



## Chemical disinfection of high-consequence transboundary animal disease viruses on nonporous surfaces

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### ARTICLE INFO

#### Article history:

Received 7 February 2011

Received in revised form

10 June 2011

Accepted 25 June 2011

#### Keywords:

Transboundary disease

Foot-and-mouth

Swine fever

Surface disinfection

### ABSTRACT

Disinfection is a critical part of the response to transboundary animal disease virus (TADV) outbreaks by inactivating viruses on fomites to help control infection. To model the inactivation of TADV on fomites, we tested selected chemicals to inactivate Foot and Mouth Disease virus (FMDV), African Swine Fever virus (ASFV), and Classical Swine Fever virus (CSFV) dried on steel and plastic surfaces. For each of these viruses, we observed a 2 to 3 log reduction of infectivity due to drying alone. We applied a modified surface disinfection method to determine the efficacy of selected disinfectants to inactivate surface-dried high-titer stocks of these three structurally different TADV. ASFV and FMDV were susceptible to sodium hypochlorite (500 and 1000 ppm, respectively) and citric acid (1%) resulting in complete disinfection. Sodium carbonate (4%), while able to reduce FMDV infectivity by greater than 4-log units, only reduced ASFV by 3 logs. Citric acid (2%) did not totally inactivate dried CSFV, suggesting it may not be completely effective for disinfection in the field. Based on these data we recommend disinfectants be formulated with a minimum of 1000 ppm sodium hypochlorite for ASFV and CSFV disinfection, and a minimum of 1% citric acid for FMDV disinfection.

Published by Elsevier Ltd on behalf of The International Alliance for Biologicals.

### 1. Introduction

TADV are high-consequence pathogens that can cause high morbidity and mortality in livestock resulting in severe economic losses stemming from quarantines and loss of export business [1]. While the prevention of an accidental or intentional release of these viruses is most important, once an outbreak has occurred, disinfection is critical both to stop further dissemination of infection and to bring contaminated facilities back into production. Despite the importance of this work little information is available about the efficacy of disinfectants to inactivate TADV, especially on surfaces such as those to be encountered in farm settings (e.g. walls, floors, farm equipment, etc.).

Foot and mouth disease virus (FMDV) is one of the most contagious infectious agents known due to its rapid replication cycle and its stability in the environment [2]. FMDV is non-enveloped and it maintains infectivity in the environment long after drying (reviewed in [3]), thus fomites are a major mechanism

of virus spread. For example, the difficulties associated with contamination of fomites in affected premises during and after the 2001 FMDV outbreak in the UK have been well documented [4,5]. CSFV and ASFV are both enveloped viruses and although they are less stable in the environment than FMDV, in both cases fomites are potential mechanisms of virus spread (reviewed in [6,7]). Indeed, contaminated transport vehicles are thought to have transmitted CSFV to an uninfected swine herd during the Netherlands outbreak in 1997 [8].

FMDV is known to be very sensitive to low pH [9] and thus acid-containing disinfectants have been used successfully to clean up after FMDV outbreaks; 0.2% Citric Acid is recommended by the World Organization for Animal Health (OIE) for use against FMDV [10]. In contrast ASFV is quite stable and will survive over a wide range of pH. Various disinfectants have been used during ASFV and CSFV outbreaks in recent decades, including 2% sodium hydroxide [11,12].

Most of the published disinfection data for these viruses has been generated in a liquid format. Disinfection of viruses in liquids can provide basic information about virus sensitivity to various chemicals. However, in the field virus is shed into the environment, thus disinfectants are applied to surfaces potentially contaminated with dried virus [13]. Quantitative carrier tests (QCT) have been

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used as a model to determine the efficacy of disinfectants for various microbes dried on a wide range of surfaces; this type of assay has been described in ASTM E1053 [14] and by Sattar and coworkers [15]. However, methods for modeling enveloped virus disinfection on nonporous surfaces vary, resulting in data that is difficult to compare (reviewed in [16]). A key problem with surface disinfection is that many enveloped viruses tend to lose significant infectivity during the drying process under both environmental and laboratory conditions, resulting in a very small difference between the virus that remains intact after drying and the overall limit of virus detection. The addition of calf serum or other proteins can be used to stabilize virus in the inoculum during drying and the use of higher titer stocks can increase the resultant titer after drying [17]. While potentially solving issues specific to a particular virus, modifications to the composition of the inoculum may change the parameters of the assay, leading to an inaccurate assessment of disinfectant efficacy (reviewed in [18]).

Numerous commercial disinfectants for some high-consequence animal disease viruses have been approved by the US Environmental Protection Agency (EPA); however, there is a lack of published carrier test chemical disinfection data for many TADV. In this manuscript, we present a method for nonporous surface disinfection that takes into account the problems of disinfection with enveloped viruses. This method allows for large inoculum volume but limits the downstream volumes of disinfectant and neutralizer to prevent the loss of detectable virus due to dilution. Here we applied this method to determine the efficacy of selected disinfectants to inactivate high-titer stocks of three structurally different high-consequence TADV.

## 2. Methods

### 2.1. Cells and viruses

FMDV strain A24 stocks were generated in BHK-21 cells (ATCC# CCL-10). CSFV strain Brescia and the Swine Kidney cell line SK6 were obtained from Dr. Manuel Borca (PIADC). ASFV strain BA71/v was obtained from the PIADC virus repository and grown in Vero cells (ATCC# CCL-81).

### 2.2. Virus stock production

Briefly, cells were infected at an MOI of 0.01 PFU/cell in 850 cm<sup>2</sup> roller bottles and incubated at 37 °C until either 100% cytopathic effect was observed (FMDV and ASFV) or 5 days post infection (CSFV). To harvest the FMDV stocks, the infected cell supernatants were clarified by centrifugation, aliquoted and stored at –70 °C prior to use. For CSFV and ASFV stocks, the infected cells were scraped from the roller bottles and centrifuged at low speed to remove the medium. The cell pellets were resuspended in 5 ml of fresh media and subjected to 2 cycles of freezing at –70 °C and thawing at 37 °C, then sonicated 3 times for 30 s each on ice. The cell debris was removed by clarification and the supernatants were aliquoted and stored at –70 °C prior to use.

### 2.3. Disinfectants and neutralizers

All tested concentrations of sodium hypochlorite (Baker) were neutralized with Fluid Thioglycolate Medium (FTM, Difco). All concentrations of citric acid (Acros Organics) were neutralized with sodium bicarbonate (Invitrogen) except 2% citric acid, which was neutralized with 1.25 M sodium hydroxide (Ricca Chemical). 4% Sodium carbonate (Ricca Chemical) was neutralized with 0.025 M sodium citrate pH 1.1. All disinfectants were diluted in 400 ppm calcium carbonate to simulate hard water conditions. A control consisting of a 1:1 mixture of disinfectant and neutralizer was

tested for cytotoxic effects in each disinfection assay described in section 2.4.

### 2.4. Disinfection assay

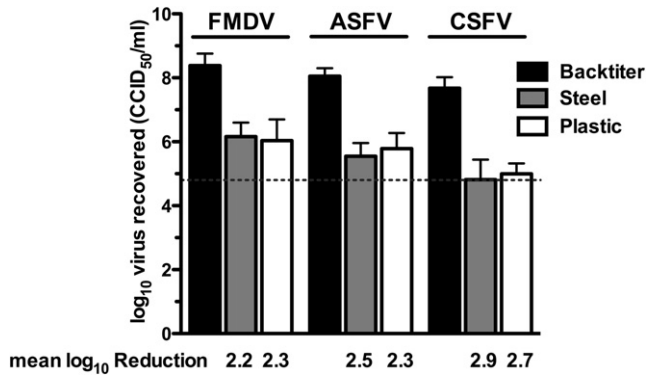
This protocol is a modification of ASTM E1053: Standard Test Method for Efficacy of Virucidal Agents Intended for Inanimate Environmental Surfaces [14]. Briefly, virus stocks were diluted in 1X phosphate buffered saline (PBS). The final concentration of calf serum in the virus inoculum was 1%. 100 µl of this mixture was pipetted on the surface coupons, either stainless steel base molds (Fisher Scientific #15182505C) or non-tissue culture treated polystyrene 6-well plates (Falcon #351146). ASFV and FMDV were dried at 30 °C in a static temperature incubator (Incufridge, Revolutionary Science). CSFV was dried at ambient temperature (20 °C–24 °C) in a biosafety cabinet with the lights off. Once dried, virus was exposed to 500 µl of the disinfectant for the indicated contact time at 22 °C in the Incufridge. At the end of the contact time, 500 µl of the appropriate neutralizer was added and the dried virus was scraped into the mixture, which was then added to 1 ml of cell culture media. In each experiment, one control coupon with dried virus was exposed to 500 µl of a 1:1 mixture of the disinfectant and neutralizer (recovery control) and one coupon without dried virus was exposed to 500 µl of cell culture media (surface cytotoxicity control). All control coupons were incubated at 22 °C for the maximum indicated contact time simultaneously with the coupons exposed to disinfectant. After the contact time was complete, the recovery control received another 500 µl of the neutralizer:disinfectant mixture prior to scraping, and then the entire mixture was added to 1 ml of cell culture media after scraping.

These post-disinfection samples were serially diluted in the appropriate media and titrated on susceptible cells in 96-well plates. FMDV infection was identified by the presence of destructive cytopathic effects 2 or 3 days post infection. ASFV was identified by the formation of plaques after 5–7 days post infection. CSFV was detected by fixing the cells 3–5 days after infection in 50% acetone/50% methanol and immunohistochemical staining with a monoclonal antibody to CSFV as described in Risatti et al. [19]. The titer of the recovered virus was calculated using the Spearman-Kärber endpoint titration method [20]. Because of virus dilution and the number of replicate wells infected per dilution, the lower limit of detection in this assay is 0.8 log<sub>10</sub> CCID<sub>50</sub>.

## 3. Results

### 3.1. Recovery of dried viruses from nonporous surfaces

In order to be registered as a disinfectant, EPA requires at minimum a 4-log reduction in virus titer in treated samples [21]. Since the lower limit of virus detection in our assay is 0.8 log<sub>10</sub> CCID<sub>50</sub>, it was necessary to confirm that after drying, a minimum of 4.8 log<sub>10</sub> CCID<sub>50</sub> could be recovered in the assay controls. High-titer stocks of FMDV, CSFV and ASFV were diluted in PBS then dried on stainless steel and plastic surfaces, according to the methods. After drying, the virus was resuspended in PBS then diluted in cell culture media and titrated in parallel with an aliquot of the undiluted inoculum. For FMDV and ASFV approximately 2 logs of virus infectivity were lost due to drying on either steel or plastic surfaces (Fig. 1). Considerably more virus was lost due to drying in the case of CSFV, with almost a 3-log reduction observed on both surfaces. This occasionally resulted in titers of less than 4.8 log<sub>10</sub> CCID<sub>50</sub> for CSFV that invalidated the results of some experiments due to insufficient virus recovery (Fig. 1).



**Fig. 1.** Recovery of test viruses from steel and plastic surfaces. Virus was dried on the on either steel or plastic surfaces, resuspended and titrated. The backtiter was a direct titration of an aliquot of the virus inoculum that was not dried. The mean log<sub>10</sub> reduction is the comparison of the virus recovery on each surface relative to that of the backtiter. Error bars indicate the mean of at least 5 experiments, ± SD. The dashed line at 4.8 indicates the minimum recovery required to achieve a 4-log reduction by disinfectant.

3.2. Dose response of disinfection

Dose-response assays were conducted to determine effective concentrations of disinfectant for each dried virus on the test surfaces. Various concentrations of disinfectants were applied to the dried virus preparations for a 10-min contact time and the titer of remaining infectious virus after neutralization was determined. Although all three viruses were effectively inactivated by sodium hypochlorite, ASFV was completely inactivated at 4–5 fold lower concentrations than FMDV or CSFV (Fig. 2). Significant differences between the surfaces were not apparent with any of the viruses tested. Interestingly, while CSFV was the least stable due to drying (Fig. 1), the infectious portion of the dried virus preparation had similar resistance to sodium hypochlorite as FMDV.

3.3. Comparison between sodium hypochlorite and citric acid

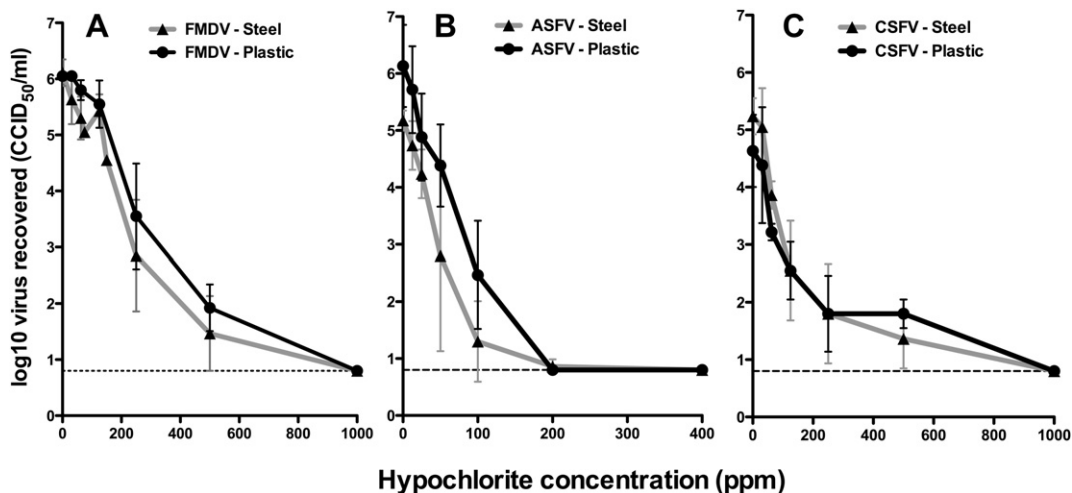
Since citric acid has been used in the field for FMDV disinfection, we wanted to compare the required effective hypochlorite concentration with that of citric acid. Disinfection assays were carried out on multiple days with multiple replicates to ensure

consistency of complete high-titer stock disinfection. Concentrations were included based on those from dose–response experiments (Fig. 2) that ensured complete disinfection in 10 min. Table 1 shows the mean log reduction between recovered dried virus and the disinfection samples for each virus on each surface with each concentration of disinfectant. For FMDV and ASFV, similar concentrations of citric acid were capable of reducing virus by greater than 4-log units on both plastic and steel surfaces. However citric acid was unable meet this criterion for CSFV on either surface even at 2% concentration. ASFV was completely inactivated by 500 ppm hypochlorite, but 1000 ppm hypochlorite was required for CSFV and FMDV.

3.4. Time-course of disinfection

Ten minutes has been a standard contact time in many disinfection protocols, but in many cases this is in excess and may lead to corrosion of surfaces. To determine the kinetics of sodium hypochlorite disinfection, assays were performed with various contact times. Sodium hypochlorite concentrations were used based on those from Table 1 that ensured complete disinfection in 10 min Fig. 3 demonstrates that 1000 ppm sodium hypochlorite completely disinfected FMDV on both surfaces by 4 min. Similarly, 500 ppm hypochlorite completely disinfected ASFV on both surfaces by 6 min. While 1000 ppm hypochlorite was able to completely disinfect CSFV on steel surfaces by 6 min, the full 10 min contact time was required on the plastic surface for complete CSFV disinfection.

Sodium carbonate (soda ash) has commonly been used as a disinfectant for transport vehicles moving between FMDV-endemic and non-endemic countries. While it is not registered by the EPA for virus disinfection, it has been given an exemption by USDA [22] and is recommended by the AUSVETPLAN [23] and FAO [24] for FMDV disinfection. To our knowledge, published data demonstrating the effectiveness of sodium carbonate against FMDV dried on surfaces does not exist. Because sodium carbonate has been demonstrated not to be highly effective against surface-dried avian influenza [25] and ASFV has been shown to be resistant to high pH [26], ASFV was included as a negative control in our experiments. While a greater than 4-log reduction of infectious FMDV was observed after sodium carbonate disinfection, the treatment did not completely disinfect the dried inoculum (Fig. 4).



**Fig. 2.** Effects of disinfectant concentration on dried virus recovery. FMDV (A), ASFV (B), CSFV (C) were dried on stainless steel or plastic coupons, exposed to the indicated concentration of sodium hypochlorite for 10 min, followed by neutralization with FTM and virus titration. Each data point is the mean of at least 3 experiments, ± SD. Dashed line indicates lower limit of virus detection.

**Table 1**  
Log<sub>10</sub> Reduction of Viruses by Chemical Disinfectants<sup>a</sup>.

Virus	Surface	500 ppm Hypochlorite	1000 ppm Hypochlorite	0.5% Citric Acid	1.0% Citric Acid	2.0% Citric Acid
FMDV	Steel	2.63 ± 0.53 n = 2(4)	5.55 ± 0.47 n = 8(12)	4.7 ± 0.88 <sup>b</sup> n = 2(4)	5.0 ± 0.35 n = 5(10)	n.d.
	Plastic	2.38 ± 0.18 n = 2(4)	5.6 ± 0.8 n = 5(10)	4.13 ± 0.72 <sup>b</sup> n = 2(4)	5.1 ± 0.49 n = 7(10)	n.d.
ASFV	Steel	4.8 ± 0.46 n = 9(9)	n.d.	n.d.	4.8 ± 0.11 n = 5(9)	n.d.
	Plastic	4.75 ± 0.61 n = 6(9)	n.d.	n.d.	4.88 ± 0.38 <sup>b</sup> n = 4(8)	5.13 ± 0.42 n = 8(9)
CSFV	Steel	n.d.	4.4 ± 0.35 n = 8(8)	n.d.	2.38 ± 0.35 n = 2(4)	3.25 ± 0.35 n = 2(4)
	Plastic	n.d.	4.25 ± 0.18 n = 5(8)	n.d.	n.d.	3.38 ± 0.53 n = 2(4)

n.d.: Not done.

<sup>a</sup> Log<sub>10</sub> reduction by indicated disinfectant ± standard deviation. Contact time was 10 min n = number of individual disinfection assays (total number of disinfection replicates).<sup>b</sup> Disinfection did not reduce virus to undetectable levels.

As expected, 4% sodium carbonate was not able to disinfect dried ASFV by the 4-log standard.

#### 4. Discussion

We present here a modified quantitative carrier test assay to test the efficacy of disinfectants against three high-consequence TADV. Because of the greater loss due to drying of enveloped viruses, we kept inoculum volumes high and volumes of disinfectant and neutralizer low to increase the lower limit of detection. The crux of this modification is effective neutralization that ensured neither virucidal nor cytotoxic effects from the disinfectant/neutralizer mixture. High-titer stocks were used to increase the virus concentration to enhance virus stability in lieu of the addition of stabilizers such as bovine serum. While this was mostly successful for both enveloped viruses in this study, in the case of CSFV the data from some disinfection assays was rejected because less than 4.8 logs of virus was recovered in the controls.

Using this method, neutralization was achieved with a volume equal to that of the disinfectant. Maintaining the virus in a small volume increases recovery after drying, but effective neutralization of highly concentrated disinfectants becomes more difficult. Therefore, this method might not be appropriate for testing classes of disinfectants that require neutralization by large dilutions. For instance, disinfectant trials of a surfactant using this method resulted in incomplete neutralization and considerable cytotoxicity in downstream cell culture detection assays (data not shown).

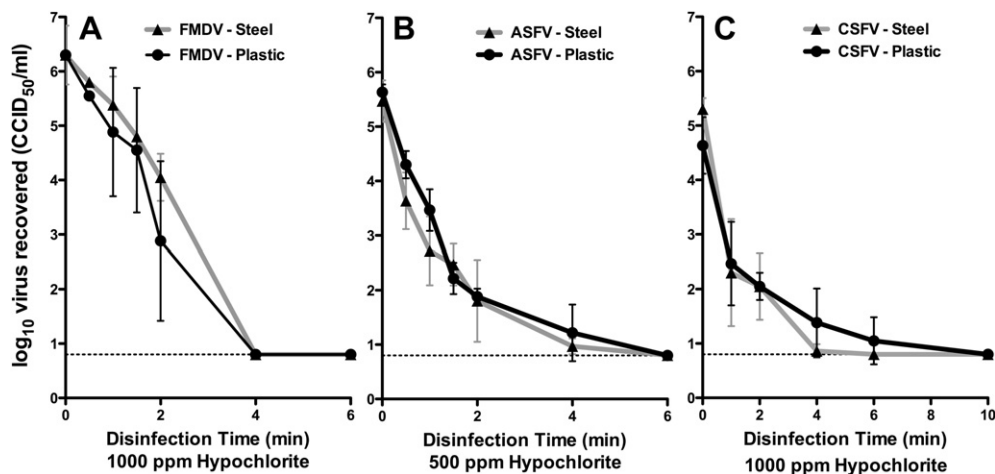
To our knowledge, this is the first study reporting ASFV surface decontamination with liquid chemicals. Heckert and coworkers [27] reported results using vapor phase hydrogen peroxide to

disinfect ASFV and CSFV in liquid suspension and dried on glass and steel surfaces. They observed less than a two-log loss of infectivity by drying ASFV and CSFV without disinfection. While this loss was slightly lower than that observed in our experiments, the inoculum used in their studies had a greater concentration of calf serum (5% vs. 1% in this manuscript).

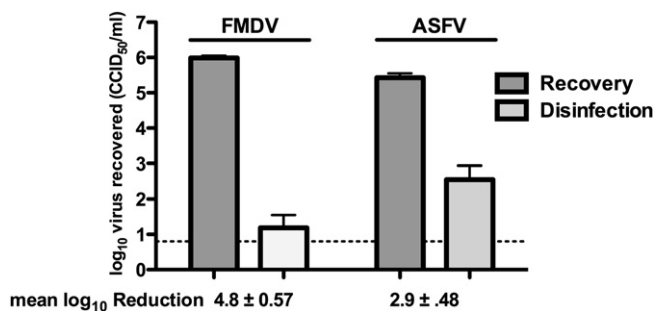
One remarkable finding was the fact that 2% citric acid was unable to completely disinfect dried CSFV in our experiments. Unlike FMDV, which is very unstable in low pH conditions, it is known that CSFV is less susceptible to acid treatment [28]. It has been suggested by Krey and coworkers [29] that this may be due to the possibility that acid-induced conformational changes in the pestivirus glycoproteins that occur during fusion are reversible and therefore virus regains infectivity when it is incubated at physiological pH with susceptible cells.

Sodium carbonate is widely recommended as a disinfectant for FMDV-contaminated surfaces in situations that citric acid might damage sensitive equipment. While greater than a 4-log reduction was observed with sodium carbonate against FMDV, in half of the experiments with this chemical we detected infectious virus after the disinfection process. The observation that ASFV was not completely disinfected by sodium carbonate is not particularly surprising since ASFV has been shown to be stable at high pH [26] and avian influenza was also resistant to sodium carbonate [25]. Because sodium carbonate is not recommended by livestock health agencies for CSFV surface disinfection, we did not test sodium carbonate against CSFV.

The data presented here extend published liquid-format disinfection data for high-consequence TADV to surface disinfection. One area of contention is that we were unable to achieve complete



**Fig. 3.** Effects of shortened contact time on dried virus disinfection. FMDV (A), ASFV (B) or CSFV (C) were dried on stainless steel or plastic coupons then exposed to the 1000 ppm (A and C) or 500 ppm (B) sodium hypochlorite for the indicated contact time, neutralized with FTM and titrated. Each data point is the mean from at least 3 experiments, ± SD. Dashed line indicates lower limit of virus detection.



**Fig. 4.** Sodium carbonate disinfection of FMDV and ASFV. The indicated viruses were dried on steel coupons then either recovered in a 1:1 mixture of sodium carbonate and citrate buffer or disinfected with 4% sodium carbonate for 10 min at 22 °C. Error bars indicate the mean of 8 replicates in 4 experiments (FMDV) or 4 replicates in 2 experiments (ASFV). The mean log reduction due to disinfection compared to the recovery is indicated ± SD. Dashed line indicates lower limit of virus detection.

disinfection of dried FMDV with 0.5% citric acid (Table 1), indicating that the 0.2% citric acid recommended by OIE [9] may not always be sufficient to completely disinfect dried FMDV in the field. Our data indicates that the use of citric acid is not effective for CSFV disinfection and that low concentrations of sodium hypochlorite are effective for ASFV disinfection.

There are many hurdles to successful disinfection testing of enveloped viruses; each virus will have its own specific difficulties in optimizing high-titer stock production and structural differences among virus families will affect survival after drying. Once these issues are resolved, this disinfection system can be used for these viruses and other chemicals. This system can also be easily modified to use other surfaces such as wood, glass or rubber. A future goal of this work is to more closely model the fomites found in the environment by using FMDV-infected cow secretions and ASFV-infected pig blood as inocula to dry and disinfect.

## Acknowledgments

This work was funded by EPA-USDA Interagency Agreement number 60-1940-8-055. L.JL, CRL and ACE were funded by the Oak Ridge Institute for Science and Education as part of the Research Participation Program at the Plum Island Animal Disease Center. We thank Jeff Kempter (EPA) and Nathan Birnbaum (USDA/APHIS) for helpful comments during the revision of this manuscript.

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