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CHARACTERIZATION OF VIRUCIDAL RESISTANCE OF DNA VIRUSES

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14. ABSTRACT:
The objective of this study was to compare the virucidal effectiveness of various disinfectants on the *Vaccinia virus* (VACV) and the *Helicoverpa zea* single-capsid nucleopolyhedrosis virus (HzSNPV) to determine whether the HzSNPV could be used as a simulant for the orthopox *Variola virus*, the causative agent of smallpox. VACV and HzSNPV were exposed to five commonly used disinfectants and three detergents. Results showed that VACV and HzSNPV exhibited similar responses to the virucidal treatments, although differences were observed as functions of concentration and exposure time. VACV showed greater susceptibility to treatment at low concentrations and lower exposure times. Host insect cells also showed direct toxic responses to some of the aldehyde and detergent solutions, and these responses were not observed in the host Vero cells that were used to propagate the VACV. We concluded that the HzSNPV demonstrated sufficient correlation with the response of the VACV to disinfectant treatment, which further warrants consideration of HzSNPV as an effective simulant for the *Variola* strain of viruses.

15. SUBJECT TERMS
Vaccinia virus (VACV)
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PREFACE

The work described in this report was supported by the Defense Threat Reduction Agency (Fort Belvoir, VA). The work was started in October 2007 and completed in September 2009.

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CONTENTS

FIGURES

TABLES

CHARACTERIZATION OF VIRUCIDAL RESISTANCE OF DNA VIRUSES

1. INTRODUCTION

For obvious health and environmental reasons, research with major Category I biological agents is confined to high-containment or biosurety laboratories. This necessary limitation significantly restricts the freedom to develop and test devices and methods for countering the threats posed by bioagents as weapons of terror or mass destruction. Consequently, non-pathogenic bioagent simulants are commonly used in testing and development efforts. The use of simulants can expedite the research, development, test, and evaluation of biodefense materials to support Army requirements for detection, decontamination, and physical protection. By definition, an effective simulant exhibits broad-spectrum similarity (with respect to biophysical and biological properties) with the actual pathogenic target. The bacteriophage MS2 is widely used as the model simulant for viral pathogens, although it exhibits little similarity in size, genome structure, or virion composition to the orthopox family of viruses that include *Variola major*, the causative agent for smallpox. We have begun investigating *Helicoverpa zea* single-capsid nuclear polyhedrosis virus (HzSNPV), a baculovirus that more closely resembles the biophysical properties of the orthopox viruses. HzSNPV has a similar virion particle size (50–100 × 400 nm) and a double-stranded DNA genome (Figure 1). HzSNPV is also generally accepted as safe for release into the environment, which increases its utility for the test and evaluation of devices and methods in real-world scenarios. The objective of this study was to compare the biological viability of HzSNPV and *Vaccinia virus* (VACV) under a number of virucidal treatment conditions. The results are also compared with the response profile of *Variola major* obtained from existing literature.

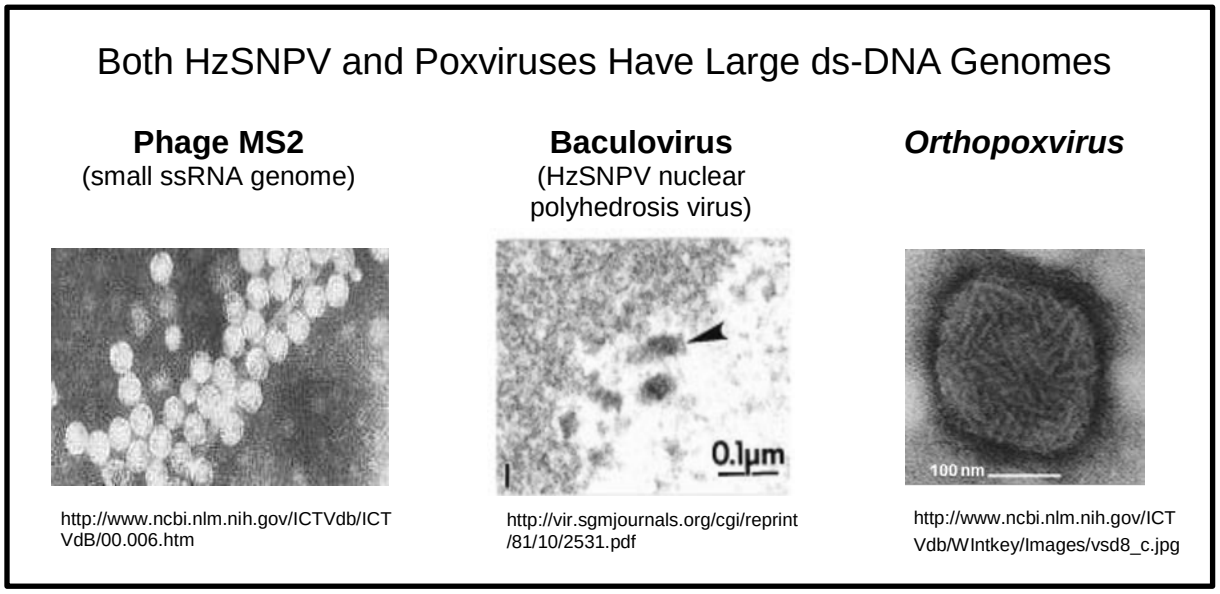

Figure 1. Comparison of the current viral simulant, the single-stranded (ss) RNA bacteriophage MS2 (left), and the proposed viral simulants, the double-stranded DNA (ds) HzSNPV (center) and *Orthopoxvirus* (right).

2. MATERIALS AND METHODS

2.1 Cell Lines and Viruses

The BCIRL-HzAM1 insect cell line, which is derived from *Helicoverpa zea* (corn earworm) pupae, was received from Chesapeake PERL, Inc. (Savage, MD). The cells were maintained at 27 ℃ in EX-CELL 420 medium (SAFC; Lenexa, KS). The medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS) with 100 U/mL penicillin and 10 µg/mL streptomycin (all from Sigma-Aldrich; St. Louis, MO). Under these conditions, cells grew in confluent, homogeneous monolayers (Figure 2). The cells were subcultured by washing with medium without supplements and were pooled for cell count. A cell count was performed using the Vi-CELL XR cell viability analyzer (Beckman Coulter Life Sciences; Indianapolis, IN). Stock cultures were seeded at 5000 viable cells/cm^2 in a total volume of 20 mL of complete medium.

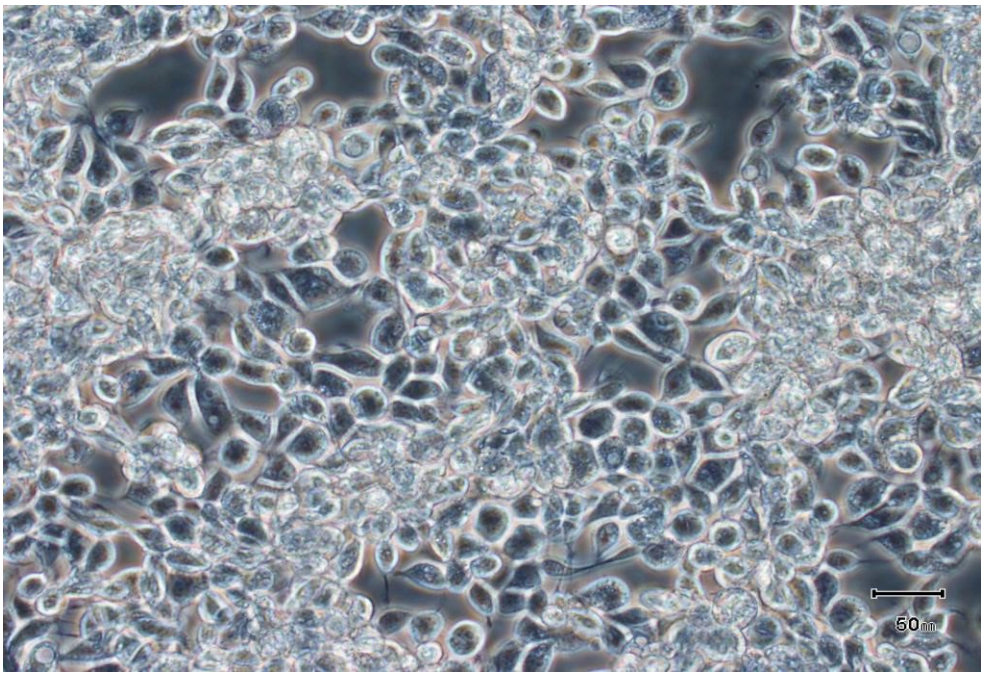

Figure 2. Light microscope image of BCIRL-HzAM1 cells grown in EX-CELL 420 media.

The HzSNPV was received from Chesapeake PERL. BCIRL-HzAM1 cells were subcultured (as described) into T25 or T75 flasks and infected with 300 µL of passage 1 HzSNPV at 7×10^5 plaque-forming units (pfu)/mL (for a 25 cm^2 flask). The flasks were incubated at 27 ℃ until the BCIRL-HzAM1 cells were infected (approximately 5–7 days). The supernatant was removed from the flasks and was filtered using a 0.2 µm syringe filter to remove cellular debris. A plaque assay was performed to determine the viral titer. Plaques manifested as a cluster of dark cells, easily differentiated from uninfected cells using a light microscope (Figure 3). The viral solution, that produced detectable plaques, was divided into sterile 2 mL aliquots and frozen and stored at –20 °C for future use.

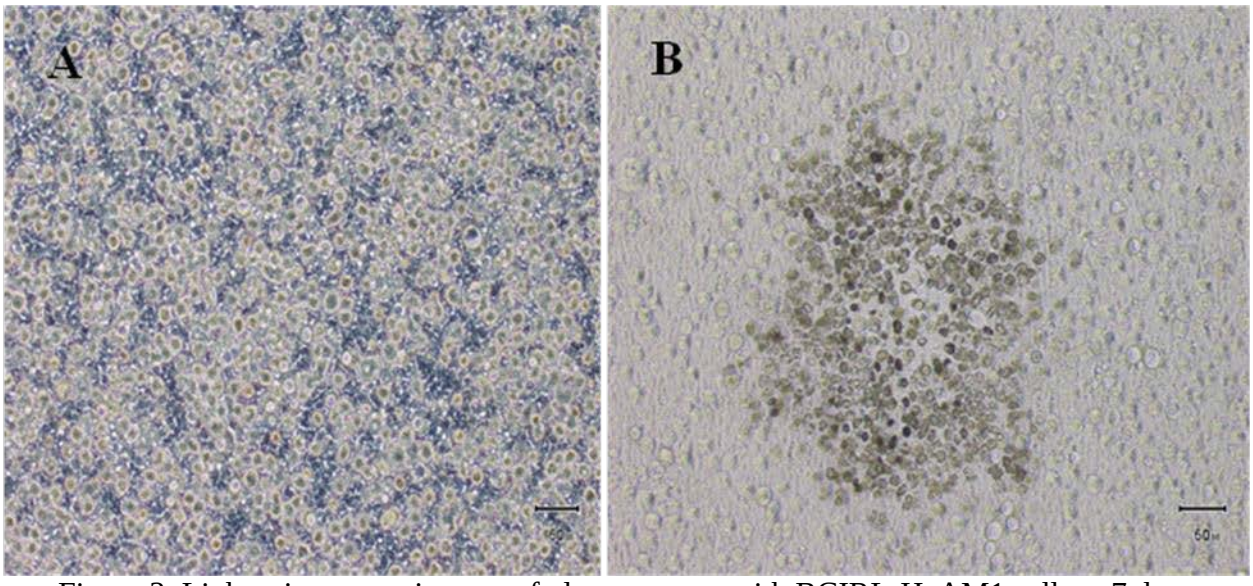

Figure 3. Light microscope images of plaque assays with BCIRL-HzAM1 cells at 7 days post-infection. (A) control cells (no virus added); and (B) cells infected with HzSNPV virions.

The Lister strain of VACV was provided by the Critical Reagents Program (Joint Project Manager for Medical Countermeasure Systems; Aberdeen Proving Ground, MD). Vero cells (ATCC CCL-81) were obtained from the American Type Culture Collection (Manassas, VA). VACV was propagated in Vero monolayer cultures in Eagle's minimum essential medium (MEM) containing 10% heat-inactivated FBS at 37 °C. Infected cells were harvested and centrifuged at 3210 ×*g* at 4 °C for 10 min. The pellets were resuspended in MEM containing 10% FBS, freeze–thawed for three cycles, sonicated for 4 min on ice, and then centrifuged at 3210 ×*g* at 4 °C for 10 min. The supernatant was collected and served as the source of virus for all of the experiments described.

2.2 Disinfectants and Virus/Disinfectant Preparations

All solutions listed in Table 1 were diluted with sterile, cell culture-grade water and were then sterile-filtered using a 0.2 µm Whatman syringe filter. A mixture of nine parts disinfectant and one part virus was used for each disinfectant for the exposure period.

Table 1. List of Disinfectants Used

Number	Solution
1	99.5% ethanol (Sigma-Aldrich)
2	95% isopropanol (Sigma-Aldrich)
3	10% sodium hypochlorite (Sigma-Aldrich)
4	30% formaldehyde (Sigma-Aldrich)
5	99% phenol (Sigma-Aldrich)
6 Mixture	6.67% cetyltrimethylammonium chloride (Sigma-Aldrich)
	3.33% benzalkonium chloride (Sigma-Aldrich)
7 Mixture	1.75% iodine (Fluka; Morris Plains, NJ)
	10% Tergitol surfactant (polyethyleneglycol nonphenyl ether; Dow Chemical Company; Midland, MI)
8	10% tetradecyltrimethylammonium bromide (Sigma-Aldrich)

2.3 Plaque Assays

2.3.1 Culture Plate Seeding

Subcultured insect cells were seeded into 60 mm Petri dishes at 2×10^6 viable cells per dish in EX-CELL 420 medium without added serum or supplements. The plates were gently rocked to evenly distribute the cells and were incubated at 27 ℃ for 1 h to allow the cells to adhere.

Vero cells were plated at a density of 1.2×10^5 viable cells per well in 24-well plates. The plates were gently rocked to evenly distribute the cells and were incubated at 37 °C in 5% CO_2 for 1 h to allow the cells to adhere.

2.3.2 Agarose Solution Preparation

BD BaculoGold agarose (BD Biosciences; San Jose, CA) was prepared by adding 0.9 g of BaculoGold agarose to 10 mL of sterile, cell culture-tested water and autoclaving for 30 min at 121 °C (liquid cycle). SeaPlaque agarose (Lonza, Ltd; Basel, Switzerland) was prepared by adding 1 g of SeaPlaque agarose to 10 mL of sterile, cell culture-tested water and autoclaving for 30 min at 121 °C (liquid cycle). After autoclaving was complete, the bottles were placed into a 42 ℃ water bath in the biosafety cabinet (BSC) to equilibrate. Next, 90 mL of complete EX-CELL 420 medium (EX-CELL 420 medium with FBS and supplements) and 90 mL of MEM that contained 10% FBS and 1× penicillin/streptomycin were placed into a sterile container and also equilibrated to 42 °C in the water bath. When all three bottles were at the same temperature, the medium was added in a sterile manner to the agarose and swirled well to mix. The bottle was then returned to the 42 °C water bath to maintain its temperature.

2.3.3 Viral Serial Dilution Preparation and Cell Line Infection

HzSNPV dilutions (Table 2) were made as follows, in media that had been warmed to 27 °C. One milliliter of each viral dilution solution was added to each 60 mm Petri dish containing BCIRL-HzAM1 for 1 h. The plates were left at room temperature (RT) in the BSC and were gently rocked by hand every 15 min.

VACV dilutions (Table 2) were also made in media that had been warmed to 37 °C. One milliliter of each viral dilution solution was added to each 60 mm Petri dish that contained Vero cells (ATCC CCL-81) for 1 h. The plates were left at RT in the BSC and were gently rocked by hand every 15 min.

Table 2. Viral Dilutions

Viral Dilution	**Volume of HzSNPV**	**Volume of Ex-Cell without Serum or Additives (mL)**	**Volume of *Vaccinia* Lister Virus**	**Volume of MEM without Serum or Additives (mL)**
10^{-2}	40 µL of HzSNPV stock	3.96	40 µL of VACV stock	3.96
10^{-3}	400 µL of 10^{-2} dilution	3.6	400 µL of 10^{-2} dilution	3.6
10^{-4}	400 µL of 10^{-3} dilution	3.6	400 µL of 10^{-3} dilution	3.6
10^{-5}	400 µL of 10^{-4} dilution	3.6	400 µL of 10^{-4} dilution	3.6
10^{-6}	400 µL of 10^{-5} dilution	3.6	400 µL of 10^{-5} dilution	3.6

2.4 Pouring Agarose

After 1 h of absorption, the HzSNPV solution was carefully removed from each Petri dish without disrupting the cell sheet. Once all of the solution was removed, 4 mL of the 0.9% agarose was slowly added to the dish and allowed to solidify. After approximately 30 min, the dishes were moved to a tray and placed into a 90% humidified, 27 °C incubator. High humidity is essential during this step, or the agarose will dry out. The Petri dishes were allowed to remain in the incubator for approximately 6 days, until plaques were visible. The plaques were then stained with a 0.9% agarose/neutral red (NR; Sigma-Aldrich) solution.

Similarly, following 1 h of absorption, the VACV medium solution was carefully removed and replaced with MEM that contained 10% FBS, 1× penicillin/streptomycin, and 1% SeaPlaque agarose. After approximately 30 min, the dishes were moved to a tray and placed into an incubator at 37 °C with 5% CO_2. The Petri dishes were allowed to remain in the incubator for approximately 3 days until plaques were visible. The plaques were then stained with a 1% agarose/NR solution.

2.5 Staining Plaques

Sterile BD BaculoGold agarose (0.9 g) was prepared as described in Section 2.3.2. After equilibration, 1.5 mL of NR was added to the bottle to yield a 50 µg/mL final solution of NR. The Petri dishes were removed from the incubator, and 3 mL of the agarose/NR solution was slowly added to each HzSNPV plate. The plates were allowed to

remain in the BSC for approximately 20–30 min and were then placed in the 90% humidified, 27 °C incubator. The dishes were left to incubate for 24 h before the plaques were counted.

Similarly, 72 h after infection, MEM containing 10% FBS, 1× penicillin/streptomycin, and 100 μg/mL of sterile NR solution was added to each well containing VACV. Cells were incubated for an additional 24 h at 37 °C in 5% CO_2. Observations were made daily: plaques were observed by eye, and cell health was observed with a microscope.

2.6 Counting Plaques

The lid was removed from each dish and placed upside down on a light box. Plaques were counted manually. PFUs were calculated as follows:

$$\frac{\text{No. of plaques counted}}{\text{Dilution factor} \times \text{volume of virus (1 mL) used per dish}} = \text{PFUs}$$

Example: For 10^{-3} (1:1000 dilution), use 0.001 as dilution factor:

$$\frac{\text{No. of plaques}}{0.001 \times 1\ \text{mL}} = \text{PFUs}$$

2.7 Virucidal Treatment Assay Design and Conditions

Cells were prepared in the same manner as for the plaque assays but with minor modifications. After the Petri dishes were seeded and incubated for 1 h, the cell culture medium was removed, and 1 mL of each virus–disinfectant solution was added to the Petri dishes or 24-well plates. A mixture of nine parts disinfectant and one part virus was used for each disinfectant for the exposure period, which correspond with the times outlined in Table 2. Immediately after each time point, fresh medium was added to dilute the virus and obtain a final dilution of 1:1000 (10^3) of the virus. Plaque assays were then performed. Each disinfectant was tested in triplicate for each experiment. Controls were treated with appropriate medium only, or for the negative controls, a dilution of each disinfectant (without virus) was used. Positive controls were treated with serial dilutions of the virus (10^{-3} to 10^{-6}) in appropriate medium, and no disinfectant was added.

Table 3 presents a summary of the virucidal treatment effects that were observed on VACV and HzSNPV. All plates were seeded at the same cell density and infected with the same amount of virus. Exposure to ethanol, isopropanol, sodium hypochlorite, phenol, and the iodine/Tergitol mixture resulted in no visible effects on the host cells, but significant virucidal activity was evident on the propagation of both VACV and HzSNPV. This effect was concentration and time dependent; VACV showed greater susceptibility to the treatments at lower concentrations and shorter exposure times. Disinfectant treatment (in the absence of virus) appeared to have no effect on cell density or morphology. However, exposure to high concentrations of formaldehyde, the cetyltrimethylammonium chloride and benzalkonium chloride mixture, or tetradecyltrimethylammonium bromide did show evidence of direct toxicity

on the host BCIRL-HzAM1 cells but not on the host Vero cells. Representative examples of the viral plaques, host cells, and cells showing toxic effects to disinfectants are shown in Figures 4–6, respectively. The exposures exhibiting the most variation among the viruses are shown graphically in Figures 7–10.

Table 3. Effects of Disinfectants Upon Infectivity of the Virus[a]

Disinfectant and Time of Exposure	***Variola Virus*[b,c]**	**VACV**	**HzSNPV**
Ethanol, 70%			
1 min	0/3	0/9	0/9
45 s	N/A	0/9	9/9
30 s	N/A	0/9	9/9
Ethanol, 40%		See Figure 7	
Ethanol, 30%			
5 min	3/3	9/9	9/9
3 min	3/3	9/9	9/9
1 min	3/3	9/9	9/9
Isopropanol, 50%			
1 min	0/3	0/9	0/9
45 s	N/A	0/9	9/9
30 s	N/A	0/9	9/9
Isopropanol, 30%		See Figure 8	
Isopropanol, 20%			
5 min	3/3	9/9	9/9
3 min	3/3	9/9	9/9
1 min	3/3	9/9	9/9
Sodium hypochlorite, 2%			
1 min	0/3	0/9	0/9
45 s	N/A	0/9	0/9
30 s	N/A	0/9	0/9
Sodium hypochlorite, 1%			
1 min	0/3	0/9	0/9
45 s	N/A	0/9	8/9
30 s	N/A	0/9	9/9
Sodium hypochlorite, 0.5%		See Figure 9	
Formaldehyde, 5%, 4%, 3%, 2%		Data not shown	
Formaldehyde, 1%, 0.05%		See Figure 10	
Phenol, 2%			
2 min	N/A	N/A	0/9
1 min	0/3	0/9	9/18
45 s	N/A	9/9	9/9
30 s	N/A	9/9	9/9
Phenol, 1%			
5 min	N/A	9/9	9/9
3 min	N/A	9/9	9/9
1 min	3/3	N/A	N/A

(continued)

Table 3. Effects of Disinfectants Upon Infectivity of the Virus (continued)

Disinfectant and Time of Exposure	*Variola Virus*[b,c]	VACV	HzSNPV
CAC–BC, 2%			
5 min	0/3	0/9	Toxic
3 min	N/A	0/9	Toxic
1 min	N/A	0/9	Toxic
CAC–BC, 0.1%			
5 min	0/3	1/9	3/9
3 min	0/3	5/9	11/12
1 min	3/3	9/9	9/9
CAC–BC, 0.05%			
5 min	0/3	0/9	9/9
3 min	N/A	0/9	12/12
1 min	N/A	0/9	11/11
IT, 0.05%			
5 min	3/3	9/9	9/9
3 min[d]	3/3	9/9	9/9
1 min[d]	3/3	9/9	9/9
TAB, 2%			
1 min	0/3	0/9	Toxic
45 s	N/A	1/9	Toxic
30 s	N/A	3/9	Toxic
TAB, 0.5%			
5 min	0/3	3/9	Toxic
3 min	2/3	5/9	Toxic
1 min	2/3	5/9	Toxic
TAB, 0.25%			
5 min	0/3	9/9	Toxic
3 min	3/3	9/9	Toxic
1 min	3/3	9/9	Toxic

[a]All data are reported as number of plates (wells) showing plaques/number plates (wells) inoculated.
[b]All data for testing the *Variola virus* were from Tanabe and Hotta (8).
[c]Bandung strain virus: 10^7 pfu/mL on Jinet cell monolayer.
CAC–BC, cetyltrimethylammonium chloride and benzalkonium chloride mixture.
IT, iodine/Tergitol mixture.
N/A, test not performed.
TAB, tetradecyltrimethylammonium bromide.
Toxic, chemical was toxic to cell line.

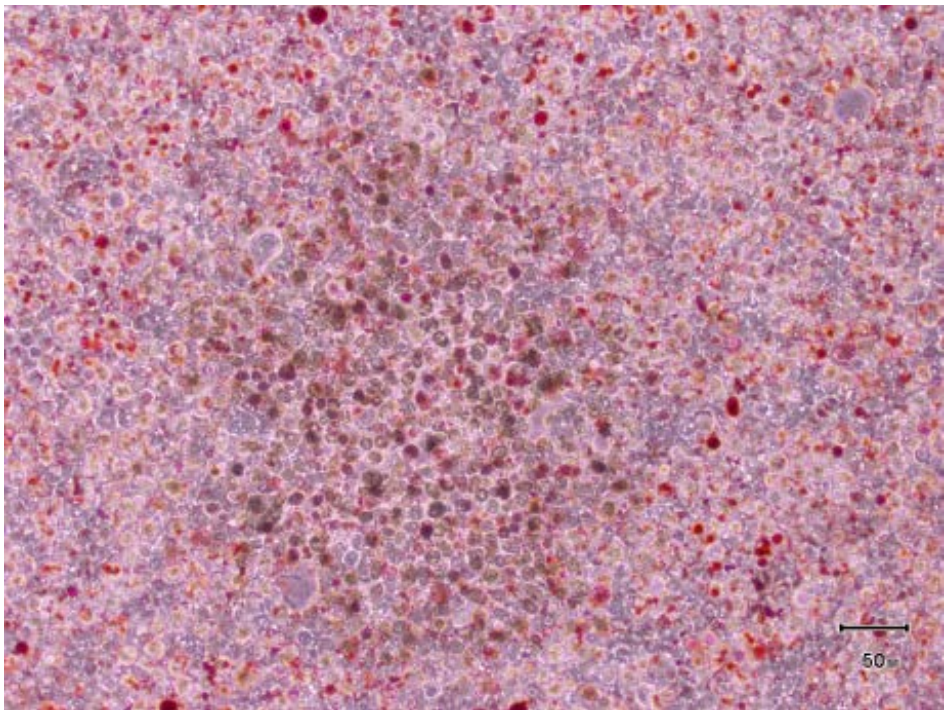

Figure 4. Light microscope image of a representative plaque seven days post-infection indicates HzSNPV resistance to 30% ethanol. Note the dark-colored cells defining the plaque.

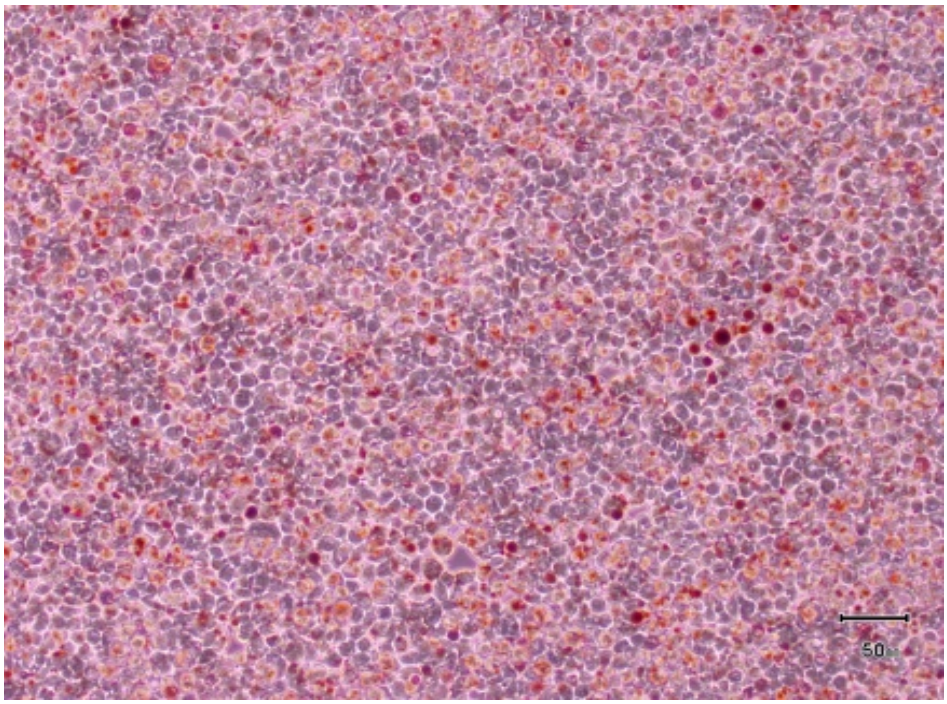

Figure 5. Light microscope image representative of *Helicoverpa zea* cells seven days post-infection with HzSNPV treated with 2% sodium hypochlorite. No plaques were visible, indicating the effectiveness of the treatment.

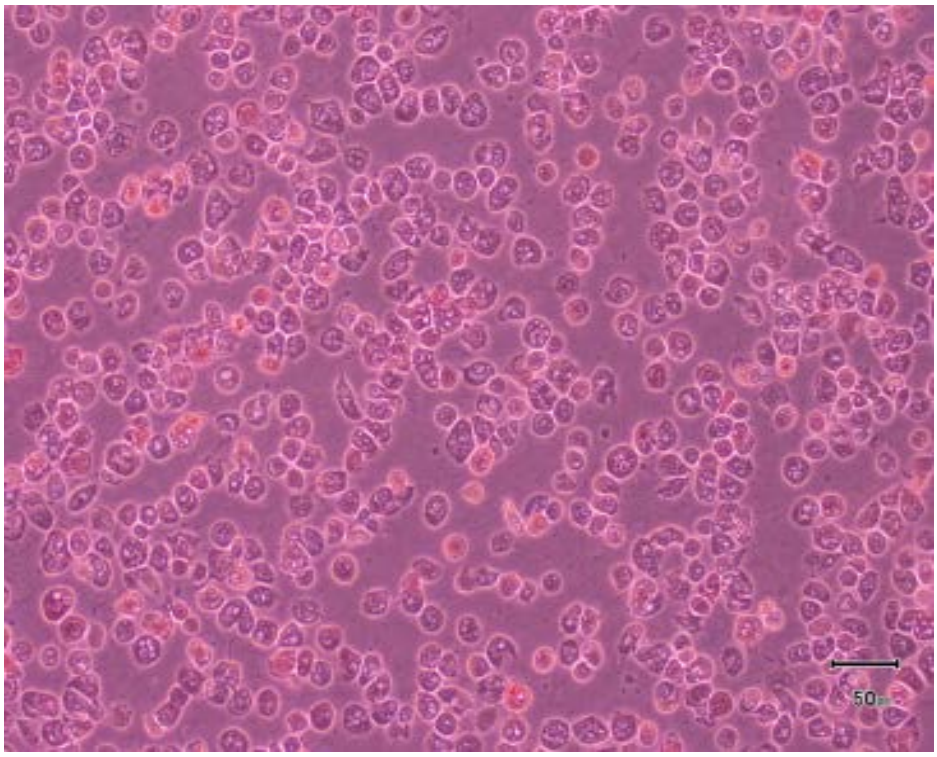

Figure 6. Light microscope image representative of *Helicoverpa zea* cells seven days post-infection with HzSNPV treated with 2% cetyltrimethylammonium chloride and benzalkonium chloride mixture. Decreased cell number and abnormal morphology indicate the toxicity of the treatment.

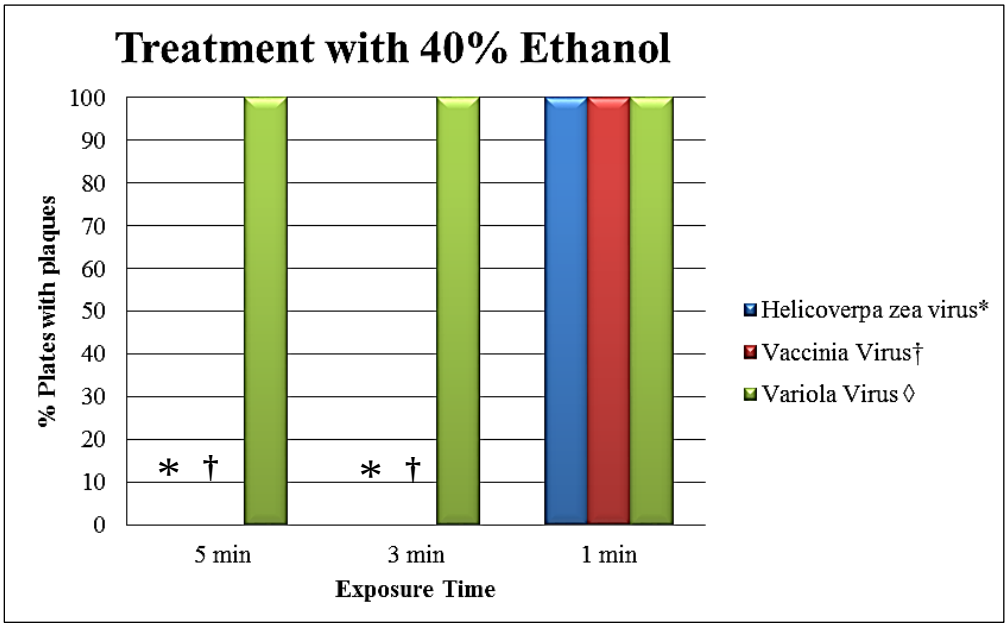

Figure 7. Graphical representation of the percentage of plates with visible plaques in the cells infected with 40% ethanol-treated virus.

In all plates containing plaques, the resistance of the virus to 40% ethanol exposure was indicated. In Figure 7, * represents a zero value for HzSNPV, and † represents a zero value for VACV, indicating the susceptibility of the virus to 40% ethanol exposure. Data for *Variola virus* were from Tanabe and Hotta (8).

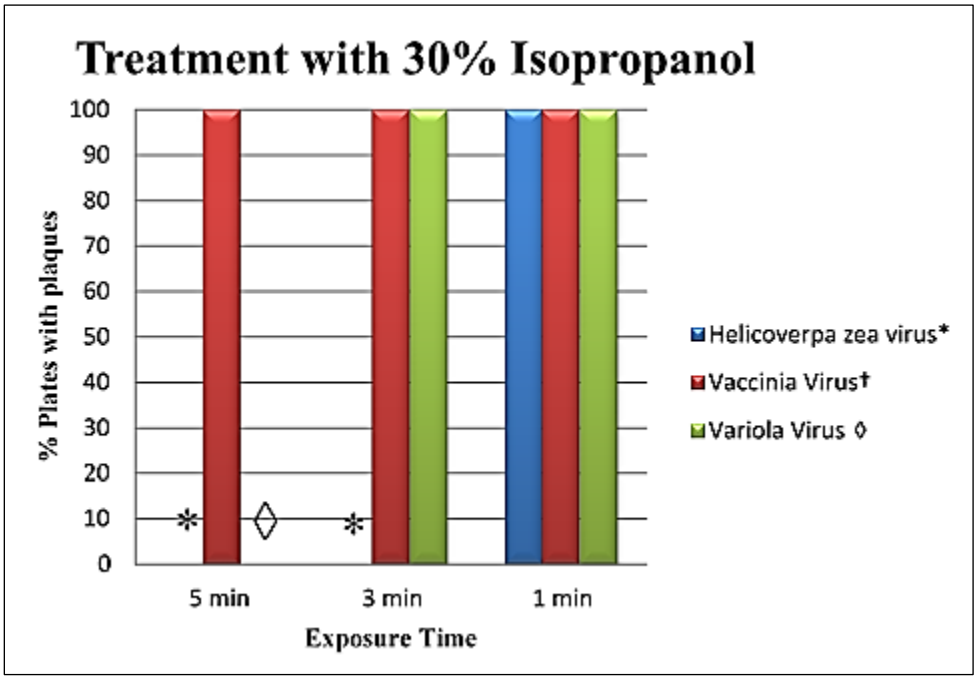

Figure 8. Graphical representation of the percentage of plates with visible plaques in the cells infected with 30% isopropanol-treated virus.

In all plates containing plaques, the resistance of the virus to 30% isopropanol exposure was indicated. Data for *Variola virus* were from Tanabe and Hotta (8). In Figure 8, * represents a zero value for HzSNPV, and ◊ represents a zero value for *Variola virus*.

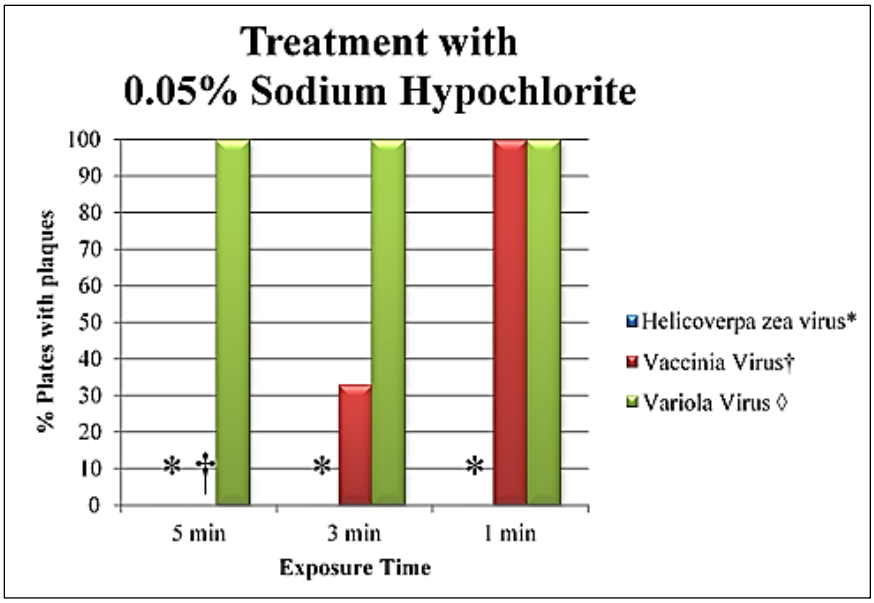

Figure 9. Graphical representation of the percentage of plates with visible plaques in the cells infected with 0.05% sodium hypochlorite-treated virus.

In all plates containing plaques, the resistance of the virus to 0.05% sodium hypochlorite exposure was indicated. Data for *Variola virus* were from Tanabe and Hotta (8). In Figure 9, * represents a zero value for HzSNPV, and † represents a zero value for VACV, indicating the susceptibility of the virus to 0.05% sodium hypochlorite exposure.

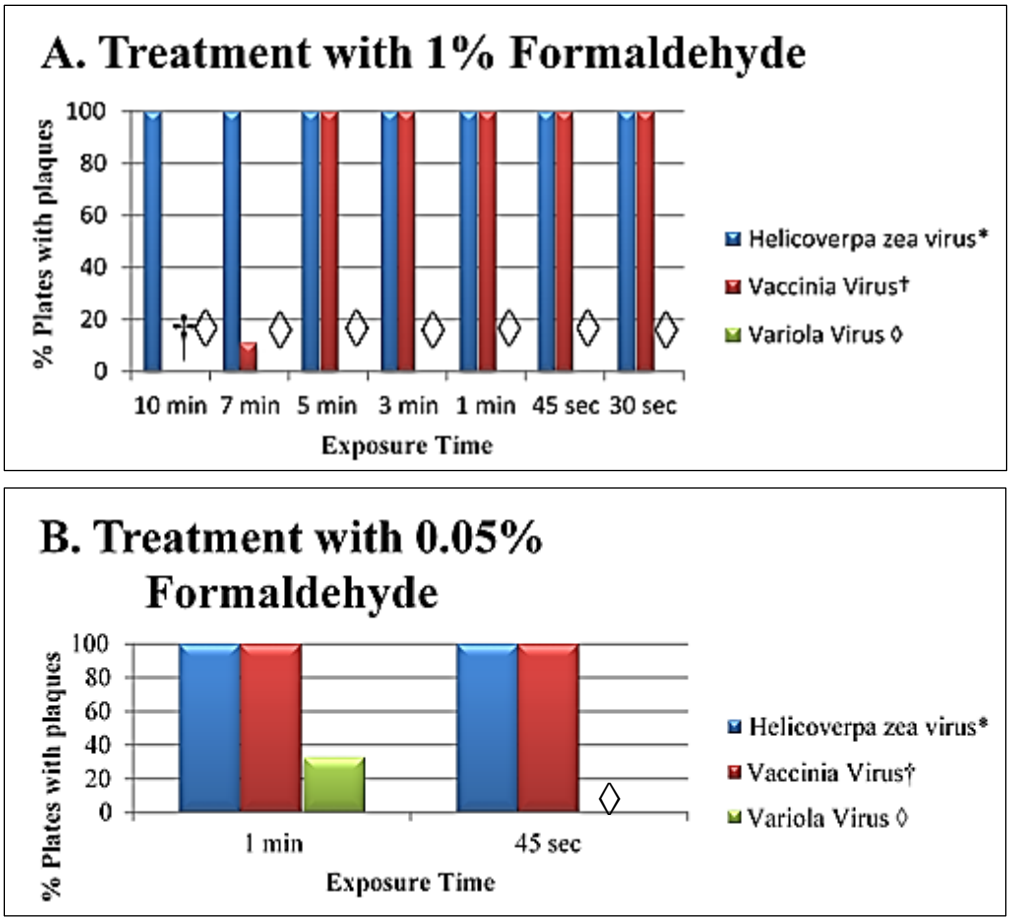

Figure 10. Graphical representation of the percentage of plates with visible plaques in the cells infected with formaldehyde-treated virus. (A) virus treated with 1% formaldehyde; (B) virus treated with 0.05% formaldehyde.

In all plates containing plaques, the resistance of the virus to formaldehyde exposure was indicated. Data for *Variola virus* were from Tanabe and Hotta (8). In Figure 10, † represents a zero value for VACV, and ◊ represents a zero value for *Variola virus* (8), indicating the susceptibility of the virus to formaldehyde exposure.

3. CONCLUSIONS

The results showed that HzSNPV closely mimics the response of VACV across a range of disinfectant solutions and, although there were some differences with the historical *Variola virus* data, HzSNPV could serve as a useful simulant for studying decontamination of the orthopox family of viruses. As shown in Table 2, however, some of the aldehyde and detergent solutions were found to be toxic to the BCIRL-HzAM1 host cell line. We conclude that the HzSNPV exhibits sufficient biophysical and chemically reactive similarities to the orthopox virus to replace MS2 as the viral simulant standard for double-stranded DNA viruses. The ability to safely release HzSNPV in the environment makes it even more attractive as a simulant for expediting the research, development, test, and evaluation of decontamination, detection, and physical protection material.

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ACRONYMS AND ABBREVIATIONS

BCIRL-HzAM1	*Helicoverpa zea* insect cell line
BSC	biosafety cabinet
CAC–BC	cetyltrimethylammonium chloride and benzalkonium chloride mixture
ds	double strand
FBS	fetal bovine serum
HzSNPV	*Helicoverpa zea* single-capsid nucleopolyhedrosis virus
IT	iodine/Tergitol mixture
MEM	Eagle's minimum essential medium
NR	neutral red
pfu	plaque-forming unit
RT	room temperature
ss	single strand
TAB	tetradecyltrimethylammonium bromide
VACV	*Vaccinia virus*

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