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Inactivation and Disinfection of Porcine Parvovirus on a Nonporous Surface

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Abstract

Animal parvoviruses have historically been accorded status as "highly resistant to inactivation". This status has been based largely on the well-known heat and chemical inactivation resistance of the animal parvoviruses (especially porcine, canine, bovine, and murine parvoviruses) in liquid inactivation settings. On the other hand, less is known about the relative resistance of parvoviruses to disinfection after being dried on surfaces. In the present article, we evaluate the ability of sodium hypochlorite and two proprietary aldehyde-based disinfectants to inactivate porcine parvovirus (PPV) dried on glass carriers in the presence and absence of varying organic load. Sodium hypochlorite and Microbide-G (a glutaraldehyde-based agent) caused rapid and complete (≥ 3 to 4 $\log_{10}$) inactivation of PPV deposited on glass carriers in a low organic load (5% serum) matrix. Microbide-G displayed the greatest inactivation efficacy for PPV deposited onto a glass surface in a blood matrix. In that case, a contact time of 10 min resulted in 3.5 $\log_{10}$ inactivation at ambient temperature.

Introduction

The *Parvoviridae* are a family of non-enveloped viruses of relatively small size (20-25 nm). Many of the animal parvoviruses (including bovine, canine, or porcine parvovirus and minute virus of mice) have been represented in the inactivation literature as exhibiting "high resistance" [1] or "very high resistance" [2] to physico-chemical inactivation. For instance, the animal parvoviruses are considered to be the most resistant of the virus families to heat inactivation [3] and only the polyomaviruses and circoviruses appear to be more resistant to gamma irradiation [4]. The parvoviruses, particularly murine minute virus, have represented a problem for the biotechnology industry, due to recurring episodes of contamination of Chinese hamster ovary cell bioreactors. The ability of rodent-derived parvovirus to survive on packaging and facility surfaces has been considered an important factor in both the recurring contamination events and the difficulty in eliminating the virus from infected facilities [5-7].

In addition to the importance of parvoviruses from a biotechnology perspective, this family of viruses has importance from a healthcare, veterinary and agricultural point of view. The human B19 virus is a parvovirus of concern for the plasma product and blood supply industries [8]. Porcine parvovirus (PPV) infection of a non-immune gilt, for instance, can cross the placenta and infect the fetuses, causing a combination of resorptions, mummifications, and stillbirths [9]. Canine parvovirus is a common and important cause of gastroenteritis and morbidity in young dogs [10].

Knowledge of the ability of disinfectants to effectively reduce the infectivity of parvoviruses on surfaces is therefore important not only in the biotech industry but also in the infection control, agricultural and veterinary arenas. Efficacy testing for disinfectants to be used for these applications is best performed in carrier studies, rather than in solution studies. The former more appropriately measure efficacy for inactivation of viruses deposited on surfaces [11-14]. In addition, the presence of organic load at the time of deposition of the virus can be investigated to realistically model the contamination of a surface by a virus suspended in blood or other organic matrices (urine, sputum, etc.) [14,15].

In the present study, we have evaluated the efficacy of three disinfectants against PPV deposited on glass carriers in the presence or absence of an organic load consisting of a 90% sheep blood solution. The efficacy of sodium hypochlorite solutions was compared with that of two proprietary aldehyde formulations from Microbide, Ltd. (Microbide-G and Microbide-S) (Table 1).

Agent	Active	Class	Mechanism of inactivation
Microbide-S	Succindialdehyde (10.0%)	aldehyde	Cross-linking of proteins and nucleic acids [16]
Microbide-G	Glutaraldehyde (3.0%)	aldehyde	Cross-linking of proteins and nucleic acids [16]
Sodium hypochlorite	Sodium hypochlorite	Oxidizing agent	Oxidation of thiol groups [16]

Table 1: Comparison of properties of the inactivating agents evaluated.

Materials and Methods

Reagents

Sodium hypochlorite solutions were prepared by dilution of Clorox® bleach. Microbide-G and Microbide-S were provided by Microbide Ltd (Dublin, Ireland).

Viruses

Porcine parvovirus (PPV) strain NADL-2 was selected as it is a commonly employed strain that is available from the American Type Culture Collection (ATCC VR-742). The virus was propagated in swine testicle (ST) cells (ATCC CRL-1746) at $36 \pm 2°C$ with $5 \pm 1\%$ CO_2 in Minimum Essential Medium (MEM) + 5% fetal bovine serum (FBS; ThermoFisher Scientific, Waltham, MA). The flasks were frozen at -80°C and then thawed at ambient

temperature. The conditioned medium was collected and clarified at 2,000 rpm for 15 min, and the resulting supernatant was purified with an ultra-centrifuge. The viral pellet was resuspended in a serum-free medium and aliquoted and stored at -80°C until use. The certified titers of the PPV stocks used in these studies ranged from 8.68 to 9.45 $\log_{10}$ 50% tissue culture infectious dose per mL ($TCID_{50}$/mL) in ST cells.

Surface inactivation studies

Solutions of the disinfectants were prepared by dilution as required with 400 ± 2.9% ppm AOAC hard water. The organic load of the virus inoculum was either low (5% fetal bovine serum, FBS) or high (90% Defibrinated Sheep's Blood; Cocalico Biologicals, Inc., Stevens, PA). Microbide-S was tested at 2,000 and 10,000 ppm and 30,000 ppm. Microbide-G was tested at 600 and 3,000 ppm and undiluted (30,000) ppm. Sodium hypochlorite was tested at 600, 3,000, and 30,000 ppm (high organic load), or 825 or 4125 ppm (low organic load). For each treatment, 0.4-mL aliquots of virus in the presence of the organic load were spread onto a 4-in^2 area of a sterile glass Petri dish and were allowed to dry at ambient temperature for 30 min.

The experimental design for the inactivation studies followed the general procedure outlined in the ASTM E1053-11 standard [17]. The studies were performed once for each experimental condition. For each disinfectant, 2.0 mL of test solution were applied directly to the virus film deposited onto a glass carrier. The solutions were allowed to completely cover the virus film for the entirety of the 15-s, 30-s, 1-min, 2-min, 5-min, or 10-min contact times at 20 ± 2°C. Following the contact times, 8.0 mL of neutralizer (MEM+10% FBS+1 % glycine for neutralizing Microbide-G and Microbide-S; MEM +10% FBS + 0.5% $Na_2S_2O_3$ for neutralizing sodium hypochlorite) were added. After neutralization, the mixtures were scraped from the surface of the glass carriers with a cell scraper. An 0.8-mL volume of each sample was transferred to a Sephacryl column and subjected to centrifugation at 1,000 rpm for 3 min. Each contact time was evaluated in a single replicate (n=1) for each disinfectant.

The post-neutralization samples (PNS) were serially diluted and selected dilutions added (0.05 mL per well) to 96-well plates containing ST cells and incubated at 36 ± 2°C with 5 ± 1% CO_2 for 8 days. Following incubation, the ST cells were examined microscopically for the presence of viral CPE or for evidence of cytotoxicity due to disinfectants. The CPE results were used to calculate the virus titer of the challenge virus stock or PNS in units of $TCID_{50}$/mL using the Spearman-Karber method.

Inactivation study controls

Plate Recovery Control (PRC): This control was performed in singlet (n=1 replicate) in a manner analogous to the disinfectant test runs, with 2.0 mL of diluent (hard water) added to the dried virus in lieu of disinfectant.

Neutralizer Effectiveness/Viral Interference Controls (NE/VI): This control was performed for each type of disinfectant as a single replicate (n=1). This control was performed in a manner identical to the test runs, except that 0.4 mL of diluent was dried onto the carriers in lieu of virus. At the completion of the contact times, the control carriers were subjected to neutralization procedures identical to those of the test carriers. This control was then divided into two portions, one for the Cytotoxicity Control and one to continue the NE/VI. An aliquot of the NE/VI PNS was diluted using serial 10-fold dilutions. Following dilution, 0.1 mL of stock PPV virus containing approximately 1,000 – 5,000 infectious units was added to each dilution and held for the longest contact time prior to inoculation of host cells.

Cytotoxicity Control (CT): This control was performed for each type of disinfectant at a single replicate (n = 1). This control was performed in a manner analogous to the NE/VI runs, except that no virus was added to the samples after serial dilution prior to the inoculation of ST cells.

Cell Viability Control (CVC): This control was performed as a single replicate (n=1). Eight wells of ST cells received only diluent to demonstrate the viability of the cells throughout the assay period.

Virus stock titer control: This control was performed as a single replicate (n=1). An aliquot of PPV stock (i.e., inoculum) was serially diluted and inoculated onto ST cells to demonstrate that the titer of the PPV virus used in the assay was approximately the same as the certified titer for the PPV stock.

Results

Inactivation efficacy and time kinetics in the presence of low organic load

The efficacies of Microbide-S, Microbide-G, and sodium hypochlorite for inactivating PPV were evaluated on glass carriers at ambient temperature in the presence of low organic load (5% FBS; Figure 1). This enabled the determination of the "best-case" surface inactivation efficacies for these disinfectants, as a higher organic load was expected to reduce the efficacy of, or alter the time kinetics for, PPV inactivation by the disinfectant agents. Data on the time kinetics of PPV inactivation under low organic load conditions were limited because of the rapid and complete inactivation that resulted, after as little as 1 min of contact time, with undiluted (30,000 ppm) Microbide-G or sodium hypochlorite solutions at 825 and 4,125 ppm. The maximal efficacies for these disinfectants were determined to be 3 $\log_{10}$ and 4 $\log_{10}$ reduction in titer, respectively. In both cases, inactivation was complete and the $\log_{10}$ reduction reported was determined after taking account of the dilution of PNS required to remove cytotoxicity to the host cells used to titrate the PPV. On the other hand, Microbide-G tested as a 3,000 ppm solution, or Microbide-S tested as 2,000 ppm or 10,000 ppm (undiluted) solutions caused incomplete and contact time-dependent inactivation of PPV. Maximal efficacies in these cases were ~ 2.4 to 3.0 $\log_{10}$ reduction in PPV titer. Insufficient data points were obtained to enable determination of the linearity of the time-dependent inactivation in the presence of low organic load.
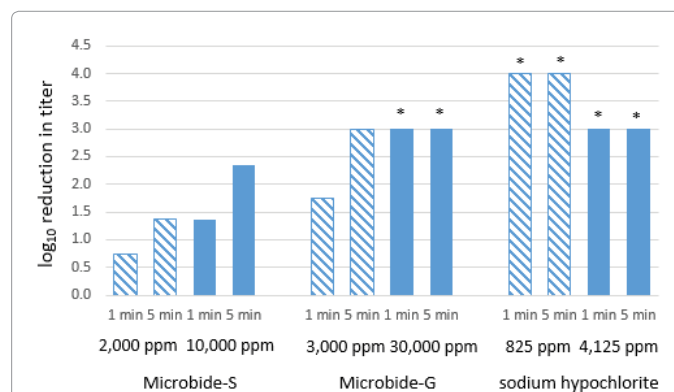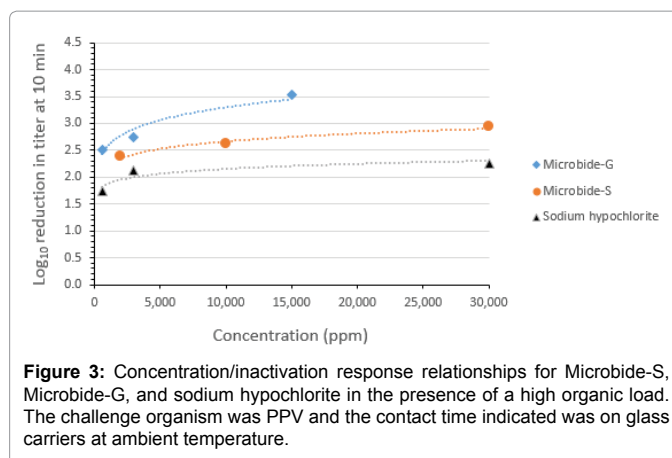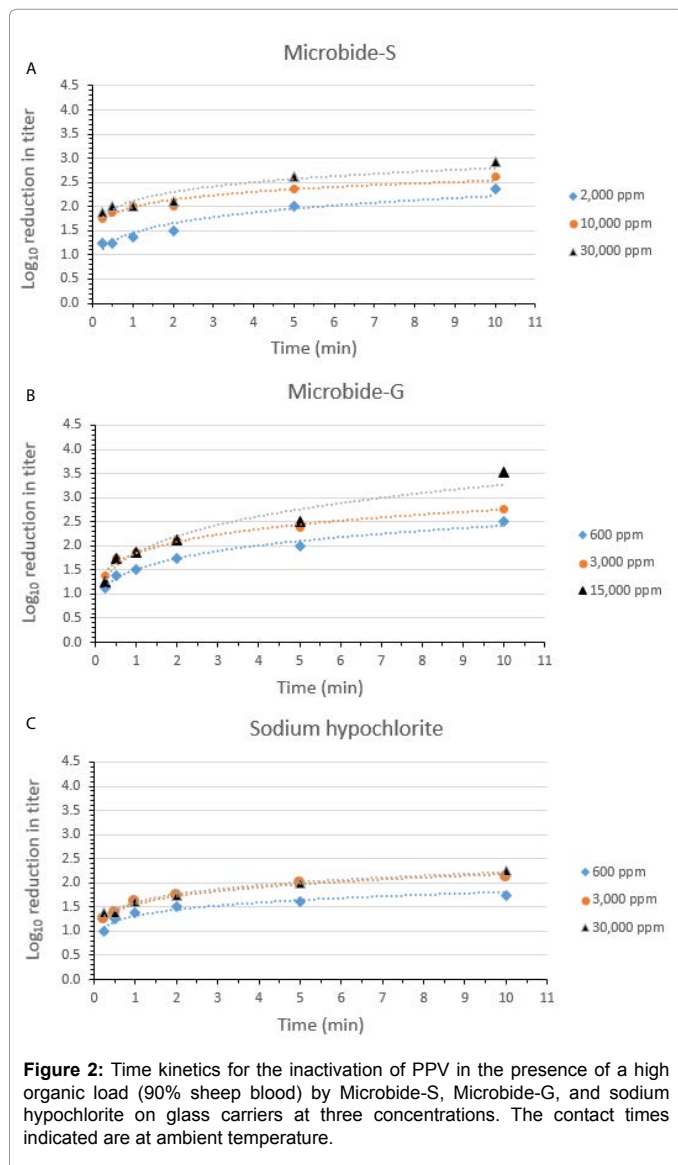

Figure 1: Efficacy of Microbide-S, Microbide-G, and sodium hypochlorite for inactivating PPV at 1 min and 5 min contact times at ambient temperature in the presence of low organic load (5% FBS). Asterisks indicate that the actual $\log_{10}$ reduction achieved was "≥" the indicated values.

Inactivation efficacy and time kinetics in the presence of high organic load

The efficacies of Microbide-S, Microbide-G, and sodium hypochlorite for inactivating PPV deposited on glass carriers in the presence of high organic load (90% sheep blood) were dependent on contact time and disinfectant concentration, although neither of these relationships was linear (Figure 2A-C and Figure 3). The PPV-inactivation time kinetics for each of the disinfectants displayed a rapid deviation from linearity within the initial few min of contact time at ambient temperature (Figure 2). The maximal inactivation efficacies were observed at the highest disinfectant concentrations at 10 min contact time (Figure 2). At 10 min of contact, Microbide-G at 15,000 ppm caused 3.5 $\log_{10}$ inactivation of PPV, while 30,000 ppm Microbide-S caused 2.9 $\log_{10}$ inactivation. A 10-min contact with sodium hypochlorite (30,000 ppm) resulted in 2.3 $\log_{10}$ inactivation of PPV. The concentration/inactivation response relationships for each disinfectant were non-linear, and in no case was the inactivation of PPV complete (Figure 3).

Figure 3: Concentration/inactivation response relationships for Microbide-S, Microbide-G, and sodium hypochlorite in the presence of a high organic load. The challenge organism was PPV and the contact time indicated was on glass carriers at ambient temperature.

Discussion

Parvoviruses exhibit high resistance to certain physical and chemical inactivation approaches [1-4] and are often used as worst-case challenge viruses in solution inactivation efficacy studies. Less is known about the relative resistance or susceptibility of parvoviruses to inactivation on surfaces (i.e., efficacy determined in carrier studies). Previous reports have demonstrated that these small-non-enveloped viruses are susceptible to surface inactivation by caustics and peroxide-generating agents [12], aldehyde disinfectants [13], and sodium hypochlorite [18,19] under certain types of organic load.

The inactivation, by glutaraldehyde, of four animal parvoviruses (mouse minute virus, PPV, bovine parvovirus, and canine parvovirus [CPV]) deposited on stainless steel surfaces was investigated by Rabenau et al. [13]. In that study, the parvoviruses were dried on carriers in a matrix containing 0.03% bovine serum albumin, and a fixed contact time of 5 min at ambient temperature was evaluated. Concentration-dependent inactivation of each virus was observed, with maximal efficacies (3 to 4 $\log_{10}$ reduction in titer) being determined at 2,500 ppm glutaraldehyde. The four parvoviruses displayed similar susceptibility to glutaraldehyde inactivation in this study.

The impact of high organic load on viral inactivation efficacy has been described previously for Zika virus and West Nile virus (two members of the flavivirus family) [14]. In the studies of Zika virus inactivation, a 90% sheep blood matrix present at the time of virus deposition onto the carriers afforded protection of the virus against the inactivating effects of heat, sodium hypochlorite, and peracetic acid. Time kinetics were not investigated in the studies, and it was hypothesized that the organic load might confer protection to the virus in two ways: 1) through stabilization of the ZIKV through protein binding, maintaining viral particle organization on the environmental surface, changing the distribution and layout of virus on the surface, or forming a protective layer on top of the virus particles; or 2) reaction of the organic material with the active ingredients of the disinfectants to reduce their effective concentrations [14].

Terpstra et al. reported that canine parvovirus (CPV) dried on stainless steel carriers in the presence of culture medium or 90% plasma (intended to model deposition in blood) displayed markedly different susceptibility to sodium hypochlorite (1,000 ppm) inactivation [19]. Namely, >4 $\log_{10}$ and >5 $\log_{10}$ inactivation of CPV dried in the presence of culture medium occurred within 1 and 10 min contact time, respectively. On the other hand, inactivation of CPV dried in the presence of 90% blood plasma was very limited (no inactivation after 1 min, ~1 $\log_{10}$

Figure 2: Time kinetics for the inactivation of PPV in the presence of a high organic load (90% sheep blood) by Microbide-S, Microbide-G, and sodium hypochlorite on glass carriers at three concentrations. The contact times indicated are at ambient temperature.

inactivation after 10 min of contact with sodium hypochlorite). The authors suggested that a blood matrix might inhibit, to an even greater extent, the inactivating effects of sodium hypochlorite, as suggested by the work of Weber et al. [20]. Although the latter authors did not specifically examine a parvovirus, they determined that the presence of 80% blood in solution inactivation of poliovirus caused reduction in efficacy and alteration in time kinetics of inactivation by sodium hypochlorite. As suggested by these and other investigators, a blood matrix is a more appropriate matrix for modeling viral inactivation in blood spills, and generally, for all bloodborne viruses.

The evaluation of the time kinetics for inactivation of PPV by each of the three disinfectants in the present study has provided additional evidence of protection of virus by a high organic load such as that represented by blood. The time kinetics plots for inactivation of PPV demonstrate a rapid deviation from linearity in the case of each disinfectant. This is highly suggestive of 1) partitioning of the active agent into the organic load; or 2) adsorption of virus to the organic, affording some degree of protection of the virus from the active; 3) a combination of the adsorption of active and virus to the organic that prevents interaction of the active with the virus. That these active agents (especially aldehydes and sodium hypochlorite) are susceptible to partitioning into organic material has been emphasized previously by Harvey [21] and McGavin [22]. Adsorption of the picornavirus poliovirus to fecal suspensions been shown to protect this non-enveloped virus from inactivation by chlorine. This protection was attributed both to increased chlorine demand as well as to occlusion of the virus from the active [23]. Another picornavirus, hepatitis A virus, has been shown to display slower chlorine inactivation kinetics when associated with organic solids, including cell membranes [24]. The decrease in disinfectant activity for any given concentration of these agents in the presence of high (Figure 2) versus low (Figure 1) organic load most likely reflects binding or reactivity of the active agent and/or virus with the organic material. In our time kinetics plots, the continued increases in accumulated viral inactivation occurring over a ten-min period suggest that such interactions of active and virus with the organic load must be reversible, subject to an equilibrium that favors binding or other interaction with the organic. According to this viewpoint, the release of active and virus from the organic load with time would result in incremental inactivation of remaining infectious virus as suggested by Figure 2.

With respect to the impact of organic load on inactivation efficacy and kinetics, the aldehyde disinfectants Microbide-G and Microbide-S and the chlorine generating agent sodium hypochlorite were similar. Of the three active agents evaluated in this study, Microbide-G (the glutaraldehyde-based agent) displayed the greatest inactivation efficacy for PPV deposited onto a glass surface in a blood matrix. A contact time of 10 min resulted in 3.5 $\log_{10}$ inactivation at ambient temperature.

Conclusion

Results of surface inactivation studies performed with PPV indicate rapid (within 5 min) and complete ($\geq 3 \log_{10}$) inactivation by Microbide-G and sodium hypochlorite in the absence of a blood matrix. However, inactivation in the presence of high organic load (90% sheep blood) required higher concentrations and/or greater contact times and exhibited non-linear time kinetics that were suggestive of binding or other chemical reaction of the active agents with the organic load. In addition, in the presence of high organic load, each of the active agents displayed direct, albeit non-linear, concentration-response relationships. Of the three active agents, Microbide-G (the glutaraldehyde-based agent) displayed the greatest inactivation efficacy for PPV deposited onto a

glass surface in a blood matrix. A contact time of 10 min resulted in 3.5 $\log_{10}$ inactivation at ambient temperature. The results should aid in the development of appropriate disinfection and sanitization regimens for PPV and other bloodborne viruses of concern.

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