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Survival of surrogate coronaviruses in water

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ABSTRACT

The emergence of a previously unknown coronavirus infection, Severe Acute Respiratory Syndrome (SARS), demonstrated that fecally contaminated liquid droplets are a potential vehicle for the spread of a respiratory virus to large numbers of people. To assess potential risks from this pathway, there is a need for surrogates for SARS coronavirus to provide representative data on viral survival in contaminated water. This study evaluated survival of two surrogate coronaviruses, transmissible gastroenteritis (TGEV) and mouse hepatitis (MHV). These viruses remained infectious in water and sewage for days to weeks. At 25 °C, time required for 99% reduction in reagent-grade water was 22 days for TGEV and 17 days for MHV. In pasteurized settled sewage, times for 99% reduction were 9 days for TGEV and 7 days for MHV. At 4 °C, there was $<1\ \log_{10}$ infectivity decrease for both viruses after four weeks. Coronaviruses can remain infectious for long periods in water and pasteurized settled sewage, suggesting contaminated water is a potential vehicle for human exposure if aerosols are generated.

1. Introduction

The Coronaviridae have been recognized for many years as a cause of common-cold-like, self-limiting respiratory infections (Monto, 1998), but the 2003 emergence of Severe Acute Respiratory Syndrome (SARS) brought new recognition that coronavirus infection could result in serious, even fatal, disease. The etiologic agent of SARS was quickly identified as a previously unknown coronavirus (Drosten et al., 2003). Emerging in an age of global travel, large healthcare facilities, and high-density housing developments, SARS coronavirus (SARS-CoV) was not only a novel human pathogen, but one that spread by novel routes. A respiratory agent transmitted from person-to-person by droplets and aerosols, SARS-CoV spread from passenger to passenger on an airplane (Olsen et al., 2003) and from patients to healthcare workers and visitors in hospitals (Seto et al., 2003; Varia et al., 2003; Chen et al., 2004). As efforts increased to halt further person-to-person spread of the disease from travelers and in healthcare facilities, an outbreak of SARS in a high-density Hong Kong housing complex led to the discovery of a new route of transmission, previously unknown for a respiratory virus.

In this outbreak, SARS-CoV shed in the feces of an infected building visitor may have spread disease to other occupants of the building via droplets and aerosols of virus-contaminated commode water, which entered multiple apartments through faulty toilet plumbing and floor drains (McKinney et al., 2006). This outbreak scenario suggests that if SARS were to reemerge in the future, water contaminated with the fecal waste of infected individuals could be a vehicle for transmission. This unique fecal droplet–respiratory route is potentially important, but aspects of it remain poorly understood. One such aspect is the role of viral stability: if SARS-CoV is capable of surviving for relatively long periods of time in water, exposure

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and transmission via fecally contaminated droplets of water may be more likely. In order to better assess the risks posed by this novel exposure pathway, data are needed on the survival and persistence of SARS-CoV in water and sewage. Because working with SARS requires specially trained personnel working in BSL-3 laboratory containment, there are significant challenges involved in studying the survival of this virus, and very few data are currently available. The use of surrogate viruses to overcome these challenges and expand the available data on coronavirus survival and persistence in water was the focus of this study.

Other members of the Coronaviridae may be appropriate surrogates for SARS-CoV, providing representative survival data that can be used to conduct risk assessments of SARS transmission via water-related pathways. Choosing a surrogate virus most similar to SARS-CoV is challenging because there is still disagreement about the exact placement of this virus within the Coronaviridae. The family is divided into three groups: 1 and 2 include human and other mammalian coronaviruses, and Group 3 consists of avian viruses. SARS is thought to be related to the Group 2 coronaviruses (Jackwood, 2006), and phylogenetic analyses have indicated it may be closely related to mouse hepatitis virus (MHV) (Lio and Goldman, 2004). However, its exact relationship to the other coronaviruses is still unclear (Gorbalenya et al., 2004). Therefore, two potential surrogates were evaluated in this study, representing both groups of mammalian coronaviruses. The two viruses chosen for study were transmissible gastroenteritis virus (TGEV), a diarrheal pathogen of swine and a member of the Group 1 coronaviruses, and mouse hepatitis virus (MHV), a respiratory and enteric pathogen of laboratory mice and a member of the Group 2 coronaviruses (Jackwood, 2006). The survival and persistence of these viruses was observed in reagent-grade water, lake water, and settled human sewage at two temperatures over a period of weeks to provide estimates of how long members of the coronavirus family, as potential surrogates for SARS-CoV, can remain infectious in these waters.

2. Materials and methods

2.1. Test waters

Reagent-grade water (pH 6.0, turbidity 0.1 NTU) was produced from laboratory tap water by a Dracor™ water purification system (Dracor, Durham, NC) which includes treatment by reverse osmosis and ultraviolet light irradiation. Lake water (pH 7.5, turbidity 1.73 NTU) came from University Lake, an impoundment that serves as the drinking water source for the town of Chapel Hill, NC, and was obtained from the raw water inlet of the Orange Water and Sewer Authority (OWASA) drinking water treatment plant. The wastewater used to produce pasteurized settled sewage was obtained from the OWASA wastewater reclamation facility. Settled sewage is raw sewage that has undergone an initial settling step after entry into the plant to separate large solids from the liquid. The resulting liquid (pH 7.6, turbidity 17.6 NTU) was pasteurized in a waterbath at 70 °C for 3 h to inactivate other microorganisms that would interfere with cell culture infectivity assays of coronaviruses.

2.2. Preparation of viral stocks

TGEV and MHV were kindly provided by R. Baric, University of North Carolina, Chapel Hill. TGEV was grown in swine testicular (ST) cell cultures, and MHV was grown in delayed brain tumor (DBT) cell cultures. Viral stocks were propagated by infecting confluent layers of host cell cultures in flasks, harvesting cell lysates, clarifying by centrifugation ($3000\times g$, 30 min, 4 °C), and storing resulting supernatants as virus stock at −80 °C. Viral titers were determined by quantal assays for cytopathic effect (CPE) and expressed as the most probable number (MPN). Assays were on confluent host cell layers in 24-well plates containing maintenance medium consisting of Eagle's minimum essential medium (MEM), 10% bovine serum replacement (Fetal Clone II, Hyclone, Logan, UT), 10% lactalbumin hydrolysate, and gentamicin (0.1 mg/mL)/kanamycin (0.05 mg/mL).

2.3. Survival experiments

For each virus, 5 mL of clarified virus stock was spiked into duplicate 45 mL aliquots of test water. A positive control sample for measuring the initial virus concentration in water at time 0 was taken and assayed immediately after spiking. One aliquot of test water was held at room temperature (23–25 °C), and another one was held at refrigerator temperature (4 °C). Both samples were held without agitation for the duration of the experiment. At each time point, samples were taken and assayed for virus infectivity on the appropriate host cell line. Four replicate samples were assayed at each time point. Virus survival at each time point was expressed as $\log_{10}(N_t/N_0)$, where N_t is the virus concentration in MPN/mL at time t, and N_0 is the initial virus concentration in MPN/mL in the control sample at time 0.

2.4. Statistical analysis

Analysis was done using Excel 2003 (Microsoft Corp.) and GraphPad Prism 5 (GraphPad, San Diego, CA). The parameter $\log_{10}(N_t/N_0)$ vs. time was used to perform regression analysis for each virus and water type. Coefficients from regression analysis were used to predict times needed for 90, 99, 99.9, and 99.99% reduction of each virus at each temperature in test waters.

3. Results

The change in infectious titer of TGEV and MHV in reagent-grade water over 49 days at 25 °C and 4 °C is summarized in Fig. 1a and b. There was a progressive decline in the infectivity of both TGEV and MHV over 49 days at 25 °C, and this reduction follows typical first-order kinetics (Fig. 1a). The infectivity of TGEV declined by approximately 0.6 $\log_{10}$ per week, and infectious MHV declined by approximately 0.8 $\log_{10}$ per week. Time required for 99% reduction in infectious titer in reagent-grade water at 25 °C was 22 days for TGEV and 17 days for MHV. There was no significant decline in infectious titer of either virus over 49 days at 4 °C (Fig. 1b).

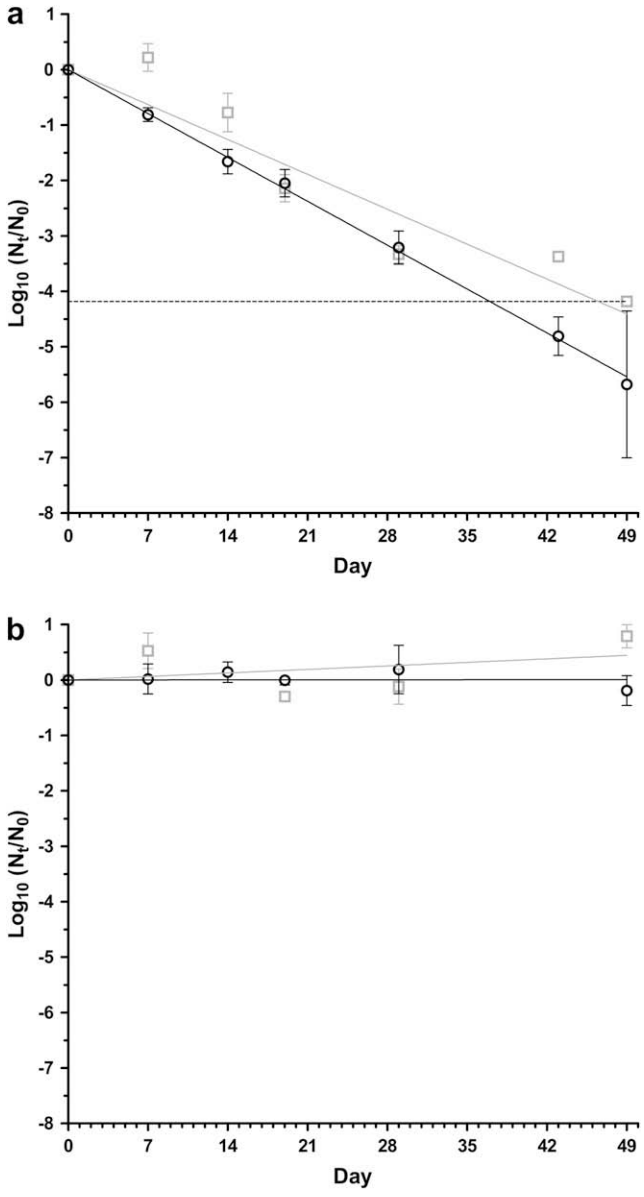

Fig. 1 – (a) Infectivity of TGEV (time 0 titer 4.5 $\log_{10}$ MPN/mL) and MHV (time 0 titer 6.5 $\log_{10}$ MPN/mL) over 49 days in reagent-grade water at 25 °C, 4 trials per point. Observed data = points; predicted values from regression analysis = lines (gray squares and lines = TGEV; black circles and lines = MHV; dashed line = TGEV detection limit). (b) Infectivity of TGEV (time 0 titer 4.8 $\log_{10}$ MPN/mL) and MHV (time 0 titer 6.5 $\log_{10}$ MPN/mL) over 49 days in reagent-grade water at 4 °C, 4 trials per point. Observed data = points; predicted values from regression analysis = lines (gray squares and lines = TGEV; black circles and lines = MHV).

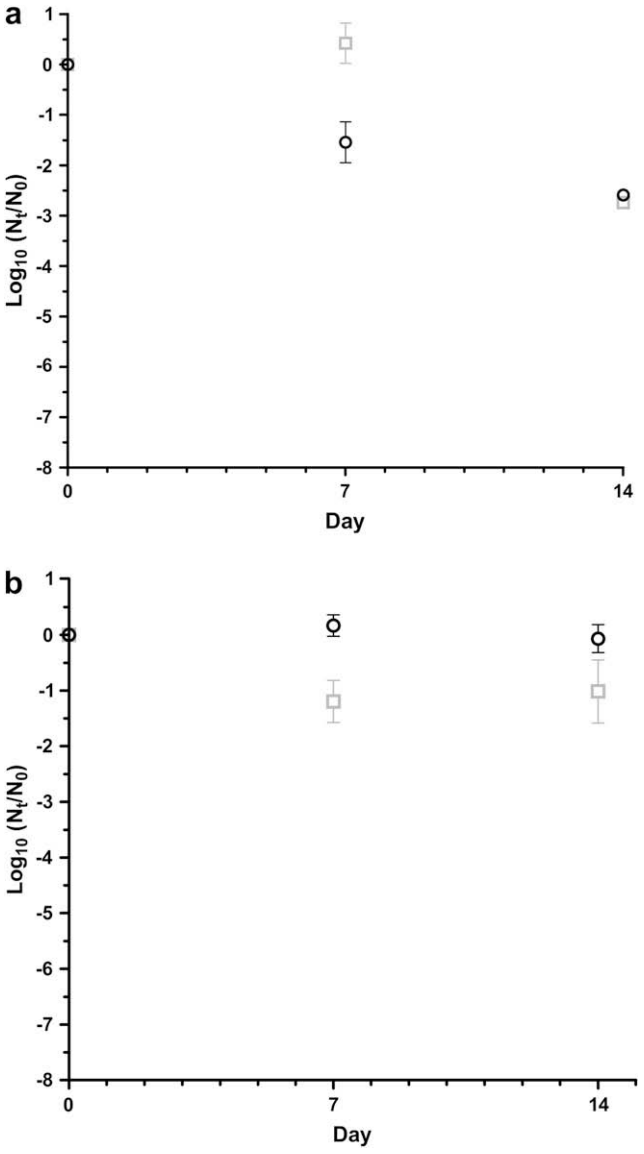

Fig. 2 – (a) Infectivity of TGEV (time 0 titer 5.0 $\log_{10}$ MPN/mL) and MHV (time 0 titer 6.9 $\log_{10}$ MPN/mL) over 14 days in lake water at 25 °C, 4 trials per point (gray squares and lines = TGEV; black circles and lines = MHV). (b) Infectivity of TGEV (time 0 titer 5.2 $\log_{10}$ MPN/mL) and MHV (time 0 titer 6.6 $\log_{10}$ MPN/mL) over 14 days in lake water at 4 °C, 4 trials per point (gray squares and lines = TGEV; black circles and lines = MHV).

Fig. 2a and b shows the infectious TGEV and MHV titers in lake water over 14 days at 25 °C and 4 °C. Time required for 99% reduction in infectious titer in lake water at 25 °C was 13 days for TGEV and 10 days for MHV. At 4 °C, TGEV infectivity declined by approximately 1 $\log_{10}$ by day 14; in contrast, MHV infectivity persisted with no decline in titer after 14 days at 4 °C. Because there were only two time points (7 and 14 days), regression analysis was not performed on data from lake water.

The change in infectious titer of TGEV and MHV in pasteurized settled sewage (pH 7.6, turbidity 17.6 NTU) over 35 days at 25 °C and 4 °C is summarized in Fig. 3a and b. There was a progressive decline in the infectivity of both TGEV and MHV at 25 °C. The reduction in infectivity of both viruses at 25 °C follows typical first-order kinetics (Fig. 3a). There was a more rapid decline in infectivity titer of both viruses at 25 °C than at 4 °C (the experiment at 25 °C was terminated at day 21 due to subsequent growth of contaminating microorganisms in the test water). Regression analysis showed that infectivity of TGEV at 25 °C

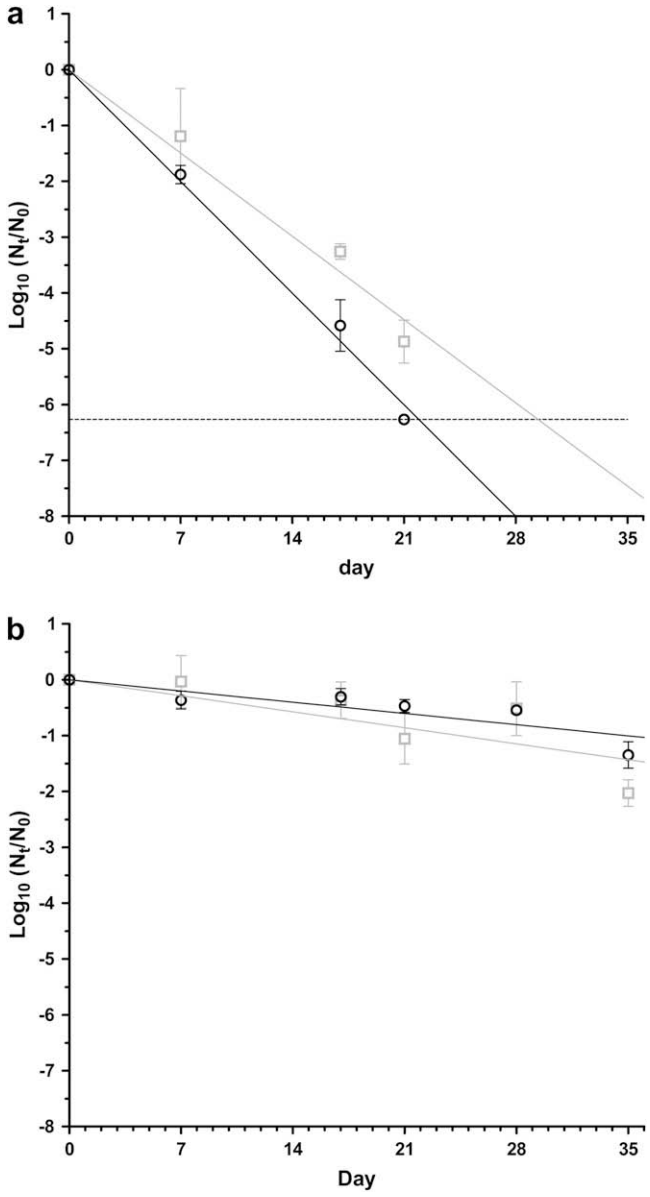

Fig. 3 – (a) Infectivity of TGEV (time 0 titer 5.8 $\log_{10}$ MPN/mL) and MHV (time 0 titer 6.6 $\log_{10}$ MPN/mL) over 21 days in pasteurized settled sewage at 25 °C, 4 trials per point. Observed data = points; predicted values from regression analysis = lines (gray squares and lines = TGEV; black circles and lines = MHV; dashed line = MHV detection limit). (b) Infectivity of TGEV (time 0 titer 5.5 $\log_{10}$ MPN/mL) and MHV (time 0 titer 6.8 $\log_{10}$ MPN/mL) over 35 days in pasteurized settled sewage at 4 °C, 4 trials per point. Observed data = points; predicted values from regression analysis = lines (gray squares and lines = TGEV; black circles and lines = MHV).

declined by approximately 1.5 $\log_{10}$ per week, and that of MHV declined by approximately 2 $\log_{10}$ per week. Times for 99% reduction in infectious titer were 9 days for TGEV and 7 days for MHV. Both viruses exhibited a similar slow rate of decline in infectivity in settled sewage at 4 °C, with 1.3 $\log_{10}$ reduction of MHV and 2 $\log_{10}$ reduction of TGEV after 35 days (Fig. 3b). Regression analysis showed that TGEV declined by approximately 0.3 $\log_{10}$ per week, and MHV by 0.2 $\log_{10}$ per week at 4 °C.

Table 1 – Predicted times (in days) for decimal reductions of TGEV and MHV infectivity in different water types at 4 °C and 25 °C.

Reduction	Reagent-grade water				Pasteurized settled sewage			
	25 °C		4 °C		25 °C		4 °C	
($\log_{10}$ (N_t/N_0))	TGEV	MHV	TGEV	MHV	TGEV	MHV	TGEV	MHV
−1 (90%)	11	9	110	>365	4	3	24	35
−2 (99%)	22	17	220	>365	9	7	49	70
−3 (99.9%)	33	26	330	>365	14	10	73	105
−4 (99.99%)	44	35	330	>365	19	14	98	139

Regression analysis on data from reagent-grade water and pasteurized settled sewage was compared to determine if water quality has an effect on virus survival. Table 1 shows the predicted values obtained by regression analysis to achieve 90%, 99%, 99.9%, and 99.99% reduction of TGEV and MHV in each water type. The time required for 4 $\log_{10}$ (99.99%) infectivity reduction of TGEV at 25 °C is longer in reagent-grade water than in pasteurized settled sewage (44 days vs. 19 days). This is also true for MHV (35 days in reagent-grade water vs. 14 days in sewage). There is also a difference in predicted inactivation times between viruses. In both water types, the predicted time to achieve a 4 $\log_{10}$ reduction in viral infectivity titer at 25 °C is longer for TGEV than for MHV (44 days vs. 35 days in reagent-grade water and 19 days vs. 14 days in pasteurized settled sewage). At 4 °C, the time required to achieve a 4 $\log_{10}$ reduction in infectivity titer in pasteurized settled sewage was 98 days for TGEV vs. 139 days for MHV, and predicted times for 4 $\log_{10}$ infectivity reduction of both viruses in reagent-grade water were approximately 1 year. Because viral titer declined so slowly at 4 °C, regression analysis based on this data set (where the longest elapsed time was 49 days) may not be a reliable way to predict viral reduction over long periods at this temperature.

Analysis was also done using a linear regression model with variables for virus type, water type, temperature, and incubation time. Virus type (TGEV or MHV), water type (reagent-grade water or pasteurized settled sewage) and temperature (4 °C or 25 °C) were dichotomous variables, and incubation time was a continuous variable. Water type ($p = 0.0071$), incubation time ($p < 0.0001$) and temperature ($p < 0.0001$) were significant predictors of $\log_{10}$ viral reduction. Virus type was not a significant predictor ($p = 0.28$). Inclusion of an interaction variable for water type and temperature did not show significant interaction between these parameters ($p = 0.47$).

4. Discussion

This study observed the stability of coronaviruses in water and sewage over long periods of time, and quantified the kinetics of viral inactivation in these media. The coronaviruses studied were capable of remaining infectious in reagent-grade waters, natural environmental waters, and waters

contaminated with human fecal waste (sewage) for periods of weeks. This long-term survival was seen at both low (4 °C) and ambient (25 °C) temperatures. In all water types, the titer of infectious virus declined more rapidly at 25 °C than at 4 °C. Infectivity titer reductions over about 6 weeks ranged from none, to slight (<1 $\log_{10}$) to modest (1–2 $\log_{10}$) at 4 °C, depending on water quality and virus type. Virus inactivation was more rapid in settled sewage than reagent-grade water.

Some comparisons can be made with the limited data available on the extent of SARS-CoV survival in water, sewage and other aqueous media. Rabenau et al. (2005) found that the titer of SARS-CoV declines approximately 0.5 $\log_{10}$ over 9 days in serum-free cell culture medium at room temperature. This is a slower rate of inactivation than was observed for TGEV and MHV in reagent-grade water and pasteurized settled sewage, and may be due to protective effects of the buffers, salts and organic nutrients found in sterile cell culture medium as compared to non-sterile water or sewage. Longer virus survival in the presence of protective buffers and salts in a sterile aqueous medium is supported by data from other investigators, who found that SARS-CoV survived longer in PBS (14 days) than in dechlorinated tap water or domestic sewage (2 days) at 20 °C (Wang et al., 2005). The survival times observed by Wang et al. (2005) in tap water and sewage are shorter than those demonstrated for TGEV and MHV. Because the authors did not report the actual change in virus titer or detection limit of the assays performed, however, a quantitative comparison of viral inactivation rates between their study and other studies is not possible. Over the course of the present study, the titer of infectious TGEV and MHV remained relatively stable in all test water types at 4 °C. This is consistent with other investigations that found SARS-CoV persisted at least 14 days at 4 °C in domestic sewage and dechlorinated tap water. Again, direct quantitative comparisons of inactivation rates are difficult, because the actual changes in viral titers over time were not reported by Wang et al. (2005).

Although coronavirus inactivation rates are difficult to compare between studies, one finding this study shares with previous work is that temperature is an important factor influencing viral survival. Temperature and incubation time were significant predictors of viral reduction in this study, which is consistent with previous findings on viral survival in water (Yates et al., 1985; Hurst et al., 1989; Enriquez et al., 1995). Water type was also a significant predictor of the rate of viral reduction, with greater reduction in pasteurized settled sewage as compared to reagent-grade water. Factors that have been suggested as contributors to greater virus reduction in more contaminated water include pH extremes, the presence of other microorganisms, and certain chemical constituents, such as proteolytic enzymes (Ward et al., 1986). However, the pasteurization process used to inactivate vegetative bacteria in the pasteurized settled sewage in these experiments may have reduced proteolytic activity in the test water. The virus inactivation kinetics observed in this study may differ from those that would be seen in raw sewage, which retains the natural proteolytic activity of vegetative bacteria, and may increase rates of viral inactivation compared to pasteurized sewage.

MHV is stable over a pH range of 5–7.4 at 37 °C and 3–10 at 4 °C (Daniel and Talbot, 1987). TGEV is stable over a pH range of 5–7 at 37 °C and 5–8 at 4 °C (Pocock and Garwes, 1975). In pasteurized settled sewage spiked with MHV, pH declined over a period of weeks (data not shown), but remained within the range of stability for these viruses, suggesting that it may not have been a significant factor in declining viral infectivity. The lack of pH effect on virus survival is consistent with previous studies (Yates et al., 1985). Chemical constituents found in sewage may have antiviral activity (Sobsey et al., 1980), and previous investigations have found that virus survival in water is influenced by high molecular weight dissolved matter (Noble and Fuhrman, 1997), which is present at higher concentrations in sewage.

It has been established with other human pathogens that formation of droplets and aerosols from water contaminated with microorganisms can serve as a vehicle for transmission. Examples include *Legionella*, a respiratory pathogen acquired when contaminated water droplets are inhaled (Butler and Breiman, 1998), and *Cryptosporidium*, an enteric pathogen acquired via ingestion of contaminated droplets (CDC, 1998). Desiccation and aerosolization of body fluids and fecal matter, resulting in ingestion or inhalation of dried particles, can also serve as a source of pathogens such as norovirus (Marks et al., 2003) and hantavirus (LeDuc, 1998). SARS was spread when water contaminated with fecally shed virus was inhaled, causing respiratory infection. This person-to-person fecal droplet–respiratory transmission route was observed in the Amoy Gardens apartment building outbreak in Hong Kong, the largest point-source outbreak attributable to this type of transmission pathway. When an individual shedding infectious virus in feces used the toilet facilities in a building, a combination of faulty drain traps and powerful exhaust fans in residential units resulted in virus-laden liquid droplets being drawn from the waste system into living spaces via floor drains. The droplets were inhaled by occupants and carried on air currents to other areas of the building, resulting in a large number of SARS cases (WHO, 2003; McKinney et al., 2006). More data are needed on the survival of SARS-CoV in fecal droplets and aerosols to assess this new risk pathway in the event that SARS reemerges. The results of this study suggest that coronaviruses can survive long enough in water and sewage for these vehicles to serve as a source of exposure. The potential for long-term survival, along with the airborne fecal droplet transmission model, suggests that fecally contaminated aqueous media could pose a health risk in future outbreaks.

If water or sewage contaminated with SARS-CoV becomes aerosolized, it could potentially expose large numbers of people to infection. This could create an ongoing risk during an outbreak, even with quarantine measures to isolate infected individuals. Commercial, residential, and hospital water or sewer systems contaminated with persistent infectious SARS-CoV might defeat quarantine measures by continuing to spread virus even after infected individuals have been removed from the area. The persistence of coronaviruses in water and sewage in this study suggests that quarantine measures, which proved effective in containing the last SARS outbreak, could be seriously undermined unless adequate attention is paid to the safety and security of building plumbing systems. For assessment of these risks, further work is necessary to better define the kinetics of SARS-CoV survival and inactivation in water, sewage, and other aqueous media. The survival and persistence data presented here show that TGEV and MHV may serve as conservative indicators of the survival of SARS-CoV in water and sewage,

providing a starting point for risk assessments of water and sewage as vehicles for SARS transmission.

5. Conclusions

- The coronaviruses TGEV and MHV survived and remained infectious for long periods in different water types, including reagent-grade water, surface water, and pasteurized settled sewage.
- Both viruses survived and remained infectious at both low (4 °C) and ambient (25 °C) temperatures.
- In all water types tested (reagent-grade water, lake water and settled sewage), the titer of infectious virus declined more rapidly at 25 °C than at 4 °C.
- Water type, incubation time, and temperature were significant predictors of $\log_{10}$ viral reduction kinetics.
- The persistence of coronaviruses in water observed in this study suggests that if SARS-CoV should reemerge in human populations, water contaminated with these viruses may continue to pose an exposure risk even after infected individuals are no longer present.

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