

Inactivation of an Enveloped Surrogate Virus in Human Sewage

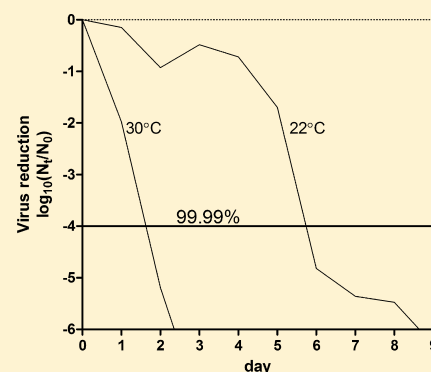
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ABSTRACT: Data are needed to provide guidance for handling of human sewage potentially containing infectious Ebola virus. The purpose of this research was to determine inactivation of enveloped viruses in sewage using bacteriophage $\Phi 6$ as a surrogate. Sewage was spiked with $\Phi 6$ and held at 22 or 30 °C, and the viral titer was measured over time. Inactivation was much more rapid at 30 °C than at 22 °C. At 30 °C, inactivation was approximately linear and reached 1.7 \log_{10} in 24 h, 5 \log_{10} by 48 h, and $>7 \log_{10}$ within 72 h. At 22 °C, the time to 5 \log_{10} inactivation was 6 days and nonlinear. In sewage, $\Phi 6$ should be considered as a potential model for survival and inactivation of enveloped human viruses. The results suggest that enveloped viruses can undergo 6–7 log inactivation in sewage in 3–7 days, depending on temperature. Longer holding times may be desirable out of an abundance of caution at lower temperatures.



INTRODUCTION

Because of the infectious nature of Ebola virus in bodily fluids,¹ data are needed to provide guidance for holding and handling of human sewage potentially containing infectious Ebola virus. Although the World Health Organization (WHO) has released interim guidance for water, sanitation, and hygiene issues related to Ebola,² there is limited information about the survival and inactivation kinetics of enveloped viruses in human sewage in general and Ebola in sewage specifically³ to guide recommendations. The purpose of this research was to determine the inactivation kinetics of enveloped viruses in sewage using bacteriophage $\Phi 6$, an enveloped dsRNA member of the *Cystoviridae*, as a potential surrogate for enveloped human viruses in sewage. This virus has been suggested as a candidate to fill the need for surrogates to help understand the survival dynamics of Ebola in the water environment.³ The survival of $\Phi 6$ has been evaluated previously as a surrogate for enveloped virus survival in water⁴ and on personal protective equipment,⁵ but its survival has not been evaluated in sewage as a potential surrogate to guide recommendations for other enveloped viruses.

EXPERIMENTAL SECTION

Virus stocks were prepared as previously described.⁵ Primary influent (having undergone no treatment) from a 40 million gallons per day (mgd) urban wastewater reclamation facility was collected and pasteurized at 70 °C for 3 h to suppress contaminating organisms that would interfere with the assay.⁶ Five replicate sewage aliquots were spiked with $\Phi 6$ to a concentration sufficient to follow 7 \log_{10} reduction and held at either 22 or 30 °C, and the viral titer was measured every 24 h.

Reduction was measured as $\log_{10}(N_t/N_0)$, where N_0 is the titer of virus at time zero and N_t that at time t . Data were analyzed using GraphPad Prism (GraphPad, San Diego, CA) and SPSS (IBM Corp.). General linear models (GLMs) for repeated-measures data were used to conduct a trend analysis via polynomial decomposition (i.e., estimate linear, quadratic, ..., n th-order polynomial effects) of time upon inactivation. Inactivation data from each temperature condition (22 and 30 °C) were examined in separate GLMs because of an unbalanced design stemming from different rates at which replicates from the different conditions reached the limit of detection.

RESULTS AND DISCUSSION

Viral inactivation was much more rapid at 30 °C than at 22 °C (Figure 1). At 30 °C, there was 2 \log_{10} inactivation in 24 h and 5.2 \log_{10} inactivation by 48 h; $>7 \log_{10}$ inactivation occurred within 72 h, for an average daily inactivation of 2.49 \log_{10} [95% confidence interval (CI) from -2.54 to -2.44]. At 22 °C, the inactivation kinetics appear to be nonlinear. There was only 0.14 \log_{10} inactivation by 24 h, and 5 \log_{10} inactivation took approximately 6 days at this temperature.

For the 30 °C condition, 7 \log_{10} reduction was measured by day 3, and the limit of detection (8 \log_{10}) was reached at day 4 (data not shown). For this condition, the three measured time points per sewage aliquot after time zero (days 1–3) were used

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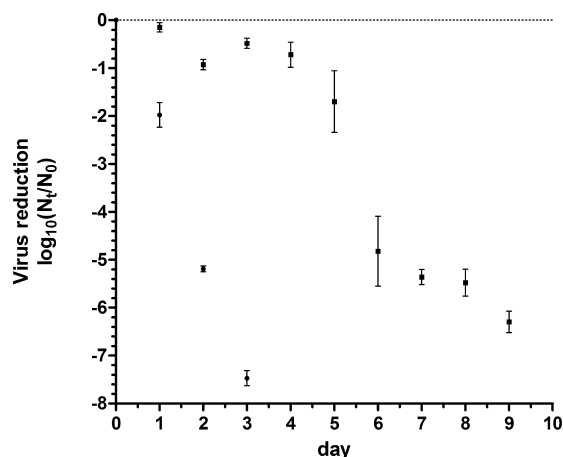


Figure 1. Inactivation of $\Phi 6$ in sewage at 22 °C (■) and 30 °C (●) (bars shows the 95% CI).

in the analysis. The GLM for this condition estimated a third-order polynomial model for the effect of time on inactivation. For the 22 °C condition, the limit of detection was reached at 10 days, for nine measurements per sewage aliquot after time zero. The GLM for this condition estimates a ninth-order polynomial model for the effect of time on inactivation. To aid interpretation, the 9 days of measurements were divided into three segments guided by visual inspection of the inactivation curve (Figure 1) over which orthogonal linear, quadric, and cubic (segment 1) polynomial contrasts were estimated: days 0–3 (segment 1), days 4–6 (segment 2), and days 7–9 (segment 3).

Inactivation at 30 °C was predominantly linear [$t(4) = 109.24$; $p < 0.001$], though a statistically significant positive cubic trend was detected [$t(4) = 7.11$; $p = 0.002$]. At 22 °C, inactivation was statistically significant but slower between day 0 and day 3 (0.31 \log_{10} reduction per day, 95% CI from -0.20 to -0.13) and nonlinear [for the linear effect, $t(4) = 18.43$ and $p < 0.001$; for the quadratic effect, $t(4) = 10.29$ and $p = 0.001$; for the cubic effect, $t(4) = 10.09$ and $p = 0.001$]. In segment 2 (days 4–6), the rate of inactivation accelerated [$2.05 \log_{10}$ per day, 95% CI from -2.35 to -1.75 ; for the linear effect, $t(4) = 18.71$ and $p < 0.001$; for the quadratic effect, $t(4) = 13.12$ and $p = 0.001$], achieving 4.8 \log_{10} inactivation by day 6. This comparatively rapid average rate of inactivation was followed by a third segment (days 7–9) with a slower average inactivation rate [$0.47 \log_{10}$ per day, 95% CI from -0.60 to -0.34 ; for the linear effect, $t(4) = 9.88$ and $p = 0.001$; for the quadratic effect, $t(4) = 6.21$ and $p = 0.003$], at which point the viral titer had declined by 6 \log_{10} .

The observed inactivation kinetics of bacteriophage $\Phi 6$ suggest that enveloped viruses can reach 6–7 \log_{10} inactivation in human sewage between 3 and 7 days, and temperature is a major influence on inactivation rate. The available data for the survival of filoviruses themselves in liquid media are limited; studies of Zaire Ebola virus and Lake Victoria Marburg virus demonstrate that the time to 2 \log_{10} inactivation in tissue culture media and guinea pig serum is 40–50 days.⁷ However, unlike sewage, these media are likely to have protective effects against viral inactivation. The data on survival of Zaire Ebola in serum and tissue culture media at room temperature⁷ may suggest a segmented inactivation curve with different rates of inactivation at different times (more rapid at earlier times and less rapid at later times), similar to what we observed with $\Phi 6$

at 22 °C. This underscores the importance of conducting survival studies of sufficient length to observe the entire shape of the inactivation curve, including possible persistent subpopulations of virus with different inactivation rates.

Previous work on enveloped virus survival in water and sewage that focused on avian influenza viruses (AIV) and coronaviruses suggests that there is variation both between viruses and within strains of the same virus.⁸ Studies consistently show that influenza and coronavirus inactivation is much slower in pure/distilled water than in natural surface waters or waters with added salinity,^{6,8–11} suggesting that physicochemical parameters as well as temperature contribute to inactivation. Because pasteurization may suppress organisms that would otherwise conduct natural predation processes in water, increasing the rate of viral inactivation, the data presented here may represent a conservative estimate of inactivation kinetics.

Previous literature reinforces our findings that temperature influences inactivation; in distilled water, the time for 1 \log_{10} inactivation of AIV increased from 11 days at 28 °C to 51 days at 22 °C¹⁰ and from 20 days at 28 °C to 71 days at 17 °C for the same strain.⁸ Avian influenza in surface water underwent a 1 \log_{10} (90%) reduction in 2 days at 30 °C but 3–4 days at 20 °C.⁹ Previous investigators have found that $\Phi 6$ itself followed this trend; in fresh water, the time for 2 \log_{10} inactivation increased from 19 days at 28 °C to 105 days at 17 °C.⁴

Inactivation of enveloped viruses in surface water appears to be slower than inactivation in sewage. At 20 °C, the time for 4 \log_{10} inactivation of AIV in surface water ranged from 10 to 23 days.^{9,11} Comparisons of distilled or tap water with sewage have also demonstrated that enveloped viruses are inactivated much more quickly in sewage than in pure water. Studies with coronavirus found that at 23 °C an $\sim 2 \log$ reduction in unfiltered primary effluent took ~ 2 days, versus 7–8 days in tap water.¹² Coronavirus demonstrated a 1–2 \log_{10} reduction in 7 days in pasteurized settled sewage at 25 °C versus 19 days in distilled water,⁶ again suggesting that physicochemical constituents in sewage may accelerate inactivation processes.

Direct comparisons of $\Phi 6$ to pathogenic human viruses are limited. Adcock et al.⁴ evaluated $\Phi 6$ as a surrogate for AIV in water; $\Phi 6$ persisted as long as or longer than AIV strains. More such direct comparisons are needed to establish $\Phi 6$ as a viable surrogate, but our results suggest that in human sewage, this virus should be considered as a potential model for the inactivation of enveloped human viruses. $\Phi 6$ has a dsRNA genome, whereas both coronaviruses and filoviruses have ssRNA genomes; this may confer additional stability, possibly making $\Phi 6$ a conservative surrogate. Results with coronaviruses suggest that inactivation in sewage follows a linear trend around 22 °C, but our results suggest a slowing of the rate of inactivation around day 6 at 22 °C. This slowing of the inactivation rate may mean that treatment and holding time recommendations need to take into account the elimination of longer-surviving subpopulations of viruses.

Initial WHO guidance recommended that “Waste, such as feces, urine, vomit, and liquid waste from washing, can be disposed of in the sanitary sewer or pit latrine. No further treatment is necessary.”¹³ Current guidance provides a holding time recommendation for pit latrines, recommending that “Each latrine should have two pits; once one pit is full it can be closed for a period of at least 1 week but ideally longer to allow for the virus to decline and thus reduce risks in later handling”.¹⁴ Ebola virus levels measured during active infection

have been as high as $7 \log_{10}$ RNA copies/mL in stool¹⁵ and $5\text{--}6 \log_{10}$ copies/mL in urine,^{15,16} and the blood viral load can range from 2 to $9 \log_{10}$ copies/mL.¹⁷ Patients may vomit profusely and excrete high stool volumes during acute infection.¹⁶ Ebola outbreaks can occur in settings where centralized sanitary sewer infrastructure may not exist. In these settings, where waste may be held in on-site treatment systems such as pit latrines or septic tanks, inactivation of enveloped viruses may take place naturally in sewage. However, it is vital to understand the characteristics of inactivation; Bibby and colleagues in a recent review caution against “an assumption of negligible persistence”.³ To ensure safe handling of waste, an appropriate holding time is important, and dependent both on fecal viral load that infected individuals shed into sewage and on the ambient holding temperature for human waste. These data suggest that substantial inactivation of enveloped viruses takes place in sewage over a period of 6–7 days, supporting current WHO guidance of holding latrine waste for a week or longer,¹⁴ and longer holding times may be desirable out of an abundance of caution at lower ambient temperatures.

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Notes

The authors declare no competing financial interest.

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