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Article

Evaluating the Environmental Persistence and Inactivation of MS2 Bacteriophage and the Presumed Ebola Virus Surrogate Phi6 Using Low Concentration Hydrogen Peroxide Vapor

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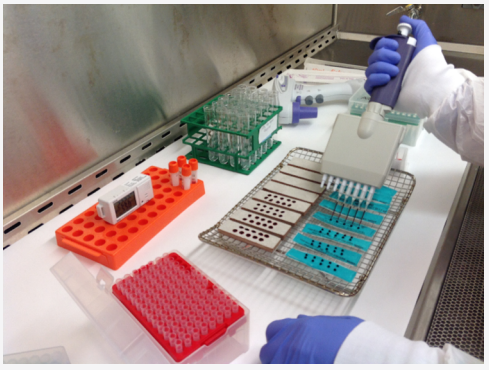
ABSTRACT: Ebola virus (EBOV) disease outbreaks, as well as the ability of EBOV to persist in the environment under certain conditions, highlight the need to develop effective decontamination techniques against the virus. We evaluated the efficacy of hydrogen peroxide vapor (HPV) to inactivate MS2 and Phi6 bacteriophages, the latter a recommended surrogate for EBOV. The phages were inoculated onto six material types with and without the presence of whole human blood. The inoculated materials were then exposed to either a high or low concentration of HPV for various elapsed times. The phages were also recovered from positive controls at these same elapsed times, to assess environmental persistence and decontamination efficacy. Low concentration hydrogen peroxide vapor (LCHP; 25 ppm) was effective against both phages on all materials without the presence of blood at 2 h. LCHP was ineffective against the phages in the presence of blood, on all materials, even with a 3-day contact time. Higher concentrations of HPV (>400 ppm) with contact times of 24−32 h achieved approximately 2−6 log reduction of the phages in the presence of blood.



INTRODUCTION

In the 2014−2016 Ebola virus (EBOV) disease outbreak in West Africa, there were 28 600 cases and over 11 000 fatalities.[1] The current EBOV disease outbreak in the Democratic Republic of the Congo has a fatality rate of approximately 67%[2] and highlights the concern of using the EBOV as a bioterrorism agent.[3] (Viruses producing hemorrhagic fever, such as EBOV, are considered Category A biological agents by the U.S. Centers for Disease Control and Prevention [CDC].) Moreover, environmental transmission of EBOV from fomites may be possible due to its persistence[4] and low infectious dose (10 viral particles).[5] EBOV may persist outside host cells for several days[6] or over a week,[7] depending on the environmental conditions and matrix (e.g., blood) with which the virus is associated.

Ebola virus is a filamentous, enveloped virus of the *Filoviridae* family.[8] Since EBOV is classified as a Risk Group 4 and Biosafety Level 4 agent,[9] relatively few laboratory disinfection studies using the actual EBOV[10−13] have been conducted. In lieu of evaluating EBOV, surrogate viruses have been proposed for disinfectant efficacy testing. In particular, the bacteriophage Phi6 has been recommended as an appropriate surrogate for EBOV for evaluating either environmental persistence or disinfection, based on having a similar viral structure (both EBOV and Phi6 are enveloped, RNA viruses) and empirical evidence.[8,14−19]

The presence of a lipid envelope in viruses is known to provide less resistance to disinfection compared to nonenveloped viruses.[9,20] Thus, as a precaution, the CDC recommends that disinfectants registered with the U.S. Environmental Protection Agency (EPA) for the more resistant nonenveloped viruses be used in environments where the enveloped EBOV may be present.[21] Accordingly, Sassi et al.[22] utilized the nonenveloped bacteriophage MS2 as a model virus for EBOV in their disinfection tests. In the present study we used the Phi6 bacteriophage as a surrogate for EBOV to evaluate disinfection via hydrogen peroxide vapor (HPV). But we also included MS2 as a test virus in our study, consistent with CDC recommendations and other research that included both bacteriophages to model EBOV.[23] Ideally, it would be preferable to conduct these tests alongside an actual EBOV strain to confirm the phage's suitability as a surrogate (to the best of our knowledge such tests have never been

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Table 1. Study Test Matrix

virus	test materials	diluent	target decontamination conditions	time points assessed (h)
Phi6	glass, stainless steel, ceramic tile, N95 media, painted joint tape, wood	blood	25 ppm, 75% RH	2, 4, 24, 72
		PBS	25 ppm, 75% RH	2, 4, 6, 8
		blood	400 ppm, 75% RH	4, 8, 24, 32
MS2	glass, stainless steel, ceramic tile, N95 media, painted joint tape, wood	blood	25 ppm, 75% RH	2, 4, 8, 24, 32, 72
		PBS	25 ppm, 75% RH	2, 4, 6, 8
		blood	400 ppm, 75% RH	4, 8, 24, 32

undertaken), and this is suggested as further research to close this data gap.

While HPV has been demonstrated to be effective in the inactivation of viruses (e.g., refs 24−26), only a few studies evaluating HPV efficacy have included Phi6 or MS2,[27,28] and none with EBOV. Further, the present study focused on using a relatively low concentration of hydrogen peroxide vapor (LCHP; which, for the purpose of this study, we selected as ≤25 ppm [ppm]), based on previous research which was shown to be effective against influenza viruses[29] as well as for inactivating *Bacillus anthracis* spores,[30,31] provided sufficient contact time. The benefit to using LCHP is that it may be generated through inexpensive means such as off-the-shelf humidifiers using off-the-shelf 3 or 8% aqueous solutions of hydrogen peroxide, and be more compatible with materials.[30] This "low-tech" approach would be advantageous where specialized equipment and financial resources are limited. To assess the decontamination efficacy of HPV, the phages were recovered from positive controls (not exposed to HPV) at the same elapsed times as the decontaminated coupons; these data are also presented to provide an indication of the environmental stability of the phages.

MATERIALS AND METHODS

Bacteriophage Propagation. In general, the methods described here for inoculation and recovery of Phi6 and MS2 phage from material coupons are consistent with previous related research investigating environmental disinfection and persistence of viruses.[14,15,19,32,33]

The two bacteriophage viruses, MS2 (ATCC 15597-B1) and Phi6 (The EPA water treatment laboratory, Cincinnati, OH) were stored at −80 °C until testing. Working stocks of each phage were prepared using a top agar overlay technique.[15] Briefly, agar plates using Tryptic Soy Agar with Magnesium for Phi6 (Hardy Diagnostics, Santa Clara, CA; Model G341) and LB Agar (Hardy Diagnostics, G77) for MS2 were inoculated with 100 μL (μL) of stock phage, 100 μL of bacterial culture (*Pseudomonas syringae* LM2489 for Phi6 and *Escherichia coli* C-3000 [ATCC 15597] for MS2), and 5 mL (mL) of molten top agar (50 ± 5 °C) of the same media type. Following overnight incubation, top agar layers from plates with visible clearing of host bacteria were aseptically scraped into conical tubes containing 5 mL SM buffer (Teknova Inc., Hollister, CA; Model S0249) and centrifuged at 7000 rpm for 15 min. The supernatant was removed and filtered through a 0.2-μm syringe filter (Corning Inc., Corning, NY; model no. 431229).

Test Materials. Since decontamination efficacy is strongly dependent on the material with which the microorganism is associated,[34,35] five common indoor surface materials were used: glass (ASTM C1036, Brooks Brothers Glass & Mirror, Columbus, OH), painted joint tape (PJT; United States Gypsum Company, Chicago, IL; model no 382198), 304 stainless steel (SS; ASTM A240; McMaster Carr, Aurora, OH), glazed ceramic tile (Lowes, Hilliard, OH model PWHITW91L01), and bare pine wood (model 142−8PINE Lowes, Hilliard, OH). These materials were selected to include both porous and nonporous, organic and inorganic substrates, to provide a wide range of challenge to HPV. Additionally, due to its medical relevance, an N95 filter medium (used for respiratory protection; 3M, St. Paul, MN; model 1860) was included. Test coupons (1.9 × 7.5 cm^2) were cut from a larger piece of each representative material. The N95 filter medium was heat sealed along the edges to bind the three layers together, representative of the larger filtering facepiece respirator. Prior to testing, glass, SS, and ceramic tile coupons were sterilized by autoclaving at 121 °C for 15 min in sterilization pouches (Fisher Cat. No. NC9241087, Pittsburgh, PA). Painted joint tape, N95 filter media, and bare pine wood were sterilized by γ-irradiation with a dose of approximately 40 kilogray.

Environmental Conditions and Test Matrix. Overall, seven experiments were conducted in the study. Each experiment included all six test materials exposed to one HPV concentration, for up to four time points. While the focus of the study was on the use of LCHP (target of 25 ppm of HPV, based on an approximate average concentration that could be obtained using a humidifier in a large test chamber), we also conducted two experiments at a target of 400 ppmv HPV, since the higher concentration is more typical of what is used in medical or hospital environments,[36] or for decontamination of biocontainment laboratories.[37] The study design included the use of two bacteriophages (MS2 or Phi6), the stock solutions of which were diluted (1 in 10) with either phosphate buffered saline (PBS) or human whole blood, since blood has been shown to diminish efficacy.[12] When either phage was used with just the PBS diluent, we evaluated only the lower HPV concentration of 25 ppm, since the lower concentration was efficacious within a reasonable time. See Table 1, below, which provides an overview of the test matrix. An adaptive experimental test approach was taken in some cases, i.e., some time points were adjusted based on previous interim results, to better assess improvement in efficacy.

Sample Processing. Each day of testing, a 1:10 dilution of the stock solution of phage was prepared using either PBS (HyClone Laboratories, Cat. No. SH302560.2, Logan, UT) or human whole blood [BioIVT, Wesbury, NY; Cat. No. HUMANWBK2UZN]), to achieve a target titer of 5 × 10^7 plaque forming units (PFU)/mL. The actual inoculum titer was verified each day coupons were inoculated, using the phage quantification techniques discussed below. Sterile coupons were laid flat in a Class II Biological Safety Cabinet (BSC) and inoculated with approximately 5 × 10^6 PFU per coupon by dispensing 100 μL as 10 droplets (10 μL per droplet) across the surface of the test coupons. For each material type, three replicate coupons were used at each time point during decontamination. The maximum number of time

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points that could be assessed in one experiment (test condition) was four, to allow 72 coupons in the test chamber. Three additional replicate coupons were used as positive controls (inoculated, exposed to ambient conditions, no exposure to HPV) for each time point and material. (The positive controls used for each time point allowed us to determine efficacy of the HPV treatment but also to assess persistence of the phage as well.) Following inoculation, all material coupons were dried for approximately 1 h in the BSC under ambient environmental conditions, prior to commencing exposure to HPV or the initial recovery of the phage at time zero. Additionally, two coupons of each material were used as blanks (not inoculated) and included for each time point tested. The blank coupons controlled for potential cross-contamination during testing as well as the sterility of the test coupons.

Decontaminant Testing. Decontamination testing was conducted inside a 498-L (L) acrylic glovebox (Plaslabs, Lansing, MI) at ambient temperatures. Fixed humidity point salts[38] were used to adjust relative humidity (RH) to a target of 75% for each experiment. Temperature and RH inside the test chamber were measured using a NIST-traceable temperature and humidity data logger (Onset, Bourne, MA; MX1101). Hydrogen peroxide vapor concentration was measured using a calibrated ATI B12 2-wire gas transmitter (Analytical Technology, Inc., Collegeville, PA) and was connected to a CNI-822 process controller (Omega Engineering, Norwalk, CT), which allowed for automatic control of HPV concentration within the test chamber; HPV data were recorded using the associated iLOG software (Omega Engineering, Norwalk, CT).

Generation of the HPV was achieved using a commercial generator (Bioquell Clarus C, Horsham, PA). Since commercial generators are typically used to target higher concentrations (>200 ppm), a two-chamber approach was required to achieve the lower 25 ppm target. For this lower target concentration, the generator supplied the HPV first to a mixing chamber to achieve a concentration of approximately 350 ppm, which then fed the test chamber as needed to maintain the target concentration of 25 ppm. Experiments targeting 400 ppm exposure level were achieved by connecting the HPV generator directly to the test chamber using a stock solution of 35% aqueous hydrogen peroxide. Once target RH and HPV were achieved and stable, the inoculated coupons were placed into a sealed container and transferred to the test chamber and opened, starting the exposure. With the direct connection between the HPV generator and the test chamber for the 400 ppm tests, the temperature within the test chamber was somewhat higher than in the LCHP tests, due to the flash evaporation of aqueous hydrogen peroxide. At the selected time points, a set of coupons was removed by placing them back into a sealed container and transferring them to a Class II BSC. Once in the BSC, the coupons were transferred to the extraction tubes.

Phage Recovery from Coupons and Quantification. At each time point, test and positive control coupons were collected and extracted for the phage by placing each individual coupon in a conical tube that contained 10 mL PBS. The tubes were agitated on their sides at room temperature on an orbital shaker for 15 min at 200 rpm. A series of 10-fold dilutions was prepared in PBS. An aliquot (0.1 mL) of the selected dilution and, when necessary, the undiluted extracts were plated onto the appropriate agar plates in triplicate by the overlay plaque assay method[15] as previously described. Each prepared tube of phage and host bacteria was then poured on a single plate and allowed to solidify under ambient conditions, then incubated at 26 ± 2 °C (Phi6) or 37 ± 2 °C (MS2) for 18−24 h. After incubation, plates were observed on light boxes, and visible plaques in the bacterial lawns were counted manually. The number of PFU/coupon was calculated by multiplying the mean number of plaques per plate by the reciprocal of the dilution, and then multiplying by 100 (0.1 mL aliquot was plated from the 10 mL sample used for each coupon extraction).

Decontamination Efficacy Calculations and D-values. For each test (coupons exposed to HPV, noted with subscript t) and positive control coupon (subscript pc), the number of PFU recovered was transformed to its $\log_{10}$ value. Then, the mean of the $\log_{10}$ values for each test coupon was subtracted from the mean of the $\log_{10}$ values from each positive control, for each time point assessed. This process is illustrated per the following equation, with efficacy reported in terms of $\log_{10}$ reduction (LR):

$$\text{efficacy} = \overline{(\log \text{PFUpc})} - \overline{(\log \text{PFUt})} \quad (1)$$

Test coupons in which there were no PFU recovered were assigned a PFU count of 1, resulting in a log PFU of zero. In such cases, the LR is reported as ≥ the value calculated by eq 1. For reference, we note that the US EPA requires that disinfectants with viricidal claims demonstrate ≥3 LR of the test virus on each surface.[39]

The LR results are reported with an associated 95% confidence interval (CI), calculated as follows:

$$95\%\text{CI} = \text{efficacy} \pm (1.96 \times \text{SE}) \quad (2)$$

The term SE is the pooled standard error, and was calculated as follows:

$$\text{SE} = \sqrt{\frac{S^2\text{pc}}{3} + \frac{S^2\text{t}}{3}} \quad (3)$$

where S is the standard deviation of the LR results for either the three positive controls (pc) or three test coupons (t) for each test condition.

In addition to calculating an LR value for each test condition and time point, we have determined inactivation rates (as D-values) for positive controls and the coupons exposed to HPV. D-values are commonly used to quantify the rate of inactivation of a microbial population, over several time points and are expressed as the time in h to reach 1 LR from the initially recovered PFU amount.[40,41] For brevity, these calculated D-values for each phage and test condition, as well as further information on the statistical methods and their detailed results, are found in the Supporting Information (SI). The D-values were calculated as the ratio of the shorter of the number of hours to achieve complete decontamination (i.e., when the phage was not detected) or the end of the decontamination test period; divided by the difference between the log recovery of the phage at the time point and the log recovery at time zero. To determine central values (e.g., median) and spread (e.g., 95% confidence interval) for D-value estimates at each condition, a resampling approach using R statistical computing software version 3.5.3 (https://www.r-project.org/) was used. From the distributions of D-values at each condition, several statistical comparisons were made to

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Table 2. Experimental Test Conditions

test number	test bacteriophage	phage stock solution diluent	actual average ± SD concentration HPV ppm	actual average ± SD temperature ° C	actual average ± SD RH%	time points (h)
1.1	Phi6	blood	24.4 ± 1.23	22.8 ± 0.24	74.7 ± 0.20	2, 4
1.2	Phi6	PBS	24.6 ± 1.38	23.3 ± 0.07	72.5 ± 0.63	2, 4
1.2	Phi6	blood	25.0 ± 0.36	22.6 ± 0.57	73.5 ± 2.19	24, 72
2	Phi6	blood	429 ± 43.0	28.1 ± 0.52	63.4 ± 2.47	4, 8, 24, 32
3	MS2	PBS	24.9 ± 0.34	22.3 ± 0.10	71.6 ± 1.32	2, 4, 6, 8
4.1	MS2	blood	24.9 ± 0.42	22.2 ± 0.13	64.6 ± 2.8	4, 8
4.2	MS2	blood	25.0 ± 0.68	22.4 ± 0.11	63.5 ± 4.3	24, 32
5	MS2	blood	454 ± 49.3	27.4 ± 0.35	74.6 ± 3.28	4, 8, 24, 32
6.1	Phi6	PBS	25.2 ± 0.6	22.0 ± 0.14	69.7 ± 1.11	6, 8
6.2	MS2	blood	25.3 ± 0.51	22.3 ± 0.3	75.8 ± 2.32	2, 72

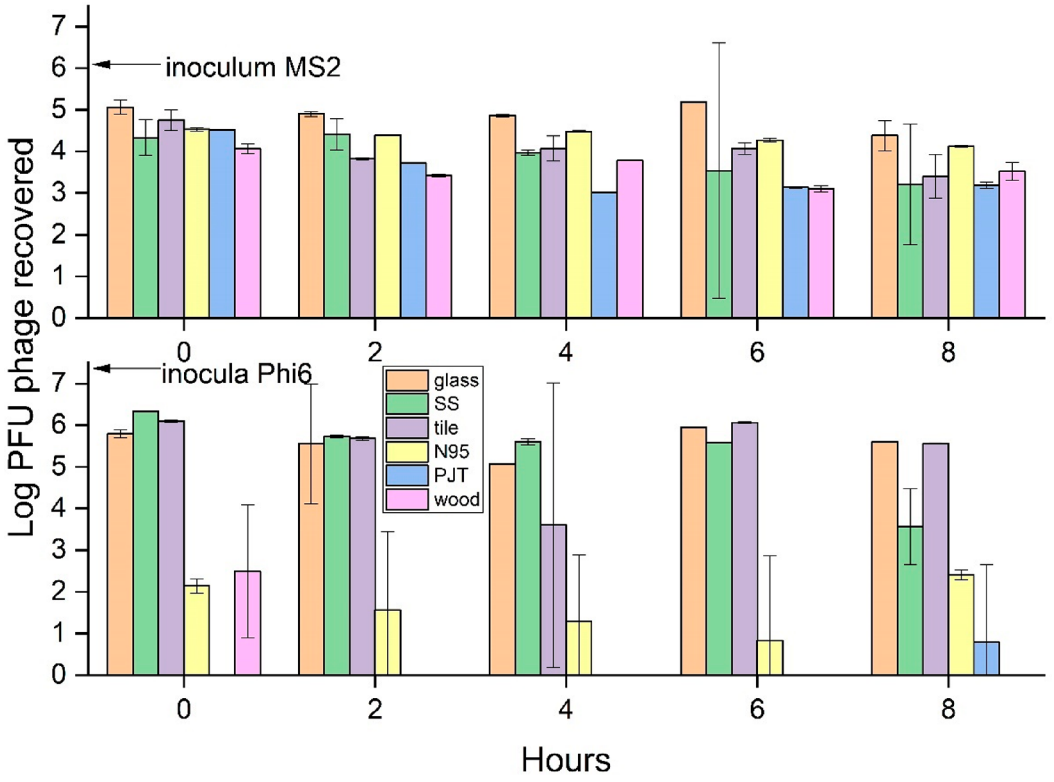

Figure 1. Inoculum and recovery of phages (average log plaque forming units ± SD) diluted in phosphate buffered saline from positive control materials, by elapsed time.

determine the significance of variables, and these are discussed along with efficacy results.

RESULTS AND DISCUSSION

Environmental Conditions. The measured air temperature, RH, and HPV concentration within the test chamber for each experiment of the study are summarized in Table 2. As Table 2 shows, some experiments such as Test 1.2 or Test 6 utilized both phages or both phage diluents (blood or PBS) concurrently in the test chamber. In Test 4, in which MS2 in blood was evaluated at 25 ppm of HPV, two subtrials were conducted because of the wide range in time points.

Air temperatures within the test chamber ranged between 22 and 28 °C, and average RH levels ranged from 63 to 76%. When fumigating at the LCHP target of 25 ppm (all tests except 2 and 5), actual concentrations were within 1 ppm of the target. In the two tests where we targeted a higher HPV concentration of 400 ppm, the actual average levels ranged from 429 to 454 ppm. While these HPV levels were somewhat higher (7−13%) than we had intended, the results are still valid and informative. The positive controls, which were kept outside the test chamber at ambient conditions, were exposed to average air temperatures ranging from 21.1−21.7 °C and average RH levels from 15−59%. A table summarizing these environmental conditions for the controls in terms of average ± standard deviation is found in the SI.

Inoculum and Recovery Levels for MS2 and Phi6 Phages from Positive Controls. The results for the inoculum titer quantification and the subsequent recoveries of the Phi6 and MS2 phages from the positive controls, presented as the average log PFU recovered from each material at each elapsed time, are shown in Figures 1 and 2. (For a more detailed listing of these results, including additional time points, refer to the corresponding tables found in the SI.) The positive control recoveries are aggregated in the figures as a function of phage and diluent and varied by several orders of

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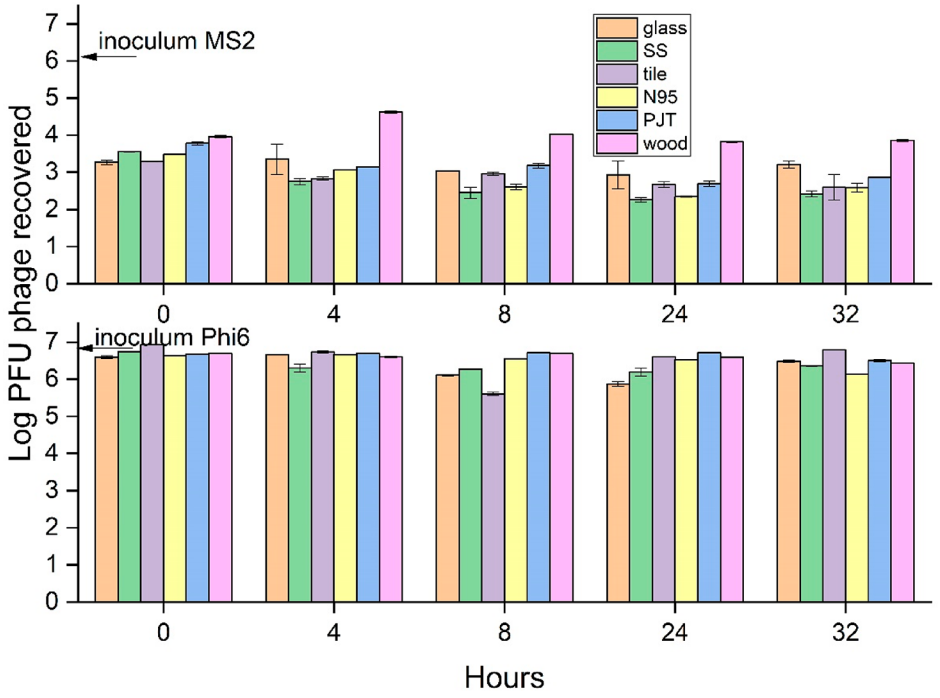

Figure 2. Inoculum and recovery of phages (average log plaque forming units ± SD) in blood diluent from positive control materials, by elapsed time.

magnitude. The inoculum levels of Phi6 averaged approximately 7.0 log PFU/coupon, whereas the MS2 inoculum levels averaged approximately a log lower, at 6.1 log PFU/coupon. While the results for the phage recoveries from positive controls are used primarily for the calculation of decontamination efficacy, the recoveries also provide insight into how the phages may persist over time outside their host cells on various materials, with or without the presence of human blood. In general, the loss in recovery or detection of a virus (or any other microorganism) from an inoculated material at ambient conditions may be due to the inability to physically extract the virus particle from the material (e.g., because of material porosity, adherence to the material) and/or that the virus was inactivated to some degree (possibly due to desiccation, chemical interaction with material).

In comparing the results for Figures 1 and 2, while the Phi6 phage was generally more persistent in blood than in PBS, the Phi6 phage in PBS was still recovered in appreciable quantities (>3.5 log PFU) from the nonporous glass, tile, and SS at the longest time point tested (8 h). With Phi6 in blood (Figure 2 and Table S9), nearly 6 log PFU was recovered from all materials at the longest time point tested of 72 h (Test 1.2), and >6 log PFU recovered at 32 h (Test 2) on all materials. The loss in recovery of the Phi6 phage in blood after 72 h was only 0.7−1.3 LR. The effect of material on the recovery of Phi6 was masked by the presence of blood. That is, the recovery of Phi6 in the PBS diluent (Figure 1, Table S8) from positive controls varied more by material than it did in blood, with generally poorer recoveries of the Phi6 phage occurring with the porous materials (PJT, N95 mask material, and wood). Further, in the PBS diluent, the Phi6 phage was not recovered from PJT at the initial "zero" time point, and was not recovered (not detected) from PJT or wood at the 2-h time point. The Phi6 phage in PBS did, however, demonstrate more stability on the nonporous materials (glass, SS, and tile), with over 5.5 log PFU recovered from glass and tile at 8 h, the longest time examined for the phages in PBS. That the Phi6 phage was generally more persistent (higher recoveries) in blood compared to PBS is generally consistent with their associated decay rates (refer to SI Table S4). Specifically, the *D*-values for Phi6 positive controls in blood ranged from 57 to 331 h, while the *D*-values for Phi6 in PBS were more variable (by material) and indeterminate, but generally lower.

In contrast to Phi6, the effect of diluent and material was not as pronounced for the MS2 phage.. When comparing the common 8-h time points for the two diluents, the range in average recovery of the MS2 phage in blood was ∼2.5−4.0 (Figure 2) and the range in recovery of the MS2 phage in PBS was 3.2−4.4 PFU. Further, the *D*-values for the MS2/blood positive controls were not significantly different from the *D*-values for the MS2/PBS positive controls, except for the N95 material (Table S4).

Thus, the presence of blood did not provide much additional protection to the MS2 phage, whereas the presence of blood does so for the enveloped Phi6 phage. This is an important finding as it contradicts the general thinking behind the disinfection hierarchy which indicates that the nonenveloped viruses (such as MS2) should be more resistant than the enveloped viruses. We were not able to locate data in the literature assessing the effect of blood on the persistence of MS2 or Phi6, to compare with our results. Research with EBOV did show that the virus was more stable in the presence of dried blood, although it depended on temperature and RH. Specifically, Schuitt et al.[7] showed that the decay rates of EBOV diluted in cell culture media and dried on materials were similar to the decay rates when EBOV was diluted in blood at room temperature (22 °C, 41% RH). However, at 28 °C and 90% RH, EBOV did persist longer in the blood matrix than in the cell culture media. Interestingly, for their 22 °C/41% RH test condition, which is comparable to our environmental condition for positive controls, Schuitt et al.[7] recovered approximately 0.8−1.5 log PFU EBOV/Mak-C05 in

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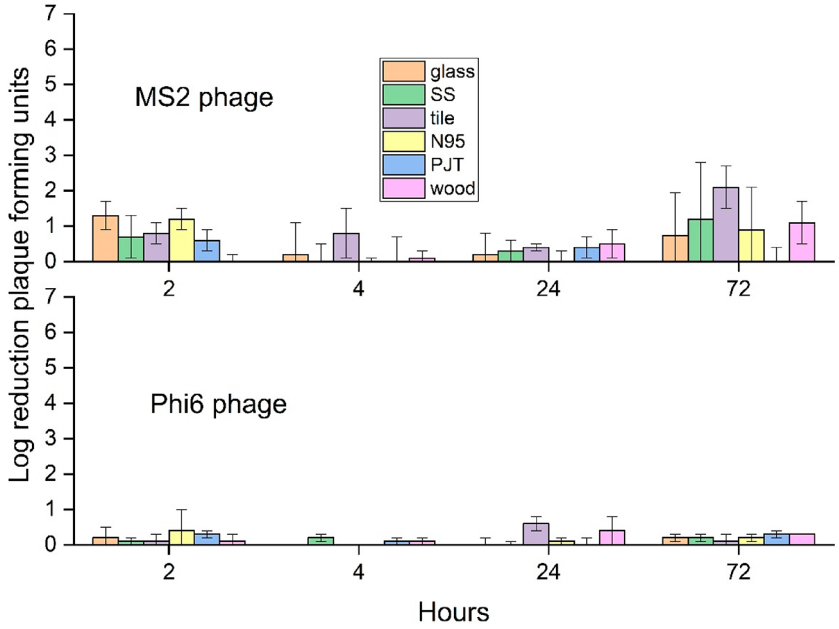

Figure 3. Decontamination efficacy results for Phi6 and MS2 diluted in blood, using low concentration hydrogen peroxide vapor (~25 ppm), as average log reduction ±95% confidence interval limits.

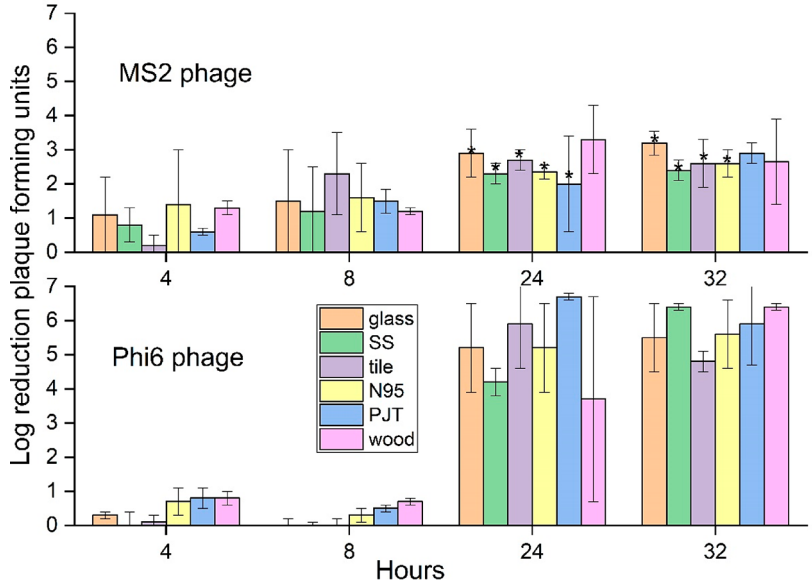

Figure 4. Decontamination efficacy results for Phi6 and MS2 diluted in blood, using hydrogen peroxide vapor >400 ppm, as average log reduction ±95% confidence interval limits. Bars with an asterisk indicate no phage were recovered from any test coupons, and thus efficacy results are determined based on recovery from positive controls.

dried blood from their four materials at 72 h (an approximately 2−3 LR). Under similar environmental conditions and elapsed time, our recovery of MS2 (1.9−3.6 log PFU) in the blood matrix was comparable to their EBOV recovery (the LR values for both were similar as well), but our recovery of the Phi6 phage was much higher (5.8−5.9 log PFU recovered; ~0.5 LR). In another study, EBOV showed greater persistence when maintained in liquid blood compared to water.[42]

In comparing the persistence of the two phages, the loss in recovery of MS2 in the presence of blood (Figure 2) was generally greater than the loss in recovery of Phi6 in blood. For example, at the common 72-h time point, only approximately 2−3 log PFU of MS2 in blood was recovered from the six positive control materials (refer to Table S11), which contrasts to the nearly 6 log PFU of the Phi6 in blood that was recovered for the same elapsed time, for every material. However, when comparing the two phages suspended in PBS (Figure 1), MS2 was recovered on all positive control materials, including PJT and wood, at 8 h, while the Phi6 in PBS was less persistent, at least on the porous materials (wood, PJT, N95). Thus, without the protection of the dried human blood, the nonenveloped MS2 phage shows more environmental persistence/stability on all materials than the enveloped Phi6, which is consistent with the general thinking that enveloped viruses are less resistant than nonenveloped viruses to disinfectants.[21,43] This finding is also consistent with a study

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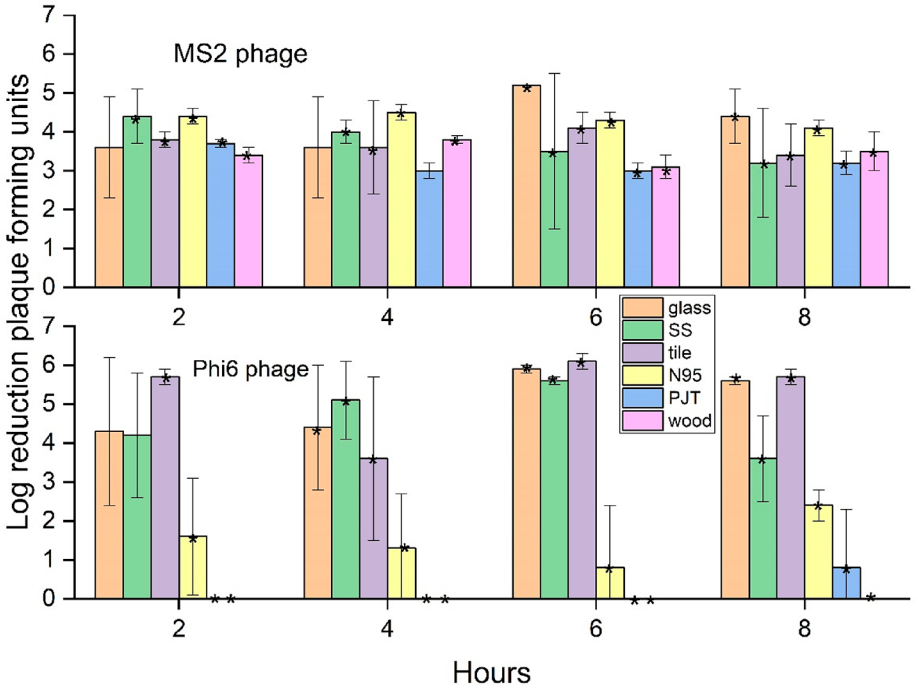

Figure 5. Decontamination efficacy results for Phi6 and MS2 diluted in phosphate buffered saline, using low concentration hydrogen peroxide vapor (~25 ppm), as average log reduction ±95% confidence interval limits. Bars with an asterisk indicate that no phages were recovered from any test coupons, and thus efficacy results are determined based on recovery from positive controls.

examining the persistence of MS2 and Phi6 (as potential surrogates for EBOV) in landfill leachate,[15] which found the enveloped Phi6 phage was much less stable than the MS2. In another study, the MS2 phage was also more stable (retained infectivity longer) than Phi6 when aerosolized and exposed to various RH levels and temperatures.[44]

Overall, the MS2 phage was recovered on all positive control materials at 8 h in the PBS diluent, and both phages were recovered at 72 h on all positive control materials in the presence of human blood. In the blood diluent, the persistence of both phages was prolonged and generally masked the effect of material, although this effect of blood was not as pronounced in the recovery of the nonenveloped MS2 phage. Relatively little decrease in the recovery of the Phi6 phage occurred at the longest time point evaluated (72 h) when dried in human whole blood, on all materials, corresponding to *D*-values up to hundreds of hours for a 1 LR.

HPV Decontamination Efficacy Results for MS2 and Phi6. The decontamination efficacy results for the study, presented in terms of the average LR of PFU, are summarized in Figures 3, 4, and 5. Select representative results are displayed for each material in each experiment, as a function of the HPV concentration (e.g., low or high level), diluent (PBS or human blood), and contact time. (All detailed decontamination efficacy results are also tabulated in the SI, Table S12.) There was minimal inactivation efficacy against both the MS2 and Phi6 phages when the phages were diluted in blood, dried onto various materials, and exposed to the LCHP of 25 ppm; refer to Figure 3. Over 90% of these average efficacy results from these test runs, for both phages, were <1 LR, while the maximum efficacy achieved overall at this condition was 2.09 LR (for MS2), which occurred at the 72-h time point on tile (Test 6.2). Although the decontamination efficacy of the LCHP was relatively low for both phages diluted in blood, statistical analysis (refer to SI) of the inactivation rates showed that the *D*-values for MS2 were generally significantly lower (approximately an order of magnitude) than for Phi6.

The presence of blood continued to preserve or shield both phages at the higher HPV concentrations (429 and 454 ppm) as well, albeit some inactivation (at least 3 to 4 LR) of both Phi6 and MS2 became evident at 24-h; refer to Figure 4. For example, at the 4- and 8-h time points for the tests >400 ppm of HPV, the LR values for the six materials were all less than 1.0 for the Phi6 phage, while the LR values for the MS2 phage were somewhat higher than the LR values for the Phi6 and ranged from 1.2 to 2.3 at the 8-h time point. At 24 h, the >400 ppm of HPV did effectively inactivate the Phi6 phage on all materials. For the MS2 phage at 24 h, none of the phage was recovered on any of the materials except wood, making decontamination efficacy values indeterminate but generally ≥2 LR. Overall, these efficacy values are still quite low compared to efficacy values achievable with similarly high HPV concentrations against other microorganisms, but without the presence of blood. For example, >6 LR was achieved against *Bacillus anthracis* spores on similar materials when using 290 ppm of HPV for 3 h.[45]

As expected, the LCHP decontamination approach (25 ppm) achieved higher efficacy with the phages inoculated in PBS (Figure 5) compared to when they were inoculated in blood. That is, without the presence of blood, the LCHP was effective (≥3 LR) for both phages on all materials at 2 h, for the materials in which phage were recovered/detected. In Test 3, with MS2/PBS at the 2-h time point, none of the materials had recoverable phage except for glass, which showed a LR of 3.58. The MS2 phage was inactivated at >5.2 LR on glass at the 6-h time point. In Test 6.1 with Phi6/PBS, decontamination at 25 ppm of HPV for 6 h resulted in no recoverable phage from all six materials. With the highest recovery of Phi6 on the nonporous material controls, this resulted in having >5.58 LR on glass, SS, and tile. The highest LR achieved against Phi6/

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PBS was >6.06 on tile at 6 h. As also expected, the *D*-values for the LCHP (25 ppm) for both phages in PBS were all ≤1 h and were significantly lower than the *D*-values for the inactivation of phages in blood.

These results demonstrating the deleterious effect of blood on the efficacy of HPV are generally consistent with the literature.[46] For example, Heckert et al. showed minimal LR against Classical Swine Fever (hog cholera) virus in the presence of porcine whole blood.[24] Pottage et al. showed that increasing the blood diluent level from 10% to 50% decreased the inactivation against the MS2 phage several orders of magnitude when using HPV.[27] The presence of blood also diminishes the efficacy of other decontaminants against other microorganisms, one example being the use of chlorine dioxide gas for the inactivation of several species of bacteria.[47] In the evaluation of several liquid disinfectants (including chlorine bleach) to assess their ability to inactivate EBOV in the presence of blood, only 5% peracetic acid was effective.[12] We would agree with Smither et al.,[12] who suggest two reasons why the presence of blood diminishes inactivation of viruses and microorganisms: that the blood may be providing a physical barrier for the decontaminant to reach the virus; and/or that the blood, an organic material, reacts with the oxidant-based decontaminant and reduces its concentration prior to reaching the virus. HPV, bleach, chlorine dioxide, and many other decontaminants rely on oxidation as the mechanism for microbial inactivation.

We were not able to locate any literature related to the use of LCHP for the inactivation of MS2, Phi6, and/or EBOV, with which to compare our results. The best comparison we can make with the literature is a study evaluating LCHP for the inactivation of another enveloped virus, i.e., several influenza viruses,[29] and which reported nearly 3.5 LR of the virus on stainless steel, using a HPV concentration of 10 ppm for only 15 min.

Regarding the effect of material on decontamination efficacy in the presence of blood, for both phages, the results indicate a lack of difference in efficacy among the six materials, most likely due to the blood overriding or masking any underlying material effects. With the phages diluted in PBS, the majority of the LCHP decontamination tests resulted in no recovery of the phages, making it difficult to make valid comparisons among the materials.

In summary, without the presence of human blood, the LCHP was effective (≥3 LR) against both phages on all materials at two h contact time, for the phages that were recoverable from controls at that time. Additionally, both the Phi6 and MS2 phages in the PBS diluent were completely inactivated (none detected) by the LCHP on all materials by 6 h. But in the blood matrix, LCHP was ineffective against both phages for all materials, even with a 3-day contact time. Thus, extrapolating from these results for both an enveloped and nonenveloped virus, we would expect LCHP would be a viable decontamination option for EBOV for relatively clean surfaces. Although we caveat that having data for an EBOV would be preferred to that of a surrogate, the evidence we proffer here and, as noted in the literature,[10] suggest that the presence/absence of a viral envelope, protective organic matrix such as blood, and the material with which the microorganism is associated may be more of a factor in decontamination efficacy (as well as environmental persistence) than the actual virus genome/biochemistry. In the presence of blood, high levels of HPV (>400 ppm) were effective in inactivating the Phi6 phage on all materials with 1 day contact time, but results were indeterminant for MS2, due to the inability to recover the MS2 phage from positive control materials ≥3 log PFU at 24 h. Further investigation of decontamination techniques that would be effective in inactivating the EBOV in a blood matrix is recommended.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b06034.

> Inactivation/decay rates (as *D*-values) for phages, for positive controls and those exposed to HPV; statistical comparison of *D*-values; positive control recovery results in visual format (figures) and detailed results in tables; environmental conditions for positive controls; and detailed decontamination efficacy results in table format (PDF)

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Notes

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