Using 96-well tissue culture polystyrene plates and a fluorescence plate reader as tools to study the survival and inactivation of viruses on surfaces

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Abstract A method for studying the behavior of viruses on surfaces has been developed and is illustrated by determining the temperatures that inactivate adsorbed viral hemorrhagic septicemia virus (VHSV) and the concentration of 1-propanol that disinfected surfaces with adsorbed VHSV and chum salmon virus (CSV). VHSV is a rhabdovirus; CSV, a reovirus, and they were detected with two fish cell lines, EPC and CHSE-214, respectively. When polystyrene tissue culture surfaces were incubated with virus, rinsed, and left to dry, they still supported the attachment and spreading of cell lines and after 7 days these cells showed the characteristic CPE of the viruses. Thus cells appeared to be infected directly from surfaces on which viruses had been adsorbed. Applying this property to 96-well plates allowed duplicate surfaces to be examined for their infectiousness or support of CPE. For each treatment 80 replicate surfaces in a 96-well plate were tested at one time and the results expressed as the number of wells showing CPE. VHSV adsorbed to polystyrene was inactivated by drying in the dark at temperatures above 14 °C, but remained infectious for at least 15 days of drying at 4 °C. For chemical sterilization of polystyrene surfaces with adsorbed virus, disinfection was achieved with 1-propanol at 40% for

VHSV and at 60% for CSV. As CPE can be conveniently monitored in 96-well plates with a fluorescence plate reader, this method can be used to rapidly evaluate a variety of treatments for their ability to inactivate surface-bound viruses.

Keywords Viral adsorption · Surface disinfection · Viral inactivation · Fomites · Fish · VHSV · CSV

Introduction

The behavior of viruses on the surfaces of man-made objects is a long-established concern in the disciplines of public health and animal husbandry. The relationships between viruses and surfaces are complex, making the establishment of standard methods for studying them difficult (Bellamy 1995). Different approaches have been used to investigate virus survival on the surfaces of many potential fomites, such as banknotes, dishpans, doorknobs, and desktops that have surfaces constructed from a range of material, such as paper, plastic, and stainless steel (Bean et al. 1982; Terpstra et al. 2007; Thomas et al. 2008). The concerns of poultry industry, as an example of animal husbandry, have led to studies on the survival of viruses on objects with surfaces of glass (windowpane), polyethylene (sacks), vinyl (gloves), metal (food hopper), and rubber (boots) (Savage and Jones 2003). Pathogen survival on

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surfaces has led to the study of disinfection and the search of chemicals that can be used to disinfect surfaces. Disinfectant tests can be generally grouped into four categories: carrier tests, suspension tests, capacity tests and practical tests (Reybrouck 1998). In the practical test, an inoculum of a pathogen is dried onto a solid surface, such as a piece of tile, stainless steel disc or microscope slide. A disinfectant is then applied over the inoculum on the contaminated surface for a certain exposure time. The amount of infectious pathogen is determined either by elution, then infection of susceptible cells, or by direct contact with susceptible cells on a different vessel (Reybrouck 1998).

The dilemma of viruses surviving on surfaces needs additional investigative approaches and one possibility is to focus the technologies of cell culture in unique ways to the problem. This is the overarching aim of the current work. Two fish viruses, viral hemorrhagic septicemia virus (VHSV) of the rhabdoviridae and chum salmon virus (CSV) of the reoviridae, have been used together with susceptible fish cell lines, EPC and CHSE-214 respectively, as the model systems. The first goal was to determine whether cells could be infected from tissue culture polystyrene surfaces to which viruses had been adsorbed, rinsed, and dried. The second goal was to use the adsorption of viruses to 96-well polystyrene plates to develop a rapid quantitative procedure to compare treatments for their ability to kill viruses on surfaces. The procedure has been used successfully to evaluate drying at different temperatures on VHSV inactivation and of 1-propanol as a disinfectant for surfaces with VHSV and CSV, but should be useful generally for any animal or human virus where the emphasis is more on the type of virus than the type of surface. In the course doing this work, original observations have been made about fish viruses. This is the first demonstration that fish viruses can survive dry on surfaces.

Materials and methods

Propagation of cells and viruses

The VHSV strain, U13653, used in this study is a type IVb that was isolated from Lake Ontario, Canada (Lumsden et al. 2007) and was obtained

from Dr J. Lumsden of the University of Guelph. The CSV strain used in this report was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The fish cell lines, EPC (*epithelioma papulosum cyprini*), which is now described by ATCC as being from fathead minnow, and CHSE-214, from Chinook salmon embryo, were used to produce and detect VHSV and CSV, respectively (Fijan et al. 1983). EPC and CHSE-214 were grown routinely in Leibovitz's L-15 medium (Fisher Scientific, Ottawa, ON) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Oakville, ON) and 1% penicillin–streptomycin (P–S) (Sigma-Aldrich, Oakville, ON). The growth temperatures were room temperature for EPC and 18 °C for CHSE-214. VHSV was propagated on confluent EPC monolayer at 14 °C in 75 cm² flasks (BD Biosciences, San Jose, CA) containing fresh L-15 with 2% FBS and 1% P–S (FBS/L15), while CSV was also propagated in 75 cm² flasks on CHSE-214 monolayers at 18 °C in L-15 with 5% FBS and 1% P–S. Supernatant containing the viruses from the inoculated flasks was collected after 10 days of incubation, pooled, and then centrifuged at >3000×g for 5 min. After centrifugation, the supernatant was syringe filtered through a 0.2 micrometer filter (Pall Corporation, Port Washington, NY), titrated, and stored at –80 °C. Viral titer was expressed as the tissue culture infectious dose (TCID₅₀ mL^{–1}), which was calculated by the Karber method (Karber 1931).

Surface adsorption of virus on plastic Petri dishes

Polystyrene 60 mm Petri tissue culture dishes (Corning Inc., Corning, NY) were seeded with 400 µL of stock VHSV (3.15 × 10⁹ TCID₅₀ mL^{–1}) and 6 mL of 2% FBS/L15 to make a final titer of 2.10 × 10⁸ TCID₅₀ mL^{–1}. Control dishes contained only 6.4 mL of 2% FBS/L15. The dishes were incubated at 14 °C for 24 h to allow VHSV to settle and to potentially attach to the surface of the dishes. After the initial incubation, medium from the dishes was removed and the dishes were washed with 6 mL of Dulbecco's phosphate buffered saline (D-PBS) (Sigma-Aldrich, Oakville, ON). Following the removal of the saline wash buffer, the dishes now potentially contained surface-bound VHSV.

Determining infectiousness of surface-adsorbed virus on plastic Petri dishes

This assay involves two different conditions: dry and wet. For both conditions, dishes were prepared with surface-bound VHSV as described in “[Materials and methods](#)”. In the dry condition, the dishes were immediately incubated to dry, after rinsing with D-PBS, at room temperature in the dark for 1, 6, 10, and 15 days. For the wet condition, 6 mL of 2% FBS/L15 were added after rinsing, then the dishes were incubated “wet” in the dark at room temperature for 1, 6, 10, and 15 days. For the dry condition, at each of the time points, 6 mL of 2% FBS/L15 containing

3.0×10^6 EPC cells were added to each dish. For the wet condition, the old medium was removed before similar addition of EPC cells. All dishes were then incubated at 14 °C for 7–10 days to allow for VHSV to infect EPC cells and develop cytopathic effect (CPE). The CPE was observed through an inverted Nikon Eclipse TS-100 microscope.

Surface adsorption of virus on 96-well plates

An overview of the procedures with 96-well plates (BD Biosciences, San Jose, CA) is shown in Fig. 1 with adsorption being outlined in steps 1–4 but fully described here. Each 96-well plate is divided into the

Fig. 1 Overview of the 96-well plate direct cellular detection method (DCDM). The arrangement of the 96-well plates and the broad flow of procedures for the adsorption, treatment, and detection of the virus are outlined. The 4 steps for adsorption are shown in the *top row* and are labeled 1–4 in *brackets* and described in more detail on the *right hand side* of the figure. At

the end of adsorption (*step 5* in the *middle* of the diagram), the plate can be used to study disinfection (*steps A1–A4*) and either dry (*D1*) or wet inactivation (*W1–W3*). After the experimental treatment, detection of virus on the surface of the wells is done using EPC cells as illustrated in the *bottom row* of the diagram and as outlined in *steps 6–9* on the *right*

control section and the virus exposed section; columns 1 and 2 (a total of 16 wells) of the 96-well plate represent the control section and columns 3–12 (a total of 80 wells) represent the virus exposed section. Stock VHSV (3.15×10^9 TCID₅₀ mL⁻¹) or CSV (1.04×10^8 TCID₅₀ mL⁻¹) was diluted to 10^{-2} in 2% FBS/L15 to 3.15×10^7 TCID₅₀ mL⁻¹ and 1.04×10^6 TCID₅₀ mL⁻¹, respectively. To each well in columns 3–12 of each plate (80 exposed well), 100 µL of the diluted viruses (either VHSV or CSV) was added. To each well in columns 1–2 of each plate (16 control wells), 100 µL of 2% FBS/L15 (no virus) was added. All plates were then incubated at 14 °C for 24 h to allow for the virus to settle and potentially bind to the bottom or wall of the wells. After 24 h, old medium from the plates were removed and the wells in each plate were washed with 100 µL of D-PBS, followed by removal of wash solution. Control wells and exposed wells on the same plate were separately sealed, with 96-well foil seals (ATCC Bioproducts, Manassas, VA), and washed to prevent cross contamination. Removal of wash solution from 96-well plate was done by blotting the plates on paper towels. The exposed wells now potentially contained surface-bound VHSV or CSV.

Inactivation of adsorbed virus on 96-well plates with time, temperature and drying

This procedure is schematically illustrated and briefly described in Fig. 1, with inactivation on dry and wet surfaces shown in steps D1 and W1–W4, respectively. In the dry condition, 96-well plate containing surface-bound VHSV (from “[Surface adsorption of virus on 96-well plates](#)”), were immediately left to dry in the dark at 4 °C, 14 °C, room temperature, 26 and 37 °C for 1, 6, 10, and 15 days. For the wet condition, 100 µL of 2% FBS/L15 were added to each well of 96-well plates containing surface-bound VHSV before the plates were incubated at identical temperatures and times as in the dry condition. At each time point, day 1, 6, 10 and 15, EPC cells were added to each well in the dry condition to detect infectious VHSV (“[Detection of adsorbed virus in 96-well plates with EPC cells](#)”), while in the wet condition, the old medium was removed, from the control and exposed columns separately with foil seals, before addition of EPC cells.

Disinfection of adsorbed virus on 96-well plates using 1-propanol

This procedure is schematically shown and briefly described in Fig. 1 steps A1–A4. Low and high titer VHSV or CSV were surface bound to wells of 96-well plates as previous described in “[Surface adsorption of virus on 96-well plates](#)”, then left to dry in the dark at room temperature for 1 day. After 1 day of drying, 100 µL of the following concentrations, 0, 5, 10, 15, 20, 40, 60 and 80%, of 1-propanol (Fisher Scientific, Ottawa, ON) diluted in D-PBS, were added to each well of replicate 96-well plates. The wells were incubated with the 1-propanol solutions for approximately 10 min before the solutions were removed, and each well was rinsed with 100 µL of D-PBS. After removal of the wash buffer, EPC cells were added to each well of the plates to detect remaining infectious VHSV (“[Detection of adsorbed virus in 96-well plates with EPC cells](#)”).

Detection of adsorbed virus in 96-well plates with EPC cells

Detection is briefly described in steps 6–9 of Fig. 1 and more completely here. EPC or CHSE-214 cells, approximately 50,000 in 10% FBS/L15, were added into each well at a volume of 100 µL and allowed to attach and spread at 14 °C over surfaces to which the virus had potentially adsorbed. The plates were incubated at 14 °C (VHSV) or 18 °C (CSV) for 7–10 days to allow CPE to develop. Each plate was then scored for the number of viral exposed wells containing CPE (out of 80 maximum wells) either by visual observation or by the alamar Blue assay (“[Alamar Blue assay for scoring cytopathic effect \(CPE\) positive wells in 96-well plates](#)”).

Estimation of the amount of attached virus and determination of threshold amount required for cytopathic effect (CPE)

To estimate the amount of VHSV attached to the well surface of 96-well plates, an indirect approach was used. In the procedure to seed the virus, the following steps occurred: (1) known amount of VHSV was added to the wells, (2) after allowing for 24 h of attachment, the initial viral solution was removed (removed portion), (3) after removal of the viral

solution, the well was washed with D-PBS (washed portion). The amount of virus that remains in the well after washing is the attached amount. The amount of virus in the “removed portion” and “washed portion” was determined using TCID₅₀ and is known as the “removed amount” and “washed amount”, respectively. The amount of virus attached was indirectly determined by taking the initial known amount and subtracting from it the removed and washed amount. The amount of virus attached was determined from using a range of dilution as the initial known amount (Table 1), for each dilution there are 3 replicate wells. To determine the threshold amount of VHSV required to produce CPE in host cells, a known titer of VHSV (1.04×10^8 TCID₅₀ mL⁻¹) was serially diluted tenfold and each dilution was added to 4 replicate wells in a 96-well plate. The plate was incubated at 14 °C for 7 days; afterward, the number of wells containing CPE for each dilution was counted.

Alamar Blue assay for scoring cytopathic effect (CPE) positive wells in 96-well plates

A high-throughput alamar Blue (AB) assay (Invitrogen, Burlington, ON) for evaluating CPE in multiwell plates that was described by Mo et al. (2008) was used with some modifications. Alamar Blue solution contains resazurin that can be reduced by fish cells during energy metabolism. The product of that reduction is resorufin, a fluorescent molecule that is detected in the assay. The healthier the cell monolayer the more resazurin is converted into resorufin resulting in a high fluorescent reading. Cell monolayers that are destroyed by virus will produce

significantly lower reading. Before assaying each 96-well plate with alamar Blue the old medium in each plate was removed by inverting the plate and blotting briefly on a paper towel. The wells in each plate were then washed with 100 µL of D-PBS per well after which 100 µL of AB working solution (5% v/v in D-PBS) was added to each well. The plates were then incubated at room temperature in the dark and after 1 h read with either the Molecular Devices SpectraMax GEMINI XS or the Perseptive Biosystems Cytofluor fluorescent plate reader at an excitation wavelength of 530 nm and emission wave length of 580 nm. The resulting output of the assay is given in relative fluorescent unit (RFU). The RFUs of the control wells in each plate were averaged. The RFU of each exposed well was compared to the average RFU values of the control wells and if the RFU value of an exposed well is lower than 10% of the control average (the cut-off criteria), the well was scored positive for infectious virus.

Results

Infectiousness of virus adsorbed to plastic surfaces in dry and wet environment

Traditionally, the study of viral inactivation on dry surfaces generally requires the elution of viruses from surfaces. However, differing and optimal solutions for each type of virus is required to ensure maximum recovery. The elution process itself would expose the virus to additional air–liquid–solid interfaces which has been previously shown to inactivate viruses (Thompson and Yates 1999). Therefore, the recovery

Table 1 Estimating the amount of attached VHSV in wells of 96-well plates

Initial amount (IA) TCID ₅₀ mL ⁻¹	Mean removed amount (RA) TCID ₅₀ mL ⁻¹	Mean washed amount (WA) TCID ₅₀ mL ⁻¹	Mean attached amount (AA)* TCID ₅₀ mL ⁻¹
4.89×10^9	2.40×10^8	4.43×10^6	4.65×10^9
4.89×10^7	6.86×10^6	2.49×10^5	4.18×10^7
4.89×10^5	2.40×10^5	4.27×10^3	2.45×10^5
4.89×10^3	6.30×10^2	ND**	4.26×10^3

For each initial starting dilution (IA), there are 3 replicate wells. The titers calculated in RA, WA, and AA are average values from 3 replicate wells. The standard deviation is all within 1 log of the average values (data not shown)

* AA = IA – (RA + WA)

** ND no data. The amount of virus is too low to determine the TCID₅₀ mL⁻¹

Fig. 2 Survival of VHSV in dry and wet condition on plastic petri dishes at room temperature. L-15 with 2% FBS (2% FBS/L15) was added either without VHSV as controls (a) and (d), or with VHSV, (b), (c), and (e) on plastic dishes. a–c Were left to dry, while d and e were incubated in 2% FBS/L15 (wet) at room temperature. Control dishes, (a) and (d), shows completely intact EPC monolayer. Plastic dishes containing adsorbed VHSV that was dried remained infectious for at least

6 days (b), while the wet counterpart remained infectious for at least 15 days (e). Cytopathic effect can be seen as either holes in monolayer (b) or complete destruction of monolayer (e) when compared to controls. The plastic dishes that were dried with adsorbed VHSV for 10 days were no longer infectious, as can be seen by perfectly intact EPC monolayers in (c). The scale bar represents 100 μ m

of infectious virus will be underestimated. The other disadvantage to methods that require elution is that the process of determining the optimal solution can be time consuming and laborious. A more robust method for detecting viruses on surfaces was sought. To this end, EPC cells were plated directly onto plastic dishes that had been incubated in FBS/L15 with VHSV or without (control), rinsed, and dried for 1, 6, 10 or 15 days at room temperature. When the virus was dried on plastic dishes, the plastic surface remained consistently infectious for up to 6 days, leading to destruction of the EPC monolayer (Fig. 2b). The monolayer destruction had the characteristic appearance of VHSV CPE and transferring medium from cultures undergoing this destruction to other cultures caused CPE. Therefore, surface-bound VHSV was able to infect EPC cells. By the 10th day of drying, VHSV was completely inactivated so the plastic surface was not infectious and EPC monolayer remained intact (Fig. 2c). By contrast, when the polystyrene surface was kept wet by the presence of FBS/L-15, the surface was still infectious even after

15 days at room temperature (Fig. 2e). These results show that at room temperature some VHSV survive on polystyrene surfaces in a dry state for almost 1 week and in a wet state for several weeks.

The above result suggests a very simple technique for adhering viruses to a solid surface which can be studied under various conditions. Cells are plated directly onto a surface on which virus has been applied and allowed to develop CPE. If CPE fails to develop, the surface must contain either no infectious virus or an amount below a threshold required for infection and is scored minus for infectivity. If CPE develops, the surface must contain enough virus (a threshold) to infect the added cells and is scored plus for infectivity. The virus would infect cells either directly from the surface and/or indirectly from the medium after being released from the surface. This method allows the infectivity ($\pm$) of a surface to be compared with time after viral adsorption. Furthermore, the use of 96-well plates provide a more quantitative study of viral adsorption to surfaces by conveniently allowing multiple replications for a

Table 2 Determining the threshold level of VHSV required to cause cytopathic effect in wells with EPC cells

Titer (TCID ₅₀ mL ⁻¹)	Mean (±SD) number of wells with CPE out of 4	Mean % of infection*
1.04×10^7	4.00 ± 0.00	100%
1.04×10^6	4.00 ± 0.00	100%
1.04×10^5	4.00 ± 0.00	100%
1.04×10^4	4.00 ± 0.00	100%
1.04×10^3	4.00 ± 0.00	100%
1.04×10^2	4.00 ± 0.00	100%
1.04×10^1	1.75 ± 0.95	44%
1.04×10^0	0.50 ± 0.57	13%

The threshold for complete infection of all wells is at 1.04×10^2 TCID₅₀ mL⁻¹. At 1.04×10^1 TCID₅₀ mL⁻¹ and below, CPE occurred in less than 50% of the exposed well. The average value is taken from 4 replicates plates with each replicate involving 4 wells

* The mean % of infection is calculated by taking the mean number of wells with CPE and divide by 4, multiply by 100%. The number is rounded to the nearest percentage

single condition within an individual experiment. A protocol for this was developed with 96-well plates (Fig. 1). The amount of VHSV attached to the wells of 96-well plate at various initial seeding titers was indirectly estimated (Table 1); at all the initial seeding titers, the attached amount is all within the same exponential power as the initial seeded amount. Once the initial attached titer was established, we proceeded to determine the threshold or minimum amount of virus required to cause CPE in reporter cells in wells as that information, along with the initial attached titer, is subsequently used to determine the amount of virus inactivated (log reduction value) by particular treatments. If a treatment prevented CPE in a well then the amount of infectious virus in that well has been reduced to below the threshold amount. The threshold amount is defined as the lowest virus titer, TCID₅₀ to the nearest exponential power, that can consistently cause CPE in 100% of exposed wells. To determine that amount, stock VHSV was serially diluted tenfold, and added to wells containing reporter cells to find the lowest dilution that can infect all of the examined wells. The threshold amount of VHSV was determined, to the nearest exponential, to be approximately 1.04×10^2 TCID₅₀ mL⁻¹; a further tenfold dilution of the virus, on average, resulted in less than 50% of the exposed well showing CPE (Table 2).

In “Inactivation of adsorbed virus with time, temperature and drying” below, for VHSV only, the plates were incubated at different temperatures either with FBS/L15 (wet) or without (dry) for up to 15 days; whereas, in “Disinfection of absorbed virus”, for both VHSV and CSV, the plates were dried for only 24 h before being treated with different concentration of 1-propanol. As a proof of principle, the temperature inactivation of VHSV and the disinfection of VHSV and CSV by 1-propanol have been studied.

Inactivation of adsorbed virus with time, temperature and drying

The inactivation of adsorbed virus with time and with temperature on either wet or dry plastic surfaces was compared using 96-well plates. Wet surfaces remained infectious for at least 15 days at 4 °C, 14 °C, room temperature, and 26 °C, whereas at 37 °C inactivation was seen as early as 1 day (Table 3). On a dry or drying surface, adsorbed virus remained infectious for all 15 days at 4 °C, but as the temperature was raised inactivation became evident and the higher the temperature the more quickly did inactivation develop. At 14 °C, room temperature, 26 °C, and 37 °C, dry inactivation became evident at 15, 10, 6, and 1 day(s), respectively (Fig. 3).

Disinfection of absorbed virus

VHSV or CSV at low and high titer were adsorbed to 96-wells plates and the ability of different concentrations of 1-propanol (0.0, 5, 10, 20, 40, 60 and 80%), to disinfect surfaces with adherent virus was examined. At low VHSV titer (1.04×10^3 TCID₅₀ mL⁻¹), 1-propanol concentration of 10% was enough to disinfect all 80 viral exposed well to allow EPC cells to develop a monolayer without the appearance of CPE. EPC cultures in low titer VHSV wells that had been treated with 0.0–5% 1-propanol were destroyed, with the damage characteristic of VHSV CPE (Table 4). At high VHSV titer (3.15×10^7 TCID₅₀ mL⁻¹), 40% concentration of 1-propanol was needed to disinfect all 80 viral contaminated wells. For both low and high titer CSV, 1.08×10^3 TCID₅₀ mL⁻¹ and 1.04×10^6 TCID₅₀ mL⁻¹, respectively, 1-propanol concentration of at least 60% was required to disinfect more than 95% of the wells

Table 3 Ability of plastic wells to remain infectious after adsorption of VHSV, rinsing, and incubation for up to 15 days either dry or wet at various temperatures

Time after viral adsorption ^a & before EPC addition ^b	Mean ($\pm$ SD) number of wells that developed CPE ^c									
	4 °C		14 °C		RT ^d		26 °C		37 °C	
	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet
Day 1	80 $\pm$ 0	80 $\pm$ 0	80 $\pm$ 0	80 $\pm$ 0	80 $\pm$ 0	80 $\pm$ 0	80 $\pm$ 0	80 $\pm$ 0	3* $\pm$ 5	19* $\pm$ 5
Day 6	80 $\pm$ 0	80 $\pm$ 0	80 $\pm$ 0	80 $\pm$ 0	76 $\pm$ 6	80 $\pm$ 0	41* $\pm$ 36	80* $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
Day 10	80 $\pm$ 0	80 $\pm$ 0	66 $\pm$ 18	80 $\pm$ 0	34* $\pm$ 18	80* $\pm$ 0	0* $\pm$ 0	80* $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
Day 15	67 $\pm$ 23	80 $\pm$ 0	39* $\pm$ 36	80* $\pm$ 0	9* $\pm$ 16	80* $\pm$ 0	0* $\pm$ 0	79* $\pm$ 2	0 $\pm$ 0	0 $\pm$ 0

For each temperature condition, 3 duplicate plates were performed

* The asterisks identify pairs of wet and dry values that were significantly different from each other as determined by ANOVA followed by the Bonferroni Multiple Comparison test ($p < 0.05$)

^a 80 wells in a 96-well plate were allowed to adsorb VHSV, rinsed, and incubated at various temperatures either dry or wet as described in the M & M

^b At various times afterwards, EPC cells were added and the plate incubated at 14 °C to allow the development of cytopathic effect (CPE)

^c CPE was scored 10 days later as described in M & M

^d RT stands for room temperature

Fig. 3 Retention of infectious surface-bound VHSV by polystyrene subjected to 15 days of drying at various temperatures. After 80 wells per plate had been allowed to adsorb VHSV at 14 °C for 24 h, the plates were washed, and allowed to dry at different temperatures for 15 days. At this point EPC cells in medium were added to the wells and the plates were returned to 14 °C for 7–10 days after which the wells were scored for CPE

and 80% 1-propanol was required to disinfect all of the wells (Table 4). VHSV appears to be more sensitive to 1-propanol inactivation than CSV. Low titer VHSV and CSV showed less wells with CPE than high titer VHSV and CSV at all concentration of 1-propanol treatment, including the no 1-propanol control (Table 4). 1-propanol, at 40% concentration,

was able to reduce high titer VHSV infection to below threshold (1.04×10^2 TCID₅₀ mL⁻¹) in all 80 exposed well, a 5 log reduction in the amount of virus. However, like more standard disinfection studies, in which the virus is eluted before quantification, log reduction values obtained by this new method should still be treated with caution as the

Table 4 Effect of 1-propanol treatments on the infectiousness of plastic wells after they had adsorbed VHSV or CSV and been rinsed and dried

1-propanol treatment ^a	Number of wells with adsorbed VHSV or CSV ^b that develop CPE ^c Means ($\pm$ SD)			
	VHSV		CSV	
	Low titer	High titer	Low titer	High titer
0% (control)	40.5 $\pm$ 3.5	80.0 $\pm$ 0.0	55.5 $\pm$ 0.7	80.0 $\pm$ 0.0
5%	23.0 $\pm$ 0.0	80.0 $\pm$ 0.0	44.0 $\pm$ 4.2	80.0 $\pm$ 0.0
10%	0.0 $\pm$ 0.0	80.0 $\pm$ 0.0	56.0 $\pm$ 4.2	80.0 $\pm$ 0.0
15%	0.0 $\pm$ 0.0	80.0 $\pm$ 0.0	55.0 $\pm$ 2.8	80.0 $\pm$ 0.0
20%	0.0 $\pm$ 0.0	14.0 $\pm$ 11.0	52.5 $\pm$ 0.7	80.0 $\pm$ 0.0
40%	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	6.0 $\pm$ 1.4	10.0 $\pm$ 1.4
60%	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	2.0 $\pm$ 0.0	1.5 $\pm$ 0.7
80%	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0

^a 100 μ L of 1-propanol in PBS was applied for 10 min to each well to which either VHSV or CSV had been applied as described in the footnote below

^b 80 wells in a 96-well plate were allowed to adsorb either VHSV (low titer: 1.16×10^3 TCID₅₀ mL⁻¹, high titer: 3.15×10^7 TCID₅₀ mL⁻¹) or CSV (low titer: 1.08×10^3 TCID₅₀ mL⁻¹, high titer: 1.04×10^6 TCID₅₀ mL⁻¹) as described in the M & M, rinsed, and dried at room temperature for 1 day prior to the application of 1-propanol as described in the above footnote

^c After the removal of 1-propanol, each well was rinsed, EPC or CHSE-214 cells added, and CPE scored 7–10 days later as described in M & M

For each 1-propanol concentration, 2 duplicate plates were performed

titres cannot be verified by approaches independent of infection. Regardless, the 96-well plate method was used successfully to identify 1-propanol concentrations that prevented formation of CPE on polystyrene surfaces and is sensitive enough to differentiate the required effective disinfectant concentration between two different types of virus.

Discussion

The purpose of this study was to determine whether the adherence of viruses to tissue culture plastic surfaces could be used to develop procedures that might be generally useful for studying viral inactivation and efficacy of chemical disinfectants on surfaces. A fish rhabdovirus (VHSV) has been used as the study virus, with affirmation of key procedural steps being sought with a member (CSV) of a different viral class, fish reoviruses. Our approach differs from traditional viral inactivation studies, as reviewed by Reybrouck (1998), in three main ways. Firstly in traditional studies a fluid with viruses is dried directly onto the inanimate surface so all the virus in the starting fluid ends up on the surface

whereas here the virus was allowed to attach from a solution onto a surface, followed by rinsing, to remove unbound virus, and drying so any resulting infection would be from surface-bound virus. Secondly, the method of detecting infectious virus on surfaces usually involves eluting the virus from the surface with a solution and applying the eluent to susceptible cells, whereas here the cells are plated onto the virus contaminated surface and become infected from the cells attaching and spreading on the surface. Thirdly, disinfection of surfaces is usually reported as a percent reduction in the amount of elutable infectious virus, whereas here the reduction in the number of infectious surfaces is the efficacy endpoint. Below, the adsorption, detection, disinfection, and inactivation of viruses on plastic surfaces are discussed along with the first demonstration of a fish virus surviving dry for a period of time.

Virus adsorption

The adsorption of the virus would involve interactions between the plastic, the virus, and the associated environment (Gerba 1984). In this case, the associated environment or viral solution was the basal

medium, L-15, with 2% FBS together with some lysate from either EPC or CHSE-214 cells, which were used to produce VHSV or CSV, respectively. Proteins are known to passively adsorb to polystyrene through not only primarily hydrophobic but also electrostatic interactions (Cantarero et al. 1980). In this study, VHSV glycoprotein ectodomain or CSV protein capsid could be binding to the plastic in a similar manner, as would the proteins from the FBS and lysate. Further anchoring of the virus to the surface could be achieved through nonspecific and/or specific interactions between the virus with FBS and lysate proteins binding to the plastic. In the case of VHSV, direct interaction also might occur between the lipid bilayer of the VHSV envelope and polystyrene, which is a possibility suggested by work on the adsorption of lipid vesicles to solid surfaces (Nollert et al. 1995; Pasquardini et al. 2008). Regardless of the way the viruses adsorbed to the surface, the attachment must have been strong enough to withstand the removal of the viral solution and the subsequent washing of the surface with buffered saline. Although the initial demonstration of viruses adsorbing to tissue culture plastic was done many years earlier with an adenovirus of mammals (Inouye 1973), the phenomenon appears to have been little studied since and this is the first report with a fish virus.

Infectiousness of virus adsorbed to plastic surfaces

As mentioned above, adsorbed viruses can be detected by allowing cells to attach and spread over the surface with virus, but the mechanisms responsible for infection from surfaces could be multiple and interacting. Firstly, the cells could participate in the elution and release viruses into the culture medium from where they infect the cells. Secondly, infection could be achieved without viruses ever being detached and released into the medium. Viral receptors on cells would be expected to be more specific and have higher affinity for virus than sites on the polystyrene. The greater affinity of the receptors on the membranes of cells can be envisioned as ‘plucking’ the viruses off the surface. This could begin the moment cells make contact with the surface and continue as they attach and spread. In this scenario the viruses would never be free in suspension but are just being passed like a baton from an inert surface to a living surface.

Only infrequently over the last 50 years has the infectiousness of adsorbed animal viruses been examined *in vitro*, and few if any studies have looked at a contribution from the plastic tissue culture vessel. In past studies, the virus was commonly adsorbed to particulate matter, which was then added to cell cultures. The particulates were natural, such as clay, or man-made, such as plastic, and were incubated with cell monolayers for just a few hours before being removed. Thus unlike the current study, the period of contact with the cells was short and the closeness of contact was unclear. Enteroviruses from mammals were most frequently studied (Lipson and Stotzky 1986; Schaub and Sagik 1975), but a fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV), also has been investigated (Yoshinaka et al. 2000). In all cases adsorbed virus remained infectious and in some cases infectivity seemed to have been enhanced (Lipson and Stotzky 1986). Although the adsorption of viruses on surfaces often leads to inactivation (Bitton 1980), the current study confirms that at least some adsorbed virus is available to infect cells. The infectiousness of adsorbed viruses provides a means of detecting viruses on plastic surface and how this can be used is discussed below.

Inactivation of adsorbed virus with time, temperature and drying

The ability to adsorb virus to 96-well plates provided a useful system for examining the effects of time, temperature, and drying on the inactivation of adsorbed viruses. Adsorbed VHSV remained infectious for at least 15 days if the surface was kept wet and the temperature was 26 °C or less while on a drying surface the virus became inactivated at all temperatures except 4 °C. Whether the wet surface continuously retained all the virus adsorbed during the 24 h adsorption period for the 15 days is unknown, but the old “wetting” medium was removed again after 15 days before the reporter cells were added so the infectiousness of only adsorbed virus was being measured. On the drying surface, inactivation likely arose through denaturation of viral proteins and/or damage to the viral envelope in the case of VHSV, initiated by loss of water together with being adsorbed to a surface. The more rapid inactivation at high temperatures could be due to the interplay of temperature, evaporation rate, and

relative humidity. The wells at low temperatures were visibly moist longer than the wells at higher temperatures, suggesting a higher relative humidity and slower evaporation rate. In the case of phages, higher relative humidity and slower evaporation rates favored survival (Ward and Ashley 1977), whereas for hepatitis A survival was better at lower rather than at higher relative humidity (Mbithi et al. 1991). Thus, the modulation of dry inactivation by environmental factors might depend on the virus type and this 96-well virus adsorption system may be one way of dissecting out the importance of each for a particular animal virus.

The results with VHSV appear to be the first demonstration of a fish virus surviving dry on a surface and also of ultimately being inactivated by drying. The temperatures at which VHSV survived are physiological (4–26 °C) for coldwater fish (Bols et al. 1992) and similar to what has been reported by others for VHSV in suspension. For example, VHSV survived in freshwater for 5 or more days at 25 °C, increasing to more than 25 days at 4 °C. However, the survival of some isolate was much lower in seawater, dropping to around 12 days at 4 °C and about 1 day at 20 °C (Hawley and Garver 2008). Interestingly, filtering freshwater but not seawater increased VHSV survival time, this hints at an association in freshwater with particles, although these might be living rather than inert (Hawley and Garver 2008).

Disinfection of adsorbed virus

Adsorption of viruses to 96-well plates provided a convenient approach for studying the actions of disinfectants on surfaces. With this system, 1-propanol at concentrations of 40% and above was shown to completely inactivate high titer VHSV adsorbed to plastic and kept dry for 1 day, whereas concentrations higher than 60% are required to completely inactivate even low titer CSV. Although the effective concentrations will depend on the starting amounts of virus, making comparisons difficult, it is interesting to note that the effective 1-propanol concentrations required to inactivate VHSV were similar to those determined by others to inactivate VHSV in suspension (Kurita et al. 2002). The similarity between surface and suspension tests need not always be the case because adsorbed viruses might be more or less

susceptible than viruses in suspension (Bellamy 1995; Ferrier et al. 2004; Gerba 1984). VHSV is more sensitive to inactivation by 1-propanol than CSV. The presence of a membrane envelope on VHSV and lack thereof on CSV, might be the reason why VHSV is more sensitive. Disruption of the envelope on VHSV will prevent the glycoprotein from binding to cell membrane receptors and therefore, prevent viral infection. Evaluation of disinfection by this method was more difficult with CSV because at low titer not all wells showed CPE, which possibly could be due to a dilution limit being reached, and disinfection was similar between low and high titer CSV. The cause of this is unknown at this time, but differences in the structural properties of different viruses might be responsible, which means more different types of viruses should be examined by this new method.

Although only allowing a plastic surface to be examined, the 96-well adsorbed virus system should be adequate to rapidly compare the effectiveness of disinfectants or virucidals for adsorbed viruses and serve as a complement to conventional suspension tests, where pathogens and disinfectant are mixed in solution (Abad et al. 1994; Bellamy 1995; Belliot et al. 2008; Berman and Hoff 1984; Macinga et al. 2008; Townsend et al. 2000), and surface disinfection tests, where pathogens are dried on a stainless steel disc, followed by elution and determination of remaining viral titer (Mbithi et al. 1990; Patnayak et al. 2008; Sattar et al. 1989). The one major difference is that the other approaches try to find the disinfectant concentration that kills 99.9 or 99.99% of the virus, whereas our approach defines the disinfectant concentration that makes the surface of plastic wells with adsorbed virus no longer infectious enough to produce CPE. This could be done by either completely inactivating all the adsorbed virus or lowering the number of infectious adsorbed virus to levels below a threshold required to cause CPE in each well. In the case of high titer VHSV, we determined that 40% 1-propanol was needed to reduce the titer of VHSV by approximately 5 logs. The influence of proteins on disinfection is an example of a problem that can be investigated by our system. Protein amount is one of the main factors affecting the efficacy of disinfectants (Ferrier et al. 2004). The type and amount of protein adsorbed with the virus and/or in solution with the disinfectant

could be varied easily. One interesting protein for VHSV would be the extracellular matrix protein, fibronectin, which has been found to bind another fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV) (Liu and Collodi 2002). Not only protein, but other particulate matters found on common surfaces could be examined to determine their effect on the concentration of disinfectants required to inactivate viruses.

Conclusion

In summary, we have made two original observations about the behavior of viruses and cells in tissue culture vessels, exploited these observations to develop a novel method for studying viruses on surfaces, and used this method to study for the first time fish viruses (VHSV and CSV) on a surface, providing new insights into the survival and inactivation of these viruses. The two original observations were that a substantial fraction of the virus added to tissue culture wells adsorbed to the plastic and that cells attached and spread on the plastic surface with adsorbed virus and became infected. Despite the use of animal cell cultures in virology for decades, these phenomena appear not to have been commented on previously, but are facts for researchers to consider in planning future in vitro experiments. Here the observations have been used to develop a new method for studying the behavior of viruses on surfaces, including their disinfection. The method uses 96-well plates and records the number of identical surfaces that remain infectious along with the Log Reduction Value (LRV) rather than just the LRV of viral titer in conventional studies. As viruses do not have to be first eluted from the surfaces, a step that can inactivate a portion of the viruses, this new approach is more sensitive than traditional ones and might be considered as a complement to conventional approaches. Currently this new method is limited to plastic surfaces, whereas many other surfaces (e.g. nylon) might be of more practical concern. However, the method allows treatments to be quickly evaluated for their ability to inactivate viruses adsorbed on a standard surface, plastic, with the recognition that their effectiveness might vary for viruses on other surfaces. In the course of this work being done, a fish virus (VHSV) has been shown for the first time to

survive for a period of time on a dry surface, but over 2 weeks the dry virus became inactivated, with the speed of inactivation depending on the incubation temperature. This information should be helpful for writing protocols to protect fish farms from viral diseases.

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