


ORIGINAL ARTICLE

Recovery and chemical disinfection of foot-and-mouth disease and African swine fever viruses from porous concrete surfaces

L.R. Gabbert¹, J.G. Neilan² and M. Rasmussen² ¹ Leidos, Plum Island Animal Disease Center, Greenport, NY, USA² United States Department of Homeland Security, Science and Technology Directorate, Plum Island Animal Disease Center, Greenport, NY, USA

Keywords

African swine fever virus, ASFV, biosecurity, carbonation, concrete, decontamination, disinfection, FMDV, foot-and-mouth disease virus, porous surface, quantitative carrier test, viral inactivation, Virkon S.

Correspondence

Max Rasmussen, United States Department of Homeland Security, Science and Technology Directorate, Plum Island Animal Disease Center, PO Box 848, Greenport, NY 11944, USA. E-mail: max.rasmussen@st.dhs.gov

2019/2272: received 17 December 2019, revised 23 April 2020 and accepted 1 May 2020

doi:10.1111/jam.14694

Abstract

Aims: Develop an effective laboratory method to consistently recover viral loads from porous concrete coupons sufficient for disinfectant efficacy testing. Investigate the role of concrete matrix pH on the recovery of foot-and-mouth disease virus (FMDV) and African Swine Fever virus (ASFV) from porous concrete. Compare parameters of FMDV and ASFV inactivation on porous and nonporous surfaces in quantitative carrier tests of a liquid chemical disinfectant.

Methods and results: Concrete test coupons were fabricated from commercial and industrial sources and carbonated by exposure to 5% CO₂ in a humidified incubator, lowering the matrix pH. Neither dried FMDV nor ASFV were recovered from high-pH concrete control coupons. Recovery of infectious virus from lower pH carbonated concrete was similar to stainless steel coupon controls. Exposure to the liquid disinfectant Virkon™ S inactivated FMDV and ASFV on porous concrete.

Conclusions: Concrete matrix pH had a greater impact than surface porosity on the ability to recover viable virus from unsealed concrete.

Significance and Impact of the Study: Concrete is commonly found in environments where virus decontamination is required. This study demonstrates a reproducible method to recover sufficient viral loads from porous concrete coupons to facilitate quantitative carrier testing. This method provides a basis for evidence-based validation testing of chemical disinfectants to inactivate pH-sensitive viruses on unsealed concrete.

Introduction

Effective disinfection of environmental surfaces contaminated with pathogenic microorganisms is crucial to prevent, respond and recover from infectious disease outbreaks. For high-consequence transboundary animal disease outbreaks due to an accidental or intentional release of agricultural pathogens, use of validated disinfection protocols ensures continuity of operations (USDA 2014, 2019). During such outbreaks, disease transmission and geographical spread are often attributed to contamination of food, fomites, facilities and transport vehicles with infectious agents. Effective cleaning and disinfection

protocols result in destruction, inactivation or elimination of the infectious agent.

Demonstration of validated disinfection procedures on porous materials such as wood, cloth, paper and others have been historically difficult. Unsealed concrete is a porous material widely used in agricultural facilities, laboratories and food processing plants where environmental contamination with microorganisms may occur. Validated decontamination and disinfection methods and protocols for unsealed concrete surfaces are notably lacking (Krug *et al.* 2018). Quantitative efficacy testing of chemical disinfectants applied to porous, unsealed concrete is often hindered by insufficient recovery of viral

loads from concrete controls, resulting in critical knowledge gaps. Potential reasons for poor recovery of viable virus from unsealed concrete include irreversible physical adhesion of virus to the porous concrete substrate and viral inactivation after contacting the substrate (Bieker 2006; Krug *et al.* 2018).

Quantitative carrier tests are commonly used to assess disinfection methods for inactivation of microorganisms dried on various materials representing environmental surfaces. These standardized tests are typically conducted using nonporous coupons such as stainless steel or glass (Sattar *et al.* 2003, Springthorpe and Sattar 2005, ASTM 2017), and success is dependent upon sufficient recovery of microorganisms from untreated, positive control coupons (Wyrzykowska-Ceradini *et al.* 2019). Insufficient recovery ($<4\text{-log}_{10}$) of viable virus from control coupons precludes demonstrating the minimum 4-log_{10} reduction in infectious titre required for virucidal efficacy determination and subsequent product registration with the U.S. Environmental Protection Agency (US EPA 1981).

Reliable experimental data for disinfection of microorganisms on unsealed concrete are limited because low recovery from concrete controls often prevents quantitative comparisons of treatments. For example, Calfee and Wendling (2013) reported statistically significant differences in the recovery of vegetative bacteria from several porous coupon materials, with concrete and wood having the lowest recovery values. Additionally, Wood *et al.* (2010) were unsuccessful in recovering viable, highly pathogenic avian influenza virus from concrete coupons by multiple extraction methods.

Foot and mouth disease virus (FMDV) and African swine fever virus (ASFV) are high-consequence transboundary animal disease pathogens that cause severe economic consequences in endemic and epizootic countries (Thompson *et al.* 2002; Sánchez-Cordón *et al.* 2018). FMDV is a small, nonenveloped, positive-strand, RNA virus in the family Picornaviridae, and ASFV is a large, enveloped, DNA virus in the Asfarviridae family. Both pathogens are highly transmissible in domestic livestock populations, and may persist in the environment for months under favourable conditions (Botner and Belsham 2012; Bravo de Rueda *et al.* 2015).

Disinfection of ASFV and FMDV on nonporous carriers, including sealed concrete has been reported, but virus recovery from porous materials remains problematic (Krug *et al.* 2011, 2012, 2018; Gabbert *et al.* 2018). Bieker (2006) reported insufficient recovery of FMDV from untreated, porous concrete that resulted in inconclusive data for the chemical disinfectants tested. Krug *et al.* (2018) reported that concrete coupons having $\text{pH} \geq 10$ were virucidal to high-titre FMDV and ASFV and

suggested that efficacy tests could only be conducted after sealing the concrete to render it nonporous.

Potential mechanisms responsible for low viral recovery from unsealed concrete include high pH, which may inactivate viruses, and the possibility that viral particles may become irreversibly bound to the concrete matrix (Bieker 2006, USDA 2015, Wyrzykowska-Ceradini *et al.* 2019). The pH of freshly prepared concrete is highly alkaline, measuring approximately pH 13 (Grubb *et al.* 2007). Both FMDV and ASFV are inactivated at pH levels ≥ 10 , presumably due to loss of the structural integrity of the FMDV capsid and ASFV envelope (Bachrach *et al.* 1957; Krug *et al.* 2011; Center for Food Security and Public Health (CFSPH) 2018; Kalmar *et al.* 2018).

Prolonged exposure to natural atmospheric and environmental conditions results in a gradual decrease in the pH of concrete over time. This process, termed carbonation, occurs due to the interaction of atmospheric carbon dioxide (CO_2) with the hydration products of cement, resulting in the formation of calcium carbonate (CaCO_3) and liberation of water: $\text{Ca}(\text{OH})_2 + \text{CO}_2 \rightarrow \text{CaCO}_3 + \text{H}_2\text{O}$ (Hussain *et al.* 2016). However, the chemical reaction is largely dependent on relative temperature and humidity, and thus may take many years to complete under natural conditions (Chen *et al.* 2018).

In this study, we sought to determine the influence of concrete pH on the recovery of infectious FMDV and ASFV. We compared virus recovery from untreated, high-pH concrete, and from concrete coupons in which the pH had been lowered through accelerated gaseous carbonation in a laboratory environment. Following demonstration of sufficient virus recovery from carbonated, pH-adjusted concrete, we conducted quantitative virucidal efficacy tests with a U.S. EPA-registered disinfectant using unsealed concrete coupons and dried inocula of FMDV and ASFV.

Materials and methods

Virus stocks and cell lines

FMDV strains A24/Cruzeiro/BRA/55 and O1 Campos were amplified in a porcine kidney cell line expressing the FMDV integrin attachment receptors αV and β_6 (LFBK- $\alpha\text{V}\beta_6$ cells), kindly supplied by M. LaRocco, U.S. Department of Agriculture (USDA) Agricultural Research Service (ARS), Plum Island Animal Disease Center (LaRocco *et al.* 2013; LaRocco *et al.* 2015, Rodriguez, 2015). ASFV strain BA71V stocks were produced by amplification in Vero cells (ATCC[®] CCL-81TM). Briefly, FMDV and ASFV were inoculated onto adherent monolayers of the appropriate cell line and cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco) supplemented with

2% foetal bovine serum (FBS), 1X Antibiotic–Antimycotic (Gibco, 15240096), sodium pyruvate (Gibco™ 11360070), and incubated at 37°C with 5% CO₂ in T150 flasks. At days three (FMDV) and five (ASFV) postinfection, cells were subjected to three freeze/thaw cycles, and the cell supernatants clarified by centrifugation and stored as single-use aliquots at –70°C.

Stainless steel coupon preparation

AISI-brushed stainless steel disks type 430, with 1 cm diameter, 0.7 mm thickness (Pegen Industries Inc., Ontario, Canada) were used as nonporous, control-surface coupons. Stainless steel coupons were washed and degreased for 30 min in a 10% (w/v) solution of Alconox detergent, rinsed three times in deionized water and dried in a biological safety cabinet prior to sterilization by autoclaving for 45 min at 121°C.

Concrete coupon production

Three concrete formulations (Concretes A, B and C) were procured from separate sources and included two industrial mixes and one off-the-shelf ready-mix product. Concrete A coupons consisted of a silicate-based food-grade concrete (TurtleCrete, Reefball Inc., Nokomis, FL). Concrete B was an industrial-grade ready-mix concrete (4500 psi) with added plasticizer (Suffolk Cement Products, Inc., Calverton, NY). Concrete C was prepared in-house, based on mixing ratios indicated on the product label, after separating the coarse aggregate from an 80-pound bag of Sakrete High-Strength Concrete Mix (Lowes, 132022) and combining 438 g of the fine cementitious material with 100 ml of tap water. For all three concrete formulations, wet concrete slurry was poured into easy-

release silicone ice cube trays to standardize size, shape and volume (one hundred and sixty 1-cm³ cubes/tray; Amazon.com) (Fig. 1). All cubes were air dried for a minimum of three days at room temperature before removal from the silicone trays. Individual concrete coupons were rinsed in deionized water and sterilized by autoclaving at 121°C for 15 min.

Concrete carbonation

Concrete coupons that received carbonation treatment were placed in a standard CO₂ incubator typically used for mammalian tissue culture (Sanyo model MCO-18AIC) for 7 days. The internal temperature was held constant at 37°C, and ambient air was continuously supplemented with CO₂ to obtain a final concentration of 5% CO₂, as measured by an infrared CO₂ sensor. Incubator humidity was regulated by natural vaporization of water in a humidifying pan. Relative humidity (rH) values, measured using a digital rH meter (ThermoPro TP-50), remained stable at 88% throughout the 7-day incubation. Carbonated coupons were sterilized by autoclaving, which did not change coupon surface pH (data not shown). As part of our methods development, we found that exposing concrete coupons to dry ice, sublimating CO₂, in a sealed container at room temperature for a period of three days, was ineffective at inducing carbonation, presumably due to the low temperature and lack of humidity.

pH Measurements

The pH of concrete matrices was measured using two methods. (i) Phenolphthalein (Sigma, 319236) is a pH indicator dye that remains colourless at pH values below 8.5, and becomes increasingly darker as pH increases



Figure 1 Production of standardized concrete coupons by filling 1-cm³ silicone ice cube moulds with ready-mix concrete. Photo courtesy of Tod Barber, Reefball Inc., Nokomis, FL. [Colour figure can be viewed at wileyonlinelibrary.com]

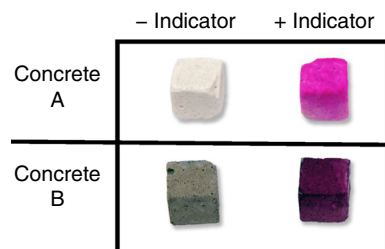


Figure 2 Surface colour of dried concrete coupons before and after the addition of phenolphthalein indicator solution depicting high initial pH of un-carbonated concrete. [Colour figure can be viewed at wileyonlinelibrary.com]

above 8.5 (CID = 4764, <https://pubchem.ncbi.nlm.nih.gov/compound/4764>. Accessed 09DEC2019). To obtain an approximate qualitative pH value of the concrete surface before and after carbonation, 50 μl of a liquid phenolphthalein solution was applied across the surface of the 1-cm³ concrete cubes and the colour change recorded. At pH values >8.5, the concrete coupon surface changed colour from grey to magenta (Fig. 2). (ii) A quantitative pH measurement was obtained by crushing the concrete coupons to generate concrete ‘dust’, which was mixed with deionized water and the solution measured directly with a digital pH meter (Grubb *et al.* 2007). Specifically, carbonated and un-carbonated coupon cubes were placed between sheets of paper, crushed with a hydraulic press and the output sifted through a wire mesh (1/8 inch) to obtain a uniform mixture of fine dust particles. Five grams of the concrete dust was combined with 10 ml of deionized water in a 50-ml conical tube and vortexed. After sedimentation of the dust particles by gravity, the pH of the supernatant liquid was measured with an Oakton hand-held digital pH meter (Cole Parmer, EW-35423-01).

Soil load preparation

The standardized soil load was prepared by combining individual component stocks of 5.0% yeast extract (Cole Parmer, BP142210), 5.0% bovine serum albumin (BSA; Cole Parmer, BP6711) and 0.4% bovine mucin (Sigma, M3895) dissolved in sterile phosphate-buffered saline (PBS; Sigma, 806552) (ASTM International 2017; OECD 2013). Component stocks were prepared in 10-ml volumes, filter-sterilized through a 0.2- μm filter, divided into single-use aliquots (1 ml) and stored at -20°C until combined into a standard soil load.

Virus inoculum preparation

Briefly, 340 μl of a thawed, single-use frozen virus stock was mixed with 25 μl of BSA stock, 35 μl of yeast extract

stock and 100 μl of bovine mucin stock, resulting in a final viral suspension containing 0.35% yeast extract, 0.25% BSA and 0.08% bovine mucin. These preparations were applied immediately to test coupons for quantitative carrier testing.

Disinfectant preparation

VirkonTM S (QC Supply, #340340) was prepared as a weight/volume solution by mixing powdered VirkonTM S in deionized water. Disinfectant solutions of 1, 2 and 5% were prepared immediately before use.

Neutralization and cytotoxicity testing

Disinfectant neutralization and cytotoxicity tests were performed in advance to rule out toxic effects on cell lines and viruses due to exposure to, or continued action of, the disinfectant after neutralization. Preparations of 2% VirkonTM S were tested. The EPA SOP MB-26-02 protocol (Environmental Protection Agency (EPA) 2017) was adapted for use with FMDV and ASFV in this study. Viral inocula were diluted to 10^2 – 10^3 infectious virus particles per 10 μl . At intervals of 30 ± 3 s, 10 μl of virus was added to flat-bottomed Nalgene vials containing either 10 ml of PBS ($n = 3$) or 10 ml of the neutralizer (DMEM + 2% FBS) ($n = 3$). Ten millilitres of the neutralizer was added to three additional vials containing 50 μl of the test chemical at 30-s intervals and allowed a 10-s contact time before 10 μl of the virus inoculum was added. Viral suspensions were serially diluted from 10^0 to 10^{-3} and plated in replicates (6 wells/dilution) on 48-well plates. The cells were incubated for 3 days (FMDV) or 7 days (ASFV) and scored to detect cell cytopathic effects (CPE). CPE values were used to calculate the 50% Tissue Culture Infective Dose (TCID₅₀) endpoint titre (Reed & Muench 1938). Neutralizer effectiveness was determined by comparing the infectious virus titre of PBS controls and the vials containing the test chemical. A reduction of $>0.5 \log_{10}$ between the control group and the treatment containing the disinfectant was considered a failed neutralization. For cytotoxicity testing, we mixed 50 μl of disinfectant with 10 ml of neutralizer, added the suspension to both cell lines and observed cell monolayers for 3 days (LFBK- $\alpha_V\beta_6$) or 7 days (Vero) to determine if cell growth was impacted.

Viral recovery and disinfection experiments

All experiments were conducted based on the Organisation for Economic Cooperation and Development (OECD) method ‘Quantitative method for evaluating virucidal activity of microbicides used on hard

nonporous surfaces' (OECD 2013). To assess virus survival and recovery, 10 μl of the prepared virus inoculum was deposited on the stainless steel and concrete coupon surfaces and dried under desiccation in a class II biosafety cabinet for approximately 1 h. Individual coupons were transferred to Nalgene™ straight-side polypropylene jars with Screw Caps (Fisher Scientific, 11-815-10G), and 10 ml of neutralizer was added. Vials were vortexed for 30 s (± 3 s) to dislodge virus from the coupon surfaces prior to quantitation of viable virus (as described above).

For the quantitative carrier disinfection tests, 50 (stainless steel) or 100 μl (concrete) of the test chemical was overlaid on the dried virus inoculum for either 5 or 10 min (± 3 s) prior to elution in 10 ml of neutralizer. All eluates were serially diluted from 10^{-1} to 10^{-6} , and plated in replicates ($n = 8$) on adherent monolayers of LFBK- $\alpha_V\beta_6$ (FMDV) or Vero (ASFV) cells in 96-well plates. CPE was used to calculate \log_{10} TCID₅₀ ml⁻¹. Any eluates negative for infectious virus were blind-passaged three additional times to confirm virus inactivation. The assay lower limit of detection was 1.0 \log_{10} TCID₅₀ ml⁻¹, which was used to calculate a geometric mean for those samples. Samples were processed in replicates for untreated control coupons ($n = 3$) and for coupons receiving disinfectant ($n = 4$). The mean values include repeat tests from different test days.

Results

Concrete carbonation

The precarbonation pH values of crushed concrete coupons were Concrete A, pH 11.1; Concrete B, pH 12.4; and Concrete C, pH 12.6 (Table 1). Following CO₂ treatment, pH values for crushed coupons formulated with Portland cement-based concretes (Concretes B and C) were lower (pH 9.4 and 9.1, respectively) compared to their respective pretreatment values. The pH of the silica-based Concrete A remained unchanged at 11 post-CO₂ exposure (Table 1).

Qualitative analysis of coupon surface pH by application of the phenolphthalein pH-indicator dye demonstrated that pH values for carbonated Concretes B and C were below 8.5, whereas the surface pH of Concrete A remained unchanged (Fig. 3). Moreover, the surface pH

Table 1 pH values of crushed concrete coupons pre- and post-carbonation

Concrete formulation	pH precarbonation	pH postcarbonation
A	11.1	11.0
B	12.4	9.4
C	12.6	9.1

of carbonated cubes of Concretes B and C remained stable for at least 12 months as shown by periodic re-application of phenolphthalein (data not shown).

Cross-section analyses of the pH of the concrete cube interior matrix showed that carbonation permeated throughout the entirety of the coupons (Fig. 4). This is based on the absence of a colour change when phenolphthalein was applied to interior surfaces of Concretes B and C (Fig. 4), and by pH values obtained from crushed concrete coupons (Table 1). The pH of Concrete A remained stable at 11, and phenolphthalein treatment of interior surfaces produced a markedly dark colour change indicating pH values well above 8.5 (Table 1, Fig. 3).

Neutralization and cytotoxicity testing

Neutralization tests demonstrated that DMEM + 2% FBS neutralized 2% Virkon™ S within 10 s following addition to the disinfectant indicated by a <0.25 \log_{10} reduction in infectious viral titres between control reactions and those including the test chemical (Table 2). In previous studies, DMEM + 2% FBS neutralized 1% Virkon™ S (Gabbert *et al.* 2018).

No cytotoxicity was observed on LFBK- $\alpha_V\beta_6$ or Vero cell lines after neutralization of 1% and 2% Virkon™ S, as indicated by the absence of cell death and lack of changes in cell morphology after plating the neutralized test chemical. Use of 5% Virkon™ S elicited pH changes

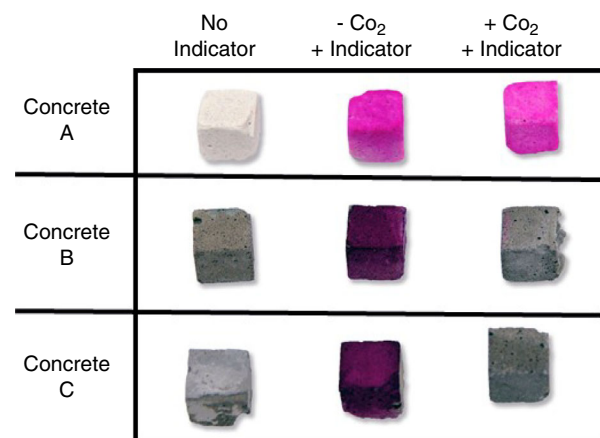


Figure 3 Colour of concrete coupons after application of pH-indicator dye phenolphthalein showing effect of carbonation on surface pH. Column 1: initial appearance of un-carbonated concrete coupons with no phenolphthalein dye. Column 2: un-carbonated concrete coupons after phenolphthalein application all showing surface pH > 8.6 . Column 3: Carbonated concrete coupons after phenolphthalein application with Concrete A showing no noticeable change in pH, and Concretes B and C showing lower pH (< 8.5) after incubation with gaseous CO₂ (5%) with humidity at 37°C for 1 week. [Colour figure can be viewed at wileyonlinelibrary.com]

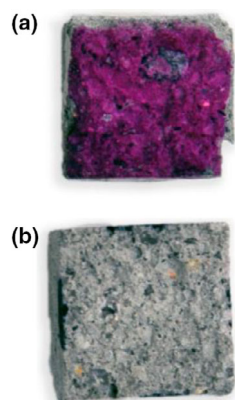


Figure 4 Colour of 1-cm³ Concrete C coupon cross-sections after application of pH-indicator dye phenolphthalein showing effect of carbonation on internal matrix pH for (a) un-carbonated Concrete C control coupon with magenta colour indicating pH > 8.6 throughout entire cement matrix and (b) Concrete C test coupon cross-sectioned after 1-week exposure to 5% CO₂ showing no colour change and matrix pH < 9.0. [Colour figure can be viewed at wileyonlinelibrary.com]

in the culture media and cell death in the 10⁰ wells of serial dilution. However, no cytotoxicity was observed after dilution to 10⁻¹, allowing for inclusion in the disinfectant testing assays.

Phase I: virus recovery experiments

Comparison of virus recovery from dried and undried (wet) inocula on nonporous stainless steel coupons indicated virus losses ranging from 0.78 to 1.3 log₁₀ due to drying (Table 3). No viable FMDV or ASFV were recovered from uncarbonated, non-pH-adjusted concrete coupons, as evidenced by the lack of CPE in all samples tested. For pH-adjusted carbonated concrete coupons (Concrete types B and C), viable virus was recovered at levels comparable to, and sometimes better than, recovery from stainless steel controls (Table 3).

Concrete A, the silica-based product, was resistant to both surface and internal pH changes following CO₂ exposure, and functioned as a high-pH carbonation control. We hypothesize this is due to the increased silica content and lack of availability of calcium hydroxide to participate in the carbonation reaction, as explained by Wang and Lee, 2008. FMDV A24 virus was recovered at low levels from Concrete A coupons ($n = 1/6$), whereas

infectious FMDV O1 Campos and ASFV were not detected.

All three viruses were recovered from carbonated Concrete C (Sakrete brand concrete mix) with FMDV O1 Campos titres exceeding recovery from stainless steel controls (Table 3). This result prompted the use of Concrete C coupons for all subsequent virus disinfection experiments.

Phase II: Virus disinfection experiments

Initial quantitative carrier disinfection experiments were conducted with FMDV A24 and ASFV BA71V on stainless steel and pH-adjusted Concrete C with VirkonTM S using a 5-minute contact time. Subsequent experiments included a 10-minute contact time for ASFV because the 5-min contact time was insufficient to completely inactivate ASFV. A FMDV A24 titre of >5 log₁₀ TCID₅₀ ml⁻¹ was recovered from control stainless steel and pH-adjusted concrete coupons (Table 4). Disinfection with 1% VirkonTM S on stainless steel resulted in a complete kill on all eight coupons (two test days). Disinfection on pH-adjusted Concrete C with 1, 2 and 5% VirkonTM S also resulted in complete virus inactivation based on the absence of detectable CPE in all replicates. Two additional blind passages of FMDV-negative eluates resulted in no additional positive samples.

An average of 3.2 (±0.2 SD) log₁₀ TCID₅₀ ml⁻¹ infectious ASFV was recovered from untreated stainless steel and pH-adjusted Concrete C controls (Table 5). Despite this lower initial starting titre compared to FMDV, infectious ASFV was detectable on 25% (1/4) of the stainless steel coupon control replicates and on all four concrete coupons after a 5-min contact time with 1% VirkonTM S. Following extension of the contact time to 10 min (per product label recommendation), ASFV was completely killed on stainless steel using 1% or 2% VirkonTM S. However, 75% (3/4) of the replicates were CPE positive when 1% VirkonTM S was applied to concrete coupons. Exposure to 2% VirkonTM S resulted in no initial detection of live virus. However, 25% (1/4) of the negative eluates were CPE positive following three blind passages in the innocuity test, suggesting a very low level of viable ASFV remained on one of the test coupons. Five per cent VirkonTM S eliminated ASFV on all replicates on carbonated Concrete C.

Table 2 Neutralization testing results for FMDV and LFBK- α V β 6 cells and ASFV and Vero cell with 2% VirkonTM S

Test chemical	Neutralizer	Virus	Cell line	Cytotoxicity	log ₁₀ reduction
2% Virkon TM S	DMEM + 2% FBS	FMDV A24	LFBK- α V β 6	No	-0.10
2% Virkon TM S	DMEM + 2% FBS	ASFV BA71V	Vero	No	0.22

Table 3 FMDV and ASFV recovery from test coupons

Treatment Group	FMDV A24	FMDV O1	
		Campos	ASFV BA71V
Undried virus*	6.09 ± 0.50 (15/15)	5.07 ± 0.43 (4/4)	3.95 ± 0.70 (11/11)
Stainless steel	5.31 ± 0.31 (15/15)	4.24 ± 0.52 (7/7)	2.65 ± 0.71 (13/13)
Uncarbonated concrete	A <1.0 ± 0.0 (0/6)	<1.0 ± 0.0 (0/3)	<1.0 ± 0.0 (0/3)
	B <1.0 ± 0.0 (0/6)	<1.0 ± 0.0 (0/3)	<1.0 ± 0.0 (0/3)
	C <1.0 ± 0.0 (0/6)	<1.0 ± 0.0 (0/3)	<1.0 ± 0.0 (0/3)
Carbonated concrete	A 0.23 ± 0.57 (1/6)	<1.0 ± 0.0 (0/3)	<1.0 ± 0.0 (0/3)
	B 4.45 ± 0.94 (6/6)	4.54 ± 0.38 (3/3)	2.19 ± 0.37 (3/3)
	C 4.93 ± 0.70 (9/9)	5.57 ± 0.37 (3/3)	2.66 ± 0.79 (9/9)

Values reported as \log_{10} TCID₅₀ ml⁻¹ ± standard deviation (number of positive coupons/total).

*Undried virus control group was included to show the impact of drying on starting titres. Starting volumes were 10 µl of inoculum for both dried and undried virus.

Discussion

Transboundary animal disease viruses pose significant risks to food security and national economies. Determining effective chemical disinfection methods on surfaces commonly found in livestock and food-processing facilities is critically important to prevent and recover from outbreaks.

Most efficacy tests for liquid chemical disinfectants use nonporous surfaces or liquid suspensions to enable recovery of sufficient numbers of target microorganisms to calculate the extent of inactivation (OECD 2013; Environmental Protection Agency (EPA) 2016; ASTM International 2017). The use of standardized quantitative carrier assays to validate disinfection methods is

Table 5 ASFV BA71V inactivation on stainless steel and concrete following 5- and 10-min exposure to Virkon™ S

Treatment Group	5-minute contact		10-minute contact	
	Infectious virus recovered (log ₁₀ TCID ₅₀ ml ⁻¹) ± SD	Number of positive coupons/total	Infectious virus recovered (log ₁₀ TCID ₅₀ ml ⁻¹) ± SD	Number of positive coupons/total
Controls				
Undried virus	4.56 ± 0.59	3/3	3.86 ± 0.13	3/3
Dried virus on stainless steel	3.34 ± 0.3	3/3	3.2 ± 0.18	3/3
Dried virus on concrete	3.2 ± 0.2	3/3	2.93 ± 0.35	3/3
Stainless steel				
1% Virkon	<1.12 ± 0.34	¼	<1.0 ± 0.00	0/4
2% Virkon	n.d.	n.d.	<1.0 ± 0.00	0/4
Carbonated concrete				
1% Virkon	1.84 ± 0.71	4/4	1.25 ± 0.5	3/4
2% Virkon	<1.0 ± 0.00	¼	<1.0 ± 0.00	0/4
5% Virkon	<1.0 ± 0.00	0/4	<1.0 ± 0.00	0/4

challenging for porous materials that may inactivate or otherwise prevent recovery of viable target microorganisms from the test surface. Disinfectant efficacy data for porous materials such as concrete have been limited due to poor recovery of test microbes from the concrete matrix (Bieker 2006; Krug *et al.* 2018). The current study demonstrates a simple, cost-effective and reproducible method to recover sufficient viral loads from porous concrete coupons to enable quantitative evaluation of disinfectant efficacy.

From a functional standpoint, the performance of the porous, carbonated concrete testing coupons was equivalent to that of nonporous stainless steel. The carbonated concrete coupons withstood repeated rounds of

Table 4 FMDV A24 inactivation on stainless steel and concrete following a 5-min exposure to Virkon™ S

Treatment group	Infectious virus recovered (log ₁₀ TCID ₅₀ ml ⁻¹) ± SD	Number of positive coupons/total
Untreated controls		
Undried virus	5.81 ± 0.13	6/6
Dried Virus on Stainless Steel	5.27 ± 0.36	6/6
Dried Virus on Concrete	5.09 ± 0.29	6/6
Stainless steel		
1% Virkon	<1.0 ± 0.00	0/8
Carbonated concrete		
1% Virkon	<1.0 ± 0.00	0/8
2% Virkon	<1.0 ± 0.00	0/4
5% Virkon	<1.0 ± 0.00	0/8

vortexing, and the 1-cm³ dimension allowed for the use of the same-sized vials and volumes as the standard quantitative carrier test. Differences in virus inoculum drying were observed between stainless steel and concrete. The virus inoculum remained discernable on the smooth stainless steel but somewhat dissolved into the concrete surface after the one-hour prescribed drying time. Based on this observation, the disinfectant volume was increased twofold for application to concrete coupons to ensure the disinfectant remained in contact with the virus for the full contact time.

Research investigating the possible mechanism(s) responsible for poor viral recovery from porous, unsealed concrete is notably lacking. Because unsealed concrete is common in livestock agricultural and laboratory environments, we sought to evaluate disinfectant efficacy on unsealed concrete in comparison to a standardized non-porous material, stainless steel.

Virus particles vary considerably in their sensitivity to pH and environmental conditions (Maillard *et al.* 2013; Mateu 2013). We hypothesized that the high pH of the concrete coupons contributed to the poor recovery of FMDV and ASFV when dried on the surface. Fresh concrete, typically used in building construction, has a high pH (≥ 12). However, over time and prolonged exposure to CO₂, the concrete matrix will carbonate at the current atmospheric CO₂ concentration of 0.04% (NOAA 2019). This natural carbonation process is relatively slow and highly dependent on temperature, relative humidity and CO₂ concentration (Saetta *et al.* 1993; Sanjuan *et al.* 2003; Chen *et al.* 2018). It is challenging to predict actual carbonation depths in building materials due the nonlinear relationship that time and the environmental micro-variables may play (Hussein *et al.* 2016). Thus, it should not be assumed that survival of pH-sensitive microorganisms is less probable on relatively new concrete construction, or that older buildings are more susceptible to contamination. For example, as part of the methods development for the current study, we measured pH values >12 from bores of 60-year-old concrete laboratory flooring (data not shown).

Accelerated carbonation of the concrete coupons enabled quantitative carrier efficacy tests with Virkon™ S, as demonstrated by high-level virus recovery and inactivation on the pH-adjusted, porous concrete test coupons. Virkon™ S was chosen as the test disinfectant in this study as it is currently registered by the U.S. EPA as a broad-spectrum disinfectant and virucide effective against both FMDV and ASFV at a concentration of 1% and a contact time of 10 min (EPA Reg. No. 39967-137; EPA 2019). Our results demonstrated that Virkon™ S was equally effective against FMDV A24 on pH-adjusted concrete as on stainless steel after 5 min. Previous studies

with Virkon™ S reported rapid FMDV inactivation (Gabbert *et al.* 2018), and the current study is the first to report ASFV inactivation at shortened contact times with Virkon™ S.

Our results demonstrated that ASFV was slightly more resistant than FMDV to inactivation with Virkon™ S because a longer contact time was required on stainless steel, and a higher concentration of Virkon™ S (2–5%) was necessary on concrete, despite a lower starting titre of ASFV. These results also suggest that there may be slight differences in disinfectant activity when applied to porous concrete *vs* stainless steel.

In conclusion, we demonstrated recovery and disinfection of both a large enveloped (ASFV), and a small encapsidated (FMDV), virus from pH-adjusted porous concrete. Further studies are needed to determine if this method is suitable for other pathogenic microorganisms or disinfectants. The method reported here may enable future validations of virucidal efficacy for liquid disinfectants on unsealed concrete through quantitative carrier testing.

Acknowledgements

We thank the Plum Island Animal Disease Center Operations and Management Team, A/Z Corp., and Todd Barber of Reefball Inc., for providing the materials necessary for concrete carrier production. Additionally, we thank Drs Barbara Kamicker and David Brake for manuscript editing, Kathleen Apicelli for photography services, and Mariceny Zurita for providing laboratory support. The U.S. Department of Homeland Security Science and Technology Directorate (DHS S&T) funded an agreement with Leidos (HSHQDC-14-F-00035 [contract GS-23F-80006H]). DHS S&T, as the funding source, had no involvement in the study design; collection, analysis and interpretation of data; writing of the report; and in the decision to submit the article for publication. All opinions expressed are the authors' and do not necessarily reflect the policies and views of DHS S&T or Leidos.

Conflict of interest

The authors report that no conflicts of interest exist.

REFERENCES

- ASTM International (2017) ASTM E2197–17e1 standard quantitative disk carrier test method for determining bactericidal virucidal, fungicidal, mycobactericidal, and sporicidal activities of chemicals. <https://doi.org/10.1520/E2197-17E01>. Accessed 09DEC2019.

- Bachrach, H.L., Breese, S.S., Callis, J.J., Hess, W.R. and Patty, R.E. (1957) Inactivation of foot-and-mouth disease virus by pH and temperature changes and by formaldehyde. *Proc Soc Exp Biol Med* **95**, 147–152.
- Bieker, J. (2006) Chemical inactivation of viruses. Doctoral dissertation. Kansas State University. <http://krex.k-state.edu/dspace/bitstream/handle/2097/226/JillBieker2006.pdf?sequence=1&isAllowed=y>. Accessed: 09DEC2019.
- Botner, A. and Belsham, G.J. (2012) Virus survival in slurry: analysis of the stability of foot-and-mouth disease, classical swine fever, bovine viral diarrhoea and swine influenza viruses. *Vet Microbiol* **157**, 41–49. <https://doi.org/10.1016/j.vetmic.2011.12.010>
- Bravo de Rueda, C., de Jong, M.C., Eblé, P.L. and Dekker, A. (2015) Quantification of transmission of foot-and-mouth disease virus caused by an environment contaminated with secretions and excretions from infected calves. *Vet Res* **46**, 43. <https://doi.org/10.1186/s13567-015-0156-5>.
- Calfee, M.W. and Wendling, M. (2013) Inactivation of vegetative bacterial threat agents on environmental surfaces. *Sci Total Environ* **443**, 387–396.
- Center for Food Security and Public Health (CFSPH) (2018) Disease factsheet: African Swine fever. http://www.cfsph.ia.state.edu/Factsheets/pdfs/african_swine_fever.pdf. Accessed 09DEC2019
- Chen, Y., Liu, P. and Yu, Z. (2018) Effects of environmental factors on concrete carbonation depth and compressive strength. *Materials (Basel)* **11**, 2167. <https://doi.org/10.3390/ma11112167>
- Environmental Protection Agency (EPA) (2019) Labels for Virkon S. US EPA Pesticide Product Label System (PPLS). https://iaspub.epa.gov/apex/pesticides/f?p=PPLS:102::NO:P102_REG_NUM:39967-137. Accessed 06DEC2019.
- Environmental Protection Agency (EPA). (2017). Neutralization of microbicidal activity using the OECD quantitative method for evaluating bactericidal activity of microbicides used on hard, non-porous surfaces. Antimicrobial Testing Methods and Procedures: MB-26-02. <https://www.epa.gov/sites/production/files/2018-01/documents/mb-26-02.pdf>. Accessed 09DEC2019.
- Environmental Protection Agency (EPA) (2016) Antimicrobial Testing Methods and Procedures: MB-05-14: AOAC Use Dilution Method for Testing Disinfectants. <https://www.epa.gov/sites/production/files/2016-08/documents/mb-05-14.pdf> Accessed 05DEC2019.
- Environmental Protection Agency (EPA). (1981) Efficacy test requirements: virucides. Washington, DC, Designation DIS/TSS-7. <https://archive.epa.gov/pesticides/oppad001/web/html/dis-07.html> Accessed 09DEC2019.
- Gabbert, L., Neilan, J. and Rasmussen, M. (2018) Smart card decontamination in a high-containment laboratory. *Health Secur* **16**, 244–251. <https://doi.org/10.1089/hs.2018.0023>.
- Grubb, J.A., Limaye, H.S. and Kakade, A.M. (2007) Testing pH of concrete, need for a standardized procedure. *Concr Int* **2**, 78–83.
- Hussain, S., Bhunia, D. and Singh, S.B. (2016) Assessment of carbonation depth under natural and accelerated carbonation conditions. *Indian Concr J*, 57–64.
- Kalmar, I.D., Cay, A.B. and Tignon, M. (2018). Sensitivity of African swine fever virus (ASFV) to heat, alkalinity and peroxide treatment in presence or absence of porcine plasma. *Vet Microbiol* **219**, 144–149. <https://doi.org/10.1016/j.vetmic.2018.04.025>
- Krug, P., Lee, L., Larson, C., Eslami, A. and Rodriguez, L. (2011) Chemical disinfection of high-consequence transboundary animal disease viruses on nonporous surfaces. *Biologicals* **39**, 231–235. <https://doi.org/10.1016/j.biologics.2011.06.016>.
- Krug, P., Larson, C., Eslami, A. and Rodriguez, L. (2012) Disinfection of foot-and-mouth disease and African swine fever viruses with citric acid and sodium hypochlorite on birch wood carriers. *Vet Microbiol* **156**, 96–101.
- Krug, P., Davis, T., O'Brien, C., LaRocco, M. and Rodriguez, L. (2018) Disinfection of transboundary animal disease viruses on surfaces used in pork packing plants. *Vet Microbiol* **219**, 219–225.
- Larocco, M., Krug, P.W., Kramer, E., Ahmed, Z., Pacheco, J.M., Duque, H., Baxt, B. and Rodriguez, L.L. (2013) A continuous bovine kidney cell line constitutively expressing bovine $\alpha\beta 6$ integrin has increased susceptibility to foot-and-mouth disease virus. *J Clin Microbiol* **51**, 1714–1720.
- Larocco, M., Krug, P.W., Kramer, E., Ahmed, Z., Pacheco, J.M., Duque, H., Baxt, B. and Rodriguez, L.L. (2015). Correction for LaRocco et al., A continuous bovine kidney cell line constitutively expressing bovine $\alpha\beta 6$ integrin has increased susceptibility to foot-and-mouth disease virus. *J Clin Microbiol* **53**, 755–755.
- Rodriguez, L.L. (2015) Correction for LaRocco et al., A continuous bovine kidney cell line constitutively expressing bovine $\alpha\beta 6$ integrin has increased susceptibility to foot-and-mouth disease virus. *J Clin Microbiol* **53**, 755–755.
- Maillard, J., Sattar, S. and Pinto, F. (2013) Virucidal activity of microbicides. In *Principles and Practices of Disinfection, Preservation, and Sterilization*, 5th edn, ed. Fraiese, A., Maillard, J. and Sattar, S., pp 178–198. Hoboken, NJ: Wiley-Blackwell.
- Mateu, M.G. (ed.) (2013) Structure and physics of viruses: An integrated textbook. *Subcell Biochem* **68**, 457–459. https://doi.org/10.1007/978-94-007-6552-8_1, Springer Science.
- National Oceanic Atmospheric Association (NOAA) (2019) Global carbon dioxide growth in 2018 reached 4th highest on record. <https://www.noaa.gov/news/global-carbon-dioxide-growth-in-2018-reached-4th-highest-on-record>. Accessed 09DEC2019
- Organisation for Economic Co-operation and Development (OECD) (2013) Quantitative method for evaluating virucidal activity of microbicides used on hard non-porous surfaces. OECD Environment, Health and Safety

- Publications. Series on Testing and Assessment No. 187 and Series on Biocides No. 6. [http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono\(2013\)11&doclanguage=en](http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2013)11&doclanguage=en). Accessed 09DEC2019
- Reed, L.J. and Muench, H. (1938) A simple method of estimating fifty percent endpoints. *Am J Hyg* **27**, 493–497.
- Sanjuan, M.A., Andrade, C. and Cheyrezy, M. (2003) Concrete carbonation tests in natural and accelerated conditions. *Adv Cem Res* **15**, 171–180.
- Saetta, A.V., Schrefler, B.A. and Vitaliani, R.V. (1993) The carbonation of concrete and the mechanism of moisture, heat and carbon dioxide flow through porous materials. *Cem Concr Res* **23**, 761–772.
- Sánchez-Cordón, P.J., Montoya, M., Reis, A.L. and Dixon, L.K. (2018) African swine fever: a re-emerging viral disease threatening the global pig industry. *Vet J* **233**, 41–48. <https://doi.org/10.1016/j.tvjl.2017.12.025>.
- Sattar, S.A., Springthorpe, V.S., Adegbonrin, O., Abu Zafer, A. and Busa, M. (2003) A disc-based quantitative carrier test method to assess the virucidal activity of chemical germicides. *J Virol Methods* **112**, 3–12.
- Springthorpe, V.S. and Sattar, S.A. (2005) Carrier tests to assess microbicidal activities of chemical disinfectants for use on medical devices and environmental surfaces. *J AOAC Int.* **88**, 182–201.
- Thompson, D., Muriel, P., Russell, D., Osborne, P., Bromley, A., Rowland, M., Creigh-Tyte, S. and Brown, C. (2002) Economic costs of the foot and mouth disease outbreak in the United Kingdom in 2001. *Rev Sci Tech* **21**, 675–687.
- United States Department of Agriculture (USDA) (2014) Foot and mouth disease response plan: the red book. https://www.aphis.usda.gov/animal_health/emergency_management/downloads/fmd_responseplan.pdf. Accessed 03DEC2019.
- United States Department of Agriculture (USDA) (2015) Reduction of infectious highly pathogenic avian influenza virus in animal agricultural settings. https://www.aphis.usda.gov/animal_health/downloads/animal_diseases/ai/hpai-reduction-of-infectious.pdf. Accessed 09DEC2019.
- United States Department of Agriculture (USDA). (2019). Disease response strategies: African swine fever. Foreign animal disease preparedness and response plan. https://www.aphis.usda.gov/animal_health/emergency_management/downloads/asf_strategies.pdf. Accessed 03DEC2019.
- Wang, X.Y. and Lee, H.S. (2008) A model predicting carbonation depth of concrete containing silica fume. *Mater Struct* **42**, 691–704. <https://doi.org/10.1617/s11527-008-9413-7>.
- Wood, J.P., Choi, Y.W., Chappie, D.J., Rogers, J.V. and Kaye, J.Z. (2010) Environmental persistence of a highly pathogenic Avian Influenza (H5N1) Virus. *Environ Sci Technol* **44**, 7515–7520. Supporting Information: https://pubs.acs.org/doi/suppl/10.1021/es1016153/suppl_file/es1016153_si_001.pdf
- Wyrzykowska-Ceradini, B., Calfee, M., Touati, A., Wood, J., Mickelsen, R., Miller, L., Colby, M., Slone, C. *et al.* (2019) The use of bacteriophage MS2 for the development and application of a virucide decontamination test method for porous and heavily soiled surfaces. *J Appl Microbiol* **127**, 1315–1326. <https://doi.org/10.1111/jam.14406>.