Persistence of Norwalk Virus, Male-Specific Coliphage, and Escherichia coli on Stainless Steel Coupons and in Phosphate-Buffered Saline

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ABSTRACT

Human noroviruses (NoVs) are a leading cause of acute gastroenteritis and are frequently transmitted by contaminated food, water, hands, and environmental surfaces. Little is known about their environmental stability and/or which alternative microorganisms can serve as effective surrogates. To examine whether Escherichia coli and male-specific coliphage MS2 can be appropriate surrogates for NoVs, approximately 6.8 log genomic equivalent copies of Norwalk virus (NV), and 6.0 to 6.5 log PFU or CFU of MS2 and E. coli, respectively, were inoculated onto stainless steel coupons and held at 4°C, room temperature (RT), or 37°C over a period of 75 min (E. coli and MS2) to 4 weeks. These three microorganisms were also seeded into phosphate-buffered saline (PBS) and sampled at different time intervals for up to 6 weeks. MS2 and E. coli survived approximately 15 min at 37°C, 45 min at RT, and 60 min at 4°C on the stainless steel surfaces. In contrast, NV RNA titers were reduced by only 2.4 log at 37°C, 1.5 log at RT, and 0.9 log at 4°C after 4 weeks. MS2 and E. coli were able to survive at least 5 weeks in PBS at 4°C and RT, and NV was stable in PBS at 4°C and RT for at least 6 weeks. However, E. coli, MS2, and NV were completely inactivated after 1-, 4-, and 5-week incubations in PBS at 37°C, respectively. These findings indicate that NoVs are highly persistent on environmental surfaces and in PBS solution at different temperatures. While E. coli does not appear to be an appropriate surrogate for NoVs, MS2 could be more relevant for modeling the environmental persistence of NoVs under wet conditions, but not under dry conditions.

Human noroviruses (NoVs) are recognized as the major cause of acute, nonbacterial gastroenteritis outbreaks worldwide (6). These viruses are readily transmitted in human populations by the consumption of fecal-contaminated food and water (10, 17) and by contact with contaminated surfaces and fomites (41). Recent epidemiological studies have documented that NoVs are responsible for a large proportion of foodborne illness in the United States and around the world (26, 28, 36). The epidemiological evidence of NoV transmission via contact with surfaces is also supported by laboratory data showing that these viruses can persist on food processing surfaces for extended periods after initial contamination events (12, 14, 24). For example, Isakbaeva et al. (18) indicated that the same human NoV strain caused a series of gastroenteritis outbreaks on cruise ships, even after aggressive disinfection was performed, suggesting a high degree of environmental stability for these viruses. Such environmental persistence is a likely contributor to the ease with which NoVs are transmitted (9, 18, 20, 27). When present on environmental surfaces and foods, human NoVs are undoubtedly exposed to varying environmental conditions related to temperature, pH, and relative humidity, but the behavior of NoVs under these environmental conditions is poorly characterized.

Because human NoVs are not cultivable in vitro, and small animal models are not available, detecting infectious virus remains challenging. In instances such as this, surrogate organisms, whose behavior mimics that of the pathogen, are frequently used. In studies of persistence and inactivation, Escherichia coli is sometimes used as a proxy for the presence of enteric pathogens; however, a large body of evidence has demonstrated a poor correlation between the presence of E. coli and enteric viruses including NoVs (2, 3, 19). Male-specific coliphages have also been proposed as an alternative surrogate (2, 11, 32). These are particularly appealing because they are easily propagated and quantified, but their relevance as a surrogate for human NoVs still requires further investigation. For example, a strong correlation between the concentration of the F-specific RNA coliphage MS2 in oysters and the incidence of gastroenteritis associated with NoV-contaminated oysters has been observed (8), but the fact remains that MS2 is rarely detected in human feces (16, 33).

One criterion for the success of a surrogate organism is that it should show a degree of environmental persistence exceeding that of the pathogenic organism(s) for which it is used as proxy. Hence, the purpose of this study was to compare the persistence of E. coli; the male-specific RNA

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coli phage MS2; and Norwalk virus (NV), the prototype of genogroup I, genotype 1 human NoV, on representative surfaces at three temperatures (4°C, room temperature [RT], and 37°C) and in phosphate-buffered saline (PBS) adjusted to three different pH values (3.0, 7.0, and 10.0).

**MATERIALS AND METHODS**

*E. coli, MS2, and NV inocula.* *E. coli* (ATCC 11775) stock was grown and titered by using an agar medium to enumerate fecal coliforms (Difco, BD, Sparks, MD). For enumeration, stocks were 10-fold serially diluted in PBS (0.01 M, pH 7.4, Dulbecco’s modification) supplemented with 0.01% Tween 80 (Fisher Scientific, Pittsburgh, PA) and 0.001% (wt/vol) Y-30 antifoam emulsion (Sigma, St. Louis, MO) to reduce the tendency of *E. coli* to aggregate. Male-specific coliphage MS2 (ATCC 15597-B1) stock was prepared and titered by using the two-step enrichment procedure as per U.S. Environmental Protection Agency (EPA) method 1601 (37). This stock was diluted to an appropriate titer prior to use in the experiments. The NV inoculum originated from a stool sample collected from an infected volunteer who participated in a previous human challenge study (23). The stool sample was diluted 20% (wt/vol) in RNase-free water, vortexed briefly, centrifuged at 550 × g for 30 s, and the virus-containing supernatant was collected. The titer of the supernatant was approximately 6.3 × 10⁶ genomic equivalent copies (GEC)/ml by titration by using reverse transcriptase quantitative PCR (RT-qPCR). Aliquots were prepared and stored at −80°C prior to use.

**Environmental persistence studies.** Stainless steel coupons (5 by 5 cm) were decontaminated by washing thoroughly with liquid soap, rinsing in tap water, soaking in 1% bleach and 70% ethanol (each for 30 min), followed by rinsing in deionized water and air drying. The coupons were autoclaved prior to use in the experiments.

Ten microliters of diluted *E. coli* and MS2 stocks (corresponding to approximately 6.0 to 6.5 log CFU or PFU, respectively) was inoculated onto the center of multiple stainless steel coupons under ambient temperature (70 to 74°F [21 to 23°C]). Immediately after inoculation (time 0 control), the inoculum was eluted from one coupon by using 1 ml of PBS, while the rest of the coupons were stored at 4°C, RT, or 37°C, with elution occurring at 15-min intervals, for a total exposure time of 75 min. A 10-μl aliquot of the 20% NV stool suspension (containing approximately 6.8 log of viral GEC) was also deposited onto the center of multiple stainless steel coupons, with virus elution done at weekly intervals for 6 weeks. *E. coli* and MS2 eluates were appropriately diluted and assayed immediately after collection of each eluate, while NV eluates were frozen at −80°C and assayed simultaneously. Each experiment was performed twice, and each sample was assayed in duplicate.

**Persistence in suspension.** Ten microliters of diluted *E. coli* or MS2 containing approximately 6.0 to 6.5 log CFU or PFU, respectively, was added into 990 μl of 1 × PBS (pH 7.0) under ambient temperature (70 to 74°F [21 to 23°C]). After removing a 100-μl aliquot (time 0 control), suspensions were stored at 4°C, RT, and 37°C, with aliquots removed weekly for 4 to 5 weeks. Each sample was appropriately diluted, assayed immediately after collection, and titrated at least in duplicate. The entire experiment was performed twice under the same conditions. For NV experiments, 10-μl aliquots of 20% fecal suspension (containing approximately 6.8 log NV GEC) were inoculated into 990 μl of 1 × PBS (pH 7.0) and incubated at the designated temperatures (4°C, RT, and 37°C), with 100-μl aliquots drawn weekly for 6 weeks. These aliquots were stored at −80°C until assayed. The studies to assess persistence in low- and high-pH suspensions were performed by using 1 × PBS adjusted to pH values of 3.0 and 10.0 prior to microbial spiking, with subsequent incubation at the same three temperatures (4°C, RT, and 37°C). For these experiments, a 10-μl inoculum of *E. coli*, MS2, and NV was exposed to 90°C PBS (pH 7.0) was enumerated by using 1 ml of PBS, while the rest of the coupons were assayed immediately after collection of each eluate, while NV eluates were stored at 4°C and RT for a period of 45 min; thereafter, titers

**Plaque assay for MS2.** MS2 was quantified with the single-agar layer method (EPA method 1602) (38). In brief, 100- and 500-μl aliquots of diluted samples were mixed with 30 ml of molten tryptic soy agar (Fisher Scientific) containing ampicillin and streptomycin (Sigma). This was then supplemented with 1 ml of log-phase *E. coli* Fₐₙₙ (ATCC 700891), and the entire mixture was poured into a large petri dish for solidification. After incubation overnight at 37°C, circular plaques were counted. The quantity of MS2 in a sample was calculated as the average total PFU in the initial samples from the multiple petri dishes of the 100- and 500-μl volumes.

**RT-qPCR for detection of NV.** Viral RNA was released by the heat-shock method (34). Specifically, 1 μl of eluate was diluted in 8.8 μl of diethyl pyrocarbonate–treated water, with the addition of 0.2 μl of RNase inhibitor (Promega, WI), heated at 99°C for 5 min, and chilled on ice for at least 2 min. A NV-specific, TaqMan real-time RT-qPCR method, targeting the RNA polymerase region of the viral genome, was used to detect NV RNA, as described elsewhere (25). For quantification purposes, a serially diluted NV RNA transcript (corresponding to ∼4.9 × 10⁵ GEC), produced from a full-length NV plasmid (a gift from K. Green, National Institutes of Health–National Institute of Allergic and Infectious Diseases [NIH–NIAID]), was used to produce a standard curve that described the relationship between the threshold cycle value and GEC.

**Statistical analysis.** Data for total CFU, PFU, or GEC were log transformed and averaged. For persistence of the three microorganisms on surfaces and in suspension over exposure time, a linear equation and R² value were calculated and graphed with the Line Format function in Excel (Microsoft, Bellevue, WA). *E. coli* and MS2 titers on surfaces at each time point were analyzed with analysis of variance (SAS 9.2, SAS Inc., Cary, NC) to identify significant differences among the three temperatures. The persistence of NV in PBS adjusted to low (3.0) or high (10.0) pH was expressed by subtracting the log-transformed NV GEC at each sampling time from the average of the log-transformed input control values.

**RESULTS**

**Persistence on stainless steel coupons.** An inverse relationship between temperature and survival of viable (infectious) *E. coli* and MS2 was observed in the surface persistence studies. In general, both survived quite well at 4°C and RT for a period of 45 min; thereafter, titers
dropped, and both *E. coli* and MS2 were not detected after 1 h at RT (Figs. 1 and 2). At 37°C, both MS2 and *E. coli* were completely inactivated (>4 log) within 30 min. In contrast, NV was highly persistent on stainless steel coupons, with detectable RNA after 28 days and minimal loss in titer as determined by RT-qPCR (Fig. 3). Similar to *E. coli* and MS2, NV RNA persistence on stainless steel coupons was inversely associated with temperature. NV titers were reduced by 2.4 log at 37°C, 1.5 log at RT, and 0.9 log at 4°C after 28 days, compared with the baseline NV GEC numbers (Fig. 3). In general, NV GEC decreased more rapidly when coupons were stored 37°C as compared with RT and 4°C.

**Persistence in PBS (pH 7.0).** Suspending *E. coli* and MS2 in PBS solution (pH 7.0) promoted survival of both organisms, particularly at lower temperatures (4°C and RT) (Figs. 4 and 5). At 4°C and RT, the two organisms were still detectable after 4 to 6 weeks of incubation. However, when incubated at 37°C, *E. coli* was only able to survive for 1 week, and MS2 for 3 weeks. NV showed a high degree of persistence when suspended in PBS; however, the virus was inactivated between 4 and 5 weeks at 37°C (Fig. 6).

**Persistence in PBS at reduced and elevated pH.** Experiments were also conducted to characterize the persistence of *E. coli*, MS2, and NV in PBS at low and high pH (3.0 and 10.0, respectively) at the same three temperatures. *E. coli* was inactivated quite rapidly at room temperature and 37°C, but showed relatively good survival when suspended in PBS at either pH 3.0 or 10.0 and 4°C (data not shown). The persistence of MS2 and NV was less influenced by extremes of pH (Fig. 7). In general, for both MS2 and NV, there was a gradual loss in virus titer over time, but both viruses were still detectable after 5 weeks. At pH 3.0, both MS2 and NV were stable at 4°C, and there was no statistically significant difference in log reductions when comparing the two. When the suspensions at pH 3.0 were exposed to higher temperatures (RT and 37°C), log reductions occurred more quickly for both MS2 and NV. Interestingly, MS2 tended to be more stable than NV at all the time points except for 3 and 5 weeks at 37°C in pH 3.0 PBS. At pH 10.0, with the exception of the 1-week incubation at 37°C, there were statistically significant differences in log reductions when comparing NV and MS2 over three time points (1, 3, and 5 weeks) at all three temperatures.

**DISCUSSION**

The results of this study clearly demonstrate that NV, a prototype human NoV, is able to persist for prolonged periods (weeks) under extreme environmental conditions, including high storage temperature (37°C) and low or high pH. Interestingly, NV appeared to be more stable at 4°C and RT than at 37°C, suggesting that even moderate temperature would have an impact on the persistence of NV. These laboratory findings strongly support previous epidemiological investigations (10, 29, 31) that implied prolonged environmental persistence of human NoV was associated with outbreaks of acute gastroenteritis. We hypothesize that the persistence of NV under extreme conditions is likely to be a major factor contributing to its transmission between people, by contact with the environment, and by consumption of contaminated food or water.

To our knowledge, this is the first study to examine the survival of nonpathogenic *E. coli* on a nonporous surface at three environmental temperatures. Not unexpectedly, we found nonpathogenic *E. coli* to be relatively unstable on stainless steel surfaces. Surprisingly, survival of nonpathogenic *E. coli* was considerably different when compared with studies of the persistence of pathogenic *E. coli*, such as...
E. coli O157:H7, under conditions similar to those used in our work (40). For example, we observed E. coli to be completely inactivated within an hour of inoculation on stainless steel coupons at all three temperatures. In contrast, E. coli O157:H7 was able to survive for at least 28 days on stainless steel under holding conditions of 4°C and RT (40). The prolonged environmental persistence of E. coli O157:H7 could contribute to its ease of transmission, especially given the fact that these strains have been shown to cause disease at very low doses.

When suspended in PBS, we found that nonpathogenic E. coli maintained its viability for weeks at RT and 4°C, much longer than what was observed for environmental surfaces. These findings are in agreement with previous observations of other bacteria, including E. coli, suspended in water and PBS. For example, several common foodborne pathogens such as Salmonella enterica and Listeria monocytogenes were able to survive well in sterile water and PBS at room temperature, with no added nutrients, for at least 30 weeks (21). Our results are also consistent with others who reported more rapid inactivation of pathogenic E. coli at high temperatures compared with low temperatures (1, 2, 7), similar to our observation that E. coli died off much more rapidly at 37°C relative to the lower temperatures tested. Reduced survival at high temperature was expected, because temperature increase might constitute greater stress and energy expenditure for E. coli, leading to faster cell death (39). Taken together, these data further support the widely held belief that E. coli is a poor surrogate for human NoVs, in large part because the organism lacks the high level of environmental persistence that has been documented for the human enteric viruses.
For many years, coliphages have been proposed as effective surrogates for human enteric viruses, largely because of structural similarities between these two major virus categories. In addition, the presence of coliphages in naturally contaminated environmental samples is thought to indicate the presence of human fecal contamination (13, 15).

In our study, we observed that MS2 showed relatively poor persistence when placed on stainless steel surfaces and held under ambient conditions (e.g., dried), but good survival capability when suspended in PBS. For example, previous studies indicated that NV was able to survive on environmental surfaces for at least 7 days, as evaluated by conventional RT-PCR (12), and in clean water for more than 2 months, as evaluated by RT-qPCR (35). Similar to our findings, Bae and Schwab (4) and Allwood et al. (1) have reported that MS2 concentrations fell in water samples at a similar rate as we observed in PBS solution. In fact, the survival curves of MS2 in PBS were quite similar to those of NV in PBS, regardless of suspension pH or storage temperature. Based on these results, one could conclude that MS2 is a poor surrogate for human NoV when used in studies that are intended to evaluate virus persistence under dry conditions. On the other hand, MS2 could be a useful human NoV surrogate under moist storage conditions.

Nonetheless, it is important to be cautious when relying on the behavior observed for MS2 as a predictor of how human NoVs might behave under the same conditions. One obvious issue is the fact that the methods used to quantify E. coli and MS2 in this study were culture based and hence measured viable (infectious) survivors, while RT-qPCR was used to quantify NV.

FIGURE 5. MS2 persistence when suspended in PBS solution (pH 7.0) at three temperatures (4°C, RT, and 37°C) by exposure time (weeks), as evaluated using the single-agar layer method (EPA method 1602). Samples were collected weekly, and data for each time point are expressed as mean log of total spiked MS2 ± standard deviation from two replicate experiments, with each sample assayed in duplicate.

FIGURE 6. NV persistence when suspended in PBS solution (pH 7.0) at three temperatures (4°C, RT, and 37°C) by exposure time (weeks), as evaluated with NV-specific TaqMan RT-qPCR assay. Samples were collected weekly, and data for each time point are expressed as mean log of total spiked NV GEC ± standard deviation from two replicate experiments, with each sample assayed in duplicate.
FIGURE 7. NV and MS2 log reductions when suspended in PBS solution (pH 3.0 and 10.0) at three temperatures (4°C, RT, and 37°C) by exposure time (weeks), as evaluated by TaqMan RT-qPCR assay (NV) and the single-agar layer method (MS2). Samples were collected weekly, and data are expressed as mean log reduction of total spiked NV GEC or total MS2 at each time point.

**REFERENCES**


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