectivity. Our work is novel because we used an animal model to study rotaviruses, and it has significant environmental and biological relevance. EDIM rotaviruses infecting mouse pups can best simulate human rotaviruses infecting humans, especially for infants and young children, and both infectivity and environmental impacts for spreading the viruses were characterized by virus replication in small intestines and virus shedding in the stool, respectively. All these merits are not available in cell culture studies. However, we must also admit that animal studies are expensive and time-consuming, and they require minimizing the number of animals. Therefore, it is not affordable to evaluate disinfection kinetics with a range of disinfectant dosages in vivo.

Moreover, our study also highlights a unique mechanism of the *en bloc* transmission of viruses mediated by vesicles, which promotes virus infectivity and possible virus resistance to disinfection. Although it remains elusive how a viral vesicle enters into a host cell, a vesicle with an intact membrane can bring multiple damaged virions and viral genomes into the host cell after disinfection to enable successful infection through genetic recombination and reassortment.[4,5,36,37] An increased dosage of disinfectants is needed to fully inactivate viral vesicles when compared to free viruses; however, special attention should be paid to the challenge of associated disinfection byproducts. Another approach is to destabilize the vesicles and release free viruses that are more easily inactivated. Certain disinfectants like ozone could oxidize lipids and potentially destruct the vesicle membrane structure. Detergents that are known to disrupt lipid membranes could also be considered for food and contact surface sanitation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c00732.

N,N-diethyl-*p*-phenylenediamine (DPD) colorimetric method for free chlorine quantification; characteristics of water samples; sequence information used in RT-qPCR; summary of qPCR assay efficiencies and R^2 of standard curve regression; schematic of a viral vesicle compared with an enveloped virus and a non-enveloped virus; stool of mouse pups infected with untreated rotavirus vesicles, wastewater-incubated rotavirus vesicles, and wastewater-incubated virus-free vesicles at 1 dpi; and stool of mouse pups infected with untreated rotavirus vesicles, chlorinated rotavirus vesicles, untreated free rotaviruses, and chlorinated free rotaviruses at 1 and 2 dpi (PDF)

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Notes

The authors declare no competing financial interest.

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