

Title: Bacteriophage $\Phi 6$ (Phi6) as a Surrogate for Enveloped Viral pathogens in Standard and Long-lasting Virucidal Efficacy Tests

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Title: Bacteriophage $\Phi 6$ (Phi6) as a Surrogate for Enveloped Viral pathogens in Standard and Long-lasting Virucidal Efficacy Tests

Running title: Bacteriophage $\Phi 6$ as a Surrogate for Enveloped Viruses

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Abstract

Development of novel antimicrobials capable of providing long-lasting disinfection on surfaces requires the implementation of new standardized methods to support claims recognized by regulatory authorities. Surrogates for viral pathogens are often desired in order for BSL-2 labs to conduct these new efficacy tests safely and efficiently, however, the knowledge of surrogate suitability for these tests is limited. Here, for the first time, we determine the resistance profile of the bacteriophage Phi6 to quaternary ammonium compounds (QACs) in a variety of test conditions. Additionally, we show that Phi6 can be used to demonstrate the long-lasting virucidal efficacy of a novel antimicrobial, Actizone™ F5, and that the Phi6 is more resistant to QACs following the UK standard long-lasting disinfection test BSI PAS2424:2014 than Vaccinia virus, which is the marker strain for claims of activity against enveloped viruses in Europe. Surface stability as well as benefits and limitations of Phi6 use relative to other enveloped viruses for antimicrobial testing is also discussed.

Introduction

The ongoing pandemic caused by SARS-CoV-2^{1,2} has led to increased interest in enhanced disinfection and longer lasting protection of environmental surfaces against viruses. Enveloped viruses including SARS-CoV-2 are generally considered less stable on inanimate surfaces and more susceptible to disinfectants compared to non-enveloped viruses^{3,4,5}. However, recent evidence suggests a surprising ability of enveloped viruses including coronaviruses to persist in the environment^{6,7} and remain infectious on various surfaces for up to several days^{8,9,10,11}.

Traditional disinfectants approved for use against viruses are not required to demonstrate antimicrobial efficacy beyond the time of application or ability to maintain activity through multiple dry or wet contacts over time. Consequently, field studies have demonstrated that various environmental surfaces treated with traditional products were quickly re-contaminated within a few hours post cleaning and disinfection^{12,13,14,15}. These factors combined with everyday challenges of disinfectant application^{16,17} leave surfaces and high-touch areas vulnerable to recontamination and onward transmission of surviving pathogens.

To address this technological gap in protection of environmental surfaces, a group of long-lasting disinfectant products have recently emerged for broad consumer and healthcare applications^{18,19,20}. The residual efficacy of CAD products are evaluated by the Residual Self-Disinfection (RSD) method in the US and BSI PAS 2424:2014 (2014) in the UK. In contrast to traditional challenge tests, these methods take into account surface abrasion (i.e. repeated touching) and recontamination events that could occur under real-world conditions while staying as close as possible to the practical conditions that are outlined in the standard efficacy tests such as culture preparation, strength and recovery as well as requirements for soil and test surface

characteristics. However, both these methods do not provide the option to test viruses, therefore limiting the residual claims possible in these regions mainly to bacterial and yeast species.

On October 14, 2020 the EPA introduced a much anticipated policy innovation which enabled the evaluation and registration of liquid disinfectants with a 24 hr virucidal claim²¹. While there is no universally accepted method to support a residual virucidal claim, the agency has recommended the combined principles of RSD and ASTM E1053 (standard virucidal test) methods as an interim guidance for the study design. The resulting test which we term, herein, as Residual Virucidal Test (RVT) will include the surface abrasion and re-inoculations features of the RSD method while maintaining the standards of virus cultivation, recovery and performance measurements as specified in ASTM E1053.

The objective of this study was twofold. First, to validate an appropriate, practical, and safe surrogate for viral pathogens, and second to use that surrogate to evaluate the residual virucidal efficacy of a novel disinfectant Actizone™ F5 by utilizing the RVT and BSI PAS2424 test methods. Bacteriophage Phi6, being a well-known surrogate for enveloped viruses^{22,23} was evaluated as the standard test virus. While the environmental stability and resistance of Phi6 to chemical treatments in relation to some viruses of interest has been studied^{3,4,5,24,25,26} comparative resistance of the bacteriophage to QACs has not been reported so far. To this effect, the efficacy of QACs against Phi6 relative to those reported for SARS-like viruses and other pathogenic enveloped viruses is also evaluated. Finally, surface stability as well as benefits and limitations of Phi6 use as a surrogate for antimicrobial testing is also discussed.

Results

Combining our own data regarding Phi6 with data found in the literature, we compared the resistance of Phi6 to the resistance of other enveloped viruses in the presence of a widely-used commercial QACbased disinfectant, Microchem-Plus (MCP™) (Figure 1-2). Our benchmarking of Phi6 demonstrates that the bacteriophage was more resistant to QAC than the bat SARS-like coronavirus, WIV1, when tested in a suspension-based method similar to the EU virucidal test, EN14476 (Figure 1). While the manufacturer recommended 5% concentration was able to completely deactivate both WIV1 and Phi6 as expected, a 5%/81 dilution of MCP™ was able to reduce WIV1 by between 6.35 and 7.17 logs, while the same concentration of MCP™ was only able to reduce Phi6 by less than 2.62 logs. A further dilution of MCP™, 5%/243, was ineffective against both viruses, reducing WIV1 by 1.27 logs and Phi6 by 0.04 logs.

We also examined the Phi6 resistance to QAC when tested in a surface-based method similar to ASTM 1053 (Fig. 2). In this case, we only had access to data for a single concentration of MCP™. The manufacturer recommended concentration of 2 oz/gal MCP™ was able to completely deactivate Avian Influenza A(>4.5 LR), Avian Influenza A (H3N2) (>4.5 LR), SARS-Associated Coronavirus (>4.0 LR), and Human Coronavirus (229E) (>3.0 LR). The viral control recovery was higher for Phi6 (7.8 log₁₀) than the other enveloped viruses (4.5-6.5 log₁₀), so while the log₁₀ reduction was high for Phi6 (>5.4 LR), there was not complete deactivation as was seen with the other enveloped viruses.

In addition to examining the resistance of Phi6 to QAC both in suspension and on surfaces, we also evaluated the innate stability of the bacteriophage on surfaces (Fig. 3). Following the protocol described by Chin and colleagues³¹ for determining the persistence of SARS-CoV-2 on surfaces, our data shows that in the short term (30 minutes and less), Phi6 is more stable on

stainless steel surfaces than SARS-CoV-2. SARS-CoV-2 loses 0.6 logs during the first 30 minutes on a stainless steel surface while Phi6 remains roughly constant (Fig. 3 inset). However, the persistence of Phi6 deteriorates after the first 30 minutes, and it becomes less stable than SARS-CoV-2 after 3 hours.

After determining the suitability of bacteriophage Phi6 as a surrogate for other enveloped viruses, we used Phi6 to test the residual virucidal efficacy of Solvay's long-lasting disinfectant, Actizone™ F5 following the interim guidance provided by the EPA (RSD), and the method described by the BSI (PAS2424) (Table 1). Solvay's Actizone™ F5 long lasting disinfectant passed the UK standard BSI PAS2424 test by reducing the viral titre of Phi6 on surfaces by at least 3 logs after the surfaces underwent a series of abrasions and inoculations designed to simulate use over a 24-hour period. The viral titre was reduced by an average of 3.06 log₁₀ after a five minute contact time, and by over 5.4 log₁₀ (complete deactivation) after a ten minute contact time. Actizone™ F5 also passed BSI PAS 2424 when tested against vaccinia virus, showing greater than 3.5 log₁₀ reduction and complete deactivation of the virus after a five minute contact time. In addition to testing Actizone™ F5, which is specially designed to provide long lasting disinfection, we also tested a version of Actizone™ F5 without Actizone™P5 (Actizone™P5 is a proprietary polymer which stabilizes the Actizone™ F5 active ingredients on surfaces). The formulation without the polymer did not pass the BSI PAS2424 standard and showed less than 1.43 log₁₀ reduction in viral load. Finally, we also tested Actizone™ F5 against the RVT standard described by the EPA in the recently published interim guidance. Actizone™ F5 passed the RVT protocol, showing greater than 4 log₁₀ reduction in viral titre after a 10 minute contact time.

Discussions

In this study, a novel disinfectant, Actizone™ F5 was evaluated for its long lasting, abrasion resistant virucidal activity against bacteriophage Phi6. The evaluations were conducted according to the principles of RSD (EPA US) and BSI PAS2424 (UK) methods which were modified for viruses. Unlike existing virucidal standards, both of these methods subject the disinfectant-treated surface to multiple cycles of alternating wet and dry wiping (abrasions) each interspersed with a challenge consisting of at least 3-4 log₁₀ virus particles. In order to meet the regulatory requirements, the test surface would need to demonstrate a minimum of 3 log₁₀ or 99.9% reduction of the final viral challenge within 10 minutes of exposure. Given the experimental rigor and high performance standards, the products that pass these tests are expected to deliver more durable and longer lasting disinfection of surfaces despite multiple touches or contamination events.

In order to operate with safety as our top priority, and considering our understanding of the suitability of Phi6 as a surrogate for select enveloped viruses, we used Phi6 as our standard test organism in both protocols. In general, stability of a given virus on an inanimate surface is an important prerequisite for its use in carrier based tests. The recovery of viruses need to be sufficient enough to allow investigators to measure the targeted log₁₀ reduction while accounting for the detection limit and errors associated with the assay. Secondly, the surrogate virus ideally should demonstrate similar if not higher levels of tolerance towards the biocidal active in question. However, to our knowledge, the surface stability and efficacy of QACs against Phi6 in relation to other enveloped viruses remain limited. To this effect, we first evaluated the surface stability under relevant environmental and humidity conditions mirroring the conditions tested in SARS-CoV-2 studies²⁷. Our data graphed in parallel with this study demonstrate the Phi6 is

stable within the first 2 hours, yet viability reduced over a longer period compared to SARS-CoV-2. This finding suggests that Phi6 recovery would be sufficient for most conventional virucidal tests including residual tests which do not require more than 1 hour of drying. On the other hand, challenge tests requiring, for example, more than 2 hr contact time or longer drying times may not be suitable for experimentation with Phi6 as a surrogate for enveloped viruses.

Comparative resistance of Phi6 to quaternary ammonium active was further evaluated according to the standards of ASTM1053 (carrier based) and EN14476 equivalent (suspension) tests.

Micro-Chem Plus™ is a well-known widely used disinfectant with wide spectrum efficacy data available publicly²⁸ and therefore was chosen as a representative test substance. In the suspension based test, Phi6 demonstrated a significantly higher resistance to the disinfectant compared to the SARS-like virus WIV1²⁷ and a similar level of sensitivity compared to other enveloped viruses in carrier based tests.

The Phi6 bacteriophage also demonstrated more tolerance towards the disinfectant in the residual disinfection test, BSI PAS2424, where Actizone™ F5 yielded the complete kill of vaccinia virus versus only 3 log₁₀ reduction of Phi6 following a 5 minute exposure. Demonstrating the need for specially designed formulations in order to provide lasting disinfection, Actizone™ F5 formulation without the proprietary polymer system (Actizone™P5) failed the BSI PAS2424 test with less than 3 log₁₀ reduction. It is proposed that Actizone™P5 polymer with QACs forms a phase stable fluid formulation which upon application forms an abrasion resistant film on surfaces²⁹. Upon drying the film acts as an abrasion-resistant barrier that retains actives on the surface that kill bacteria and viruses on-demand. As such, Actizone™P5 technology serves as a safe and inert additive (data on file with Solvay) that will help formulators to achieve a long

lasting surface disinfection with standard levels of QACs while minimizing the number of re-applications and risk of chemical exposure during use.

Actizone™ F5 was also tested following the interim guidance provided by the EPA (RSD (RVT)) and passed with a greater than 4 log₁₀ reduction following a 10 minute exposure. In general, the RSD protocol is more challenging than the BSI PAS2424 standard as it requires more abrasions (48 total passes over the surface) and more weight applied to the surface during the abrasions. Although the scientific rationale behind such rigorous abrasion regimen involved in the RSD method is not clear, the abrasion and inoculation procedures of BSI PAS2424 have been justified by a field testing³⁰. Regardless, Actizone F5 being efficacious against Phi6 in both RSD and BSI PAS 2424 methods, is likely to be effective against many other enveloped viruses pathogenic to animals.

This is to our knowledge, the first demonstration of the Phi6 resistance profile to QACs suggesting that the phage could be an appropriate substitute for many enveloped viruses that are otherwise difficult or highly risky to work with.

Use of safer and easier to handle strains like Phi6 provides additional advantages for investigators. More specifically, Phi6 can be cultivated in a day or two whereas most human and animal viruses require a minimum of 5-7 days of cultivation which increases cost and the experimental turnaround times. Phi6 is also easier to handle and recover: a refrigerated stock of Phi6 will last several months at least without significant reduction of titer, and for contact times of half an hour or less, recovery of viral controls is high (typically <0.5 log₁₀ difference between the applied and recovered titers). In the context of the RVT test, the Gardco® wear tester specified in the protocol (used for abrasion cycles) is a large instrument (and therefore may not be suitable for operation within the typical biosafety level 3 or level 2 cabinets without

obstructing the laminar flow or technician dexterity required for safe operation. The abrasion and re-inoculation steps, while being executed on the bench top will require extensive and repetitive contact of operators with viral laden tiles, increasing the risk of exposure to airborne pathogens such as SARS-CoV-2. Manipulation of pathogenic viruses must only be conducted within a biological laminar flow hood of a suitable biocontainment level for the virus being worked on. Safer surrogates such as bacteriophage Phi6, therefore, could be an ideal option for investigators while working under standard laboratory conditions.

In conclusion, long lasting virucidal efficacy of a novel disinfectant Actizone™ F5 was demonstrated, suggesting that the product among other similar products could be useful in continuous protection of high touch surfaces from harmful viruses. The novel disinfectant along with other infection prevention strategies may serve as a superior alternative to conventional disinfectants. Resistance towards the QAC disinfectant and surface stability profiles of Phi6 demonstrated herein suggest that Phi6 is a suitable and safer alternative for enveloped viruses like coronaviruses.

Materials and Methods

Test strain and culture conditions

Bacteriophage Phi6 (HER102) and its host *Pseudomonas syringae* HB10Y #3 (HER1102) were purchased from Universite Laval (Quebec City, Quebec, Canada). High titer ($\sim 10^9$ PFU/mL) phage stock was prepared in broth. Briefly, an aliquot of an overnight *P. syringae* culture in AOAC nutrient broth (NB) media was diluted into fresh NB and allowed to grow, shaking at room temperature for 4-8 hours. Phage freezer stock was harvested with a sterile toothpick, added to the host culture, and grown overnight at room temperature to lyse completely. The lysed culture was centrifuged at 3500g for 15 minutes and the lysate (supernatant) was filtered at 0.22 microns and transferred to a sterile tube for storage at 4°C. Strain and maintenance for Vaccinia virus

Vero cells (African green monkey kidney epithelial cells, PHE, catalogue number: 84113001) cultured in DMEM:F12 were inoculated with a stock solution of vaccinia virus Elstree strain (PHE, catalogue number: 0110282v) at a multiplicity of infection (MOI) of 0.1. Once a cytopathic effect (CPE) was observed throughout the culture after 3-4 days post infection the flask was subjected to three freeze-thaw cycles. Cell debris was removed by low-speed centrifugation. The resulting supernatant was recovered for testing, aliquoted and stored at -80°C.

Replication of the study by Zhang and colleagues²⁷ suspension based efficacy test

A 10X dilution of phage stock was prepared in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 2.5% fetal bovine serum (FBS; GE Life Sciences). A 243X dilution of 5% Microchem-Plus (MCP™) detergent disinfectant cleaner in soft water (DI water) was prepared freshly the day of the experiment. A 1 mL aliquot of phage solution was mixed with an

equal volume of 5%/243, 5%/81 or 5% MCP™ or soft water as a control. After a one minute contact time the activity of the MCP™ (or the water control) was neutralized with 2 mL of Dey Engley (DE) broth. The surviving viral particles were enumerated by the double agar overlay plaque assay: neutralized solutions were serially diluted in phage buffer (100 mM NaCl, 8 mM MgSO₄•7H₂O, 50 mM Tris-Cl), 100 μL of phage dilution was mixed with 100 μL of *P. syringae* and 4 mL of soft NBY (nutrient broth yeast extract) agar (7.5 g agar/L) and plated over hard NBY agar plates (15g agar/L). Plates were incubated for 18-24 hours at 23°C and log₁₀ reduction of Phi6 is compared to log₁₀ reduction of the bat SARS-like coronavirus, WIV16 in the same conditions.

Persistence studies following Chin et al., 2020³¹

A working phage suspension was prepared by adding 50 μL FBS and 850 μL DMEM to 100 μL phage stock. A 5 uL droplet of suspension was applied to clean stainless steel surfaces and then placed in an environmental chamber at 22°C and 65% Relative Humidity (RH) for the given time. To harvest, 200 μL phage buffer was pipetted on top of the steel disk and left to sit for 30 min. The surface was gently scraped with a pipette tip and 100 μL was used for serial dilution and plating as described above. The survival of Phi6 was compared to the survival of SARS-CoV-2 in the same conditions⁸.

Surface disinfection efficacy tests

Surface disinfection efficacy tests were based on ASTM 1053. A 2 oz/gal concentration of MCP™ was prepared in hard water as described in ASTM 1153. Inoculum was prepared by adding 125 μL BSA, 500 μL mucin, 175 μL yeast extract to 1.7 mL phage stock. 200 μL of the phage suspension was placed on a glass petri plate and dried in a biological safety cabinet.

Control films were overlaid with 2 mL phage buffer while test films were overlaid with 2 ml MCP™.

After a 10 min contact time 2 mL Dey Engley broth was added as a neutralizer and the surface was scraped with a sterile pipette tip to resuspend the phage. The neutralized solutions were serially diluted and plated as described above. The sensitivity of Phi6 was compared to the sensitivity of several enveloped viruses in similar conditions⁷.

Residual Virucidal Efficacy Tests

Residual Self-Disinfection (RSD) Activity of Dried Chemical Residuals on Hard, Non-Porous Surfaces (EPA) along with The PAS2424:2014 (the British Standard Institute) are two antimicrobial tests that take into account surface abrasion (i.e. repeated touching) and recontamination events that could occur under real world conditions while staying as close as possible to the practical conditions that are outlined in the current US EPA and European Standards.

Residual Self-Disinfection (i.e Residual Virucidal Activity)

In the RSD method, the test surface (stainless steel) tiles were exposed to 100-150 µL of the test substances and placed in a humidity (45-55% RH) controlled chamber until completely dry. Once the tiles were dry, each tile was inoculated with 10^6 to 10^7 PFU of Phi6 particles and then abraded using a standardized abrasion machine (Gardco wear tester, Gardner, Pompano Beach, FL) under multiple alternating wet and dry wiping conditions and re-inoculations with 10^5 PFU: 6 dry cycles, 6 wet cycles, for a total of 12 cycles [4 passes per cycle = 48 passes] was used with 1.0 kg of weight applied. Re-inoculations occurred after each cycle, wet and dry. Upon completion of abrasion and re-inoculation regimen each tile was challenged with the final

inoculum of 10^6 to 10^7 PFU of Phi6 particles and the ability of the disinfectant to kill >99.9% of the test microbes within 10 minutes was measured. After the exposure time of 10 minutes, each tile was submerged into 30 mL of neutralizing broth (Dey Engley Broth, BD Difco™) and sonicated for 1 minute to dislodge surviving phage particles. The resulting mixture was then serially diluted and plated using double overlay agar and incubated as described above.

BSI PAS 2424

In the PAS 2424 test, sterile stainless steel discs were initially inoculated with 10µL of the virus test suspension ($\sim 10^8$ PFU/mL in dMEM media with 3 g/L bovine serum albumin (BSA) for initial and final inoculations, $\sim 10^6$ PFU/mL in dMEM media with 3 g/L bovine serum albumin for reinoculations) and allowed to dry. Following the drying period 100 µL of disinfectant or hard water control was dosed onto the surface of the substrate and left to dry overnight. The dosed substrate then underwent a series of abrasions. A dry abrasion was applied followed by reinoculation of 10 µL of the virus test suspension followed by a drying period. A wet abrasion was then conducted using hard water (pH 7.0 ± 0.2). Once dry a further 10 µL virus reinoculation took place followed by another drying period. This was considered as one abrasion cycle. The process was conducted for a total of three abrasion cycles. The final inoculation was added at a challenge dose of 50 µL of the virus test suspension and left for a five minute contact time. Following the contact time, the disc was immediately transferred to 4950 µL of ice-cold dMEM and vortex mixed for 60 seconds. The virucidal activity of the test disinfectant was evaluated by calculating the decrease in detectable virus titre in comparison to the water control without disinfectant. In accordance with the BSI PAS 2424:2014, a disinfectant or product is deemed as having residual efficacy if the titre is reduced by at least 3 log₁₀.

BSI PAS 2424:2014 method for vaccinia virus

The method conducted to assess for residual virucidal activity was developed by combining aspects of the BS EN 16777:2018 and BS PAS 2424:2014 and is referred to as 'viral PAS 2424' hereon in. In the viral PAS 2424, sterile stainless discs were inoculated with virus test suspension (virus stock mixed 9:1 with 3g/L bovine serum albumin). After drying, within one hour, 100µL of product was dosed onto the substrate surface and left to dry for 24 hours. The dosed substrate then underwent a series of abrasions using a 100% polypropylene wipe and a 210 ± 2 gram weight. A dry abrasion was applied followed by reinoculation of 10µL of the virus test suspension and allowed to dry. A wet abrasion was then conducted using hard water (pH 7.0 ± 0.2). Once dry, a further 10µL of virus test suspension was inoculated and allowed to dry. This was considered as one abrasion cycle. The process was conducted for a total of three abrasion cycles. The final challenge inoculum was 50µL of virus test suspension which was left for a contact time of 5 minutes. Following the contact time, the substrate was immediately transferred to 4950µL of ice-cold DMEM without FBS and vortex mixed (SLS lab basics). The water virus controls were conducted in parallel under the same stated conditions. The virucidal activity of the product was calculated by the decrease in detectable virus titres from the average of the water controls in comparison to the test. A disinfectant or product was deemed as having residual activity if the titre is reduced by at least 3 \log_{10} steps.

Where cytotoxicity of the product was so high that a 3 \log_{10} reduction was not possible the large volume plating (LVP) technique was applied. Following the inactivation assay, a further 1:50 dilution was immediately performed in DMEM (total dilution 1:5000). The resulting mixture was added to eight 96-well plates per assay replicate to assess for residual virus.

Statistical analysis

Each data point generated represents the mean \pm standard deviation of multiple replicates as indicated. The software used for the data analysis was Microsoft® Excel. The student t-test was used to compute the differences between the samples and the controls with significance assigned to $P < 0.01$. The percent \log_{10} reduction was calculated using the following formula:

$PLR = (1 - 10^{-LR}) * 100$ where LR is the \log_{10} reduction.

BSI PAS 2424 statistical analysis for vaccinia virus

The tissue culture infective dose 50% per mL (TCID₅₀/mL) of the tests were calculated as a mean of the replicates using the Spearman-Kärber formula. Where LVP was used and no CPE was observed the virus titre was calculated by the Poisson distribution formula. If CPE was observed the Taylor formula was applied.

Reference

1. Gorbalenya AE, Baker SC, Baric RS. 2020. The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat Microbiol* 5:536–544.
2. Wang C, Horby PW, Hayden FG, Gao GF. 2020. A novel coronavirus outbreak of global health concern. *Lancet* 395:470-473.
3. Boehm A & Wigginton K. 2020. Overview of Research on the Fate and Behavior of Enveloped Viruses in the Environment. [Virtual issue]. *Environmental Science & Technology*.
4. Kampf G, Todt D, Pfaender S, Steinmann E. 2020. Persistence of coronaviruses on inanimate surfaces and their inactivation with biocidal agents. *J Hosp Infect* 104:246-251.
5. Wigginton KR, Ye Y, Ellenberg RM. 2015. Emerging investigators series: the source and fate of pandemic viruses in the urban water cycle. *Environ Sci Water Res Technol* 1:735.
6. Ye Y, Ellenberg RM, Graham KE, Wigginton KR. 2016. Survivability, Partitioning, and Recovery of Enveloped Viruses in Untreated Municipal Wastewater. *Environ Sci Technol* 50:5077-5085.
7. Otter JA, Donskey C, Yezli S, Douthwaite S, Goldenberg SD, Weber DJ. 2016. Transmission of SARS and MERS coronaviruses and influenza virus in healthcare settings: the possible role of dry surface contamination. *J Hosp Infect* 92:235-250.
8. Olsen SJ, Chang HL, Cheung TY, Tang AF, Fisk TL, Ooi SP, Kuo HW, Jiang DD, Chen KT, Lando J, Hsu KH, Chen TJ, Dowell SF. 2003. Transmission of the severe acute respiratory syndrome on aircraft. *N Engl J Med* 349:2416–2422.

9. Harbourt D, Haddow A, Piper A, Bloomfield H, Kearney B, Gibson K. 2020. Modeling the stability of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) on skin, currency, and clothing. *PLoS Negl Trop Dis* 14:e0008831.
10. Liu Y, Li T, Deng Y, Liu S, Zhang D, Li H, Wang X, Jia L, Han J, Bei Z, Li L, Li J. 2021. Stability of SARS-CoV-2 on environmental surfaces and in human excreta. *J Hosp Infect.* 107:105-107.
11. van Doremalen N, Bushmaker T, Morris DH, Holbrook MG, Gamble A, Williamson BN, Tamin A, Harcourt JL, Thornburg NJ, Gerber SI, Lloyd-Smith JO, de Wit E, Munster VJ. 2020. Aerosol and Surface Stability of SARS-CoV-2 as Compared with SARS-CoV-1. *N Engl J Med* 382:1564-1567.
12. Aldeyab MA, McElnay JC, Elshibly SM, Hughes CM, McDowell DA, McMahon MA, Scott MG, Kearney MP. 2009. Evaluation of the Efficacy of a Conventional Cleaning Regimen in Removing Methicillin—Resistant *Staphylococcus aureus* From Contaminated Surfaces in an Intensive Care Unit. *Infect Control Hosp Epidemiol* 30:304–306.
13. Kwan SE, Shaughnessy RJ, Hegarty B, Haverinen-Shaughnessy U, Peccia J. 2018. The reestablishment of microbial communities after surface cleaning in schools. *J Appl Microbiol.* 125:897-906.
14. Schmidt MG, Fairey SE, Attaway HH. 2019. In situ evaluation of a persistent disinfectant provides continuous decontamination within the clinical environment. *Am J Infect Control* 47:732-734.
15. Scott E, Bloomfield SF, Barlow CG. 1984. Evaluation of disinfectants in the domestic environment under 'in use' conditions. *J Hyg (Lond)* 92:193-203.

16. Boyce, J.M. 2016. Modern technologies for improving cleaning and disinfection of environmental surfaces in hospitals. *Antimicrob Resist Infect Control* 5:10
<https://doi.org/10.1186/s13756-016-0111-x>.
17. Peters A, Otter J, Moldovan A, Parneix P, Voss A, Pittet D. 2018. Keeping hospitals clean and safe without breaking the bank; summary of the Healthcare Cleaning Forum *Antimicrob Resist Infect Control* 7:132 <https://doi.org/10.1186/s13756-018-0420-3>.
18. Rutala WA, Gergen MF, Sickbert-Bennett EE, Anderson DJ, Weber DJ. 2019. Antimicrobial activity of a continuously active disinfectant against healthcare pathogens. *Infect Cont Hosp Ep* 40:1284–1286.
19. Schmidt MG, Fairey SE, Attaway HH. 2019. In situ evaluation of a persistent disinfectant provides continuous decontamination within the clinical environment. *Am J Infect Control* 47:732-734.
20. Byotrol PLC. 2021. Landmark test result released for Byotrol 4-in1 Cleaner & Disinfectant which offers COVID-19 protection over 24-hours. As retrieved on May 2021. <http://www.byotrolplc.com>.
21. Environmental Protection Agency, USA. 2020. Interim Guidance - Review for Products Adding Residual Efficacy Claims. <https://www.epa.gov/pesticide-registration/interim-guidance-review-products-adding-residual-efficacy-claims>.
22. Adcock NJ, Rice EW, Sivaganesan M, Brown JD, Stallknecht DE, Swayne DE. 2009. The use of bacteriophages of the family Cystoviridae as surrogates for H5N1 highly pathogenic avian influenza viruses in persistence and inactivation studies. *J Environ Sci Health A Tox Hazard Subst Environ Eng*. 44:1362-1366.

23. Gallandat K, Lantagne D. 2017. Selection of a Biosafety Level 1 (BSL-1) surrogate to evaluate surface disinfection efficacy in Ebola outbreaks: Comparison of four bacteriophages. *PLoS One*. 12:e0177943.
24. Li J, Dennehy JJ. 2011. Differential bacteriophage mortality on exposure to copper. *Appl Environ Microbiol*. 2011 Oct;77(19):6878-83. doi: 10.1128/AEM.05661-11. Epub 2011 Aug 12.
25. Wood JP, Richter W, Sunderman M, Calfee MW, Serre S, Mickelsen L. Evaluating the Environmental Persistence and Inactivation of MS2 Bacteriophage and the Presumed Ebola Virus Surrogate Phi6 Using Low Concentration Hydrogen Peroxide Vapor. *Environ Sci Technol* 2020 Mar 17;54(6):3581-3590.
26. Ye Y, Chang PH, Hartert J, Wigginton KR. 2018. Reactivity of Enveloped Virus Genome, Proteins, and Lipids with Free Chlorine and UV254. *Environ Sci Technol* 52:7698-7708.
27. Zhang H, Peng C, Liu B, Liu J, Yuan Z, Shi Z. 2018. Evaluation of MICRO-CHEM PLUS as a Disinfectant for Biosafety Level 4 Laboratory in China. *Applied Biosafety*. Volume 23:32-38.
28. Freedom of Information (FOIA) Online. Environmental Protection Agency. Efficacy Review #1839-95 documents as retrieved April, 2021.
29. Sehgal, A., Ramdani K., Plummer C., Evans H. 2019. Compositions and methods for long lasting disinfection. Patent WO2019199886A1.C5439676.
30. Byotrol PLC. 2013. Cleaning Behaviours in the Home, Lancaster University MNGT200 Management and Consulting Practice and Critique. www.byotrol.com.

31. Chin AWH, Chu JTS, Perera MRA, Hui KPY, Yen HL, Chan MCW, Peiris M, Poon LLM. 2020. Stability of SARS-CoV-2 in different environmental conditions. *Lancet Microbe* 1:e10. doi: 10.1016/S2666-5247(20)30003-3.

Author contributions

JBH designed and executed the tests and wrote the manuscript

CP designed Vaccinia PAS2424 methodology and contributed to technical discussions and review of the manuscript

GG designed and executed Vaccinia BSI PAS2424 test and also contributed to technical discussions and review of the manuscript

AS formulated the disinfectant and contributed to technical discussions

LBP-G co-designed the tests and wrote the manuscript

KR conceived the study

Figure 1.

Comparative Sensitivity to a Quat Disinfectant in Suspension

Concentration	Virus	Viral kill (TCID50 or PFU)
5%	WIVI	>7.17
	Phi6	>5.99
5%/81	WIVI	>6.35 and <7.17
	Phi6	<2.62
5%/243	WIVI	1.27 ± 0.13
	Phi6	0.04 ± 0.16

Viral kill as a function of MCP™ concentration in suspension test for bacteriophage Phi6 and the bat SARS-like Coronavirus, WIV1²⁷. Each data point for Phi6 data represents the mean of 3 replicates (n=3).

Figure 2

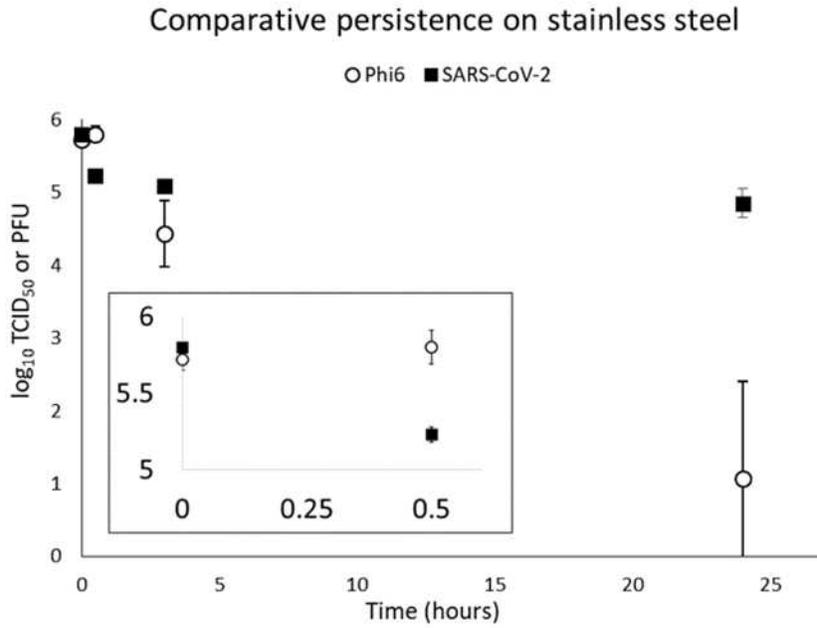
Comparative Sensitivity to a Quat Disinfectant on Glass

Virus Name	Titer of the Virus Control	Cytotoxicity	Performance
Avian Influenza A virus	6.0 log ₁₀	1.5	CD (>4.5)
Avian Influenza A (H3N2)	5.0 log ₁₀	1.5	CD (>4.5)
SARS-Associated Coronavirus	6.5 log ₁₀	2.5	CD (>4.0)
Human Coronavirus (229E)	4.5 log ₁₀	1.5	CD (>3.0)
Phi6 Bacteriophage	7.8 log ₁₀	N/A	(>5.4)

CD= Complete Deactivation

Comparative sensitivity of bacteriophage Phi6 and several other enveloped viruses to 2 oz/gal MCP™ based on carrier based efficacy evaluations (ASTM 1053). Each data point for Phi6 data represents the mean of 4 replicates (n=4).

Figure 3



Decay of virus titer of Phi6 and SARS-CoV-2³¹ on stainless steel as a function of time at 22°C and 65% Relative Humidity (RH). Inset shows an expanded view of the first 30 minutes. Each data point for Phi6 data is an average and standard deviation (mean \pm S.D.) of 3 replicate samples.

Table 1

Test	Organism	Product	Interfering substance	Contact time	Log ₁₀ reduction
BSI PAS2424	Vaccinia	Actizone™ F5	3 g/L BSA	5 min	CD (>3.5)
BSI PAS2424	Phi 6	Actizone™ F5	3 g/L BSA	5 min 10 min	3.06 ± 0.97 CD (>5.4)
BSI PAS2424	Phi 6	Actizone™ F5 w/o Actizone™ P5	3 g/L BSA	5 min	<1.43
EPA RSD(V)	Phi 6	Solvay F5	5% FBS	10 min	CD (>4.0)

Log₁₀ reduction of either Phi6 or Vaccinia Virus after residual virucidal efficacy testing following the BSI PAS2424 method or the interim guidance based on EPA RSD method. Greater than a 3.0 log₁₀ reduction in viral titer after the specified contact time is required for a regulatory approval. For Phi6 data 4 replicates (n=4) were tested for the RSD method and 5 replicates (n=5) were tested for the BSI PAS2424 method. CD denotes complete deactivation.