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Cell Culture and PCR Determination of Poliovirus Inactivation by Disinfectants

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Inactivation of poliovirus type 1 by 1 N HCl, 1 N NaOH, 0.5 and 1.0 mg of free chlorine per liter, and UV light was compared by using cell culture and seminested PCR (30 cycles of reverse transcriptase-PCR plus 30 cycles of seminested PCR). A minimum contact time of 45 min with HCl, 3 min with NaOH, 3 and 6 min with 1.0 and 0.5 mg of free chlorine per liter, respectively, was required to render 1.64×10^2 PFU of poliovirus type 1 per ml undetectable by seminested PCR. In cell culture, a minimum contact time of 5 min to HCl, 30 s to NaOH, and 1 min to either chlorine concentration was required to render the viruses undetectable by the plaque assay method. No correlation was observed between results by PCR and cell culture when viruses were exposed to UV light. These data suggest that inactivated virus with intact nucleic acid sequences can be detected by PCR.

PCR-based detection methods for enteric viruses in environmental samples, including water (1, 6), wastewater (10), sewage sludge (4), and sewage sludge-amended soils (13), have documented the usefulness of this procedure in terms of significantly reduced detection time and greater sensitivity compared with cell culture methods. This increased sensitivity is due mostly to the ability of the assay to detect intact nucleic acid sequences from viruses regardless of their infectivity (11). Therefore, equipment and materials used for sample collection and processing must be rendered free of contaminating nucleic acid sequences as well as infectious virus, for each sample collected and processed, if PCR is to be used as an integral part of an environmental monitoring program.

Current protocols for disinfecting equipment and materials used for collection and processing environmental samples include (i) autoclaving small materials, media, and reagents; (ii) disinfecting large equipment (pumps and large vessels) by using chlorine or iodine; and (iii) disinfecting sensitive equipment, such as pH electrodes, with strong acids (2). These procedures effectively inactivate virions; however, with the advent of extremely sensitive gene amplification methods (PCR), there is the possibility that viral nucleic acid sequences may not be destroyed by these disinfection practices.

The purpose of this study was to evaluate decontamination procedures currently used in environmental virology for their ability to render viral nucleic acid undetectable by PCR. This data could subsequently be used to optimize the conditions for each disinfectant's use for equipment decontamination.

Poliovirus type 1 (PV1) strain LSc was used for all disinfection experiments. It was propagated by infection of BGM cells and purified by freon extraction and ultracentrifugation (100,000 × *g* for 3.5 h) to minimize disinfectant residual demand, and titer was determined by the plaque assay method as previously described (8). Viruses were seeded into disinfectant solutions to achieve an initial concentration of 1.64×10^2 or 1.64×10^5 PFU/ml, depending on the experiment.

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The following disinfectants were tested for their ability to render viruses undetectable by both PCR and cell culture: 1 N HCl, 1 N NaOH, 1.0 and 0.5 mg of free chlorine per liter, and UV light. For the chemical disinfectants, concentrated stock solutions were diluted in reagent-grade (high-performance liquid chromatography [HPLC]) distilled water to achieve the desired starting test concentration.

All experiments were conducted at room temperature to approximate assay conditions in the field and an environmental virology laboratory. In addition, a minimum of duplicate experiments was performed for each disinfectant evaluated.

For acid or base disinfection experiments, 10 ml of either 1 N HCl or 1 N NaOH was seeded with PV1 (1.64×10^2 PFU/ml). At selected time intervals, a 0.2-ml aliquot was neutralized with 0.2 ml of the opposite chemical and buffered to pH 7.2 (± 0.2) with 0.2 ml of a 1 M HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Research Organics, Cleveland, Ohio).

Chlorine disinfection experiments were conducted at 0.5 and 1.0 mg of initial free chlorine per liter and two initial concentrations of PV1, 1.64×10^2 and 1.64×10^5 PFU/ml. Experiments determining the minimum contact time for undetectability by PCR used the lower virus input whereas comparison studies examining the correlation between cell culture infectivity versus loss of nucleic acid sequences used the higher virus input. In the latter case, only the 1-mg/liter concentration was tested. At selected time intervals, free chlorine was neutralized by the addition of sodium thiosulfate (2). The initial and final free chlorine concentrations were measured in a Hach (Loveland, Colo.) DR/2000 spectrophotometer (530 nm) by the DPD (*N,N*-diethyl-*p*-phenylenediamine) method (2).

Solutions for UV disinfection were prepared by the addition of PV1 to 50 ml of Tris-HCl-buffered water (pH 7.2 ± 0.2) to achieve a starting concentration of 1.66×10^5 PFU/ml. The 50-ml stock solution was distributed into 10 quartz glass dishes (5 ml per dish), and the intensity of the UV light was measured to calculate selected applied doses. Applied UV doses from 0 to 30,000 $\mu W/s/cm^2$ were tested.

Samples were assayed by PCR and cell culture. PCR assays were conducted by using the protocols described by Abbaszadegan et al. (1) and Straub et al. (13). For low-input-titer

TABLE 1. Degradation of PV1 nucleic acid by 1 N HCl[a]

Trial no.	Result for contact time (min) of:							
	0	5	10	15	20	30	45	60
1	+	+	+	−	−	−	−	−
2	+	+	+	+	NT[b]	NT	NT	NT
3	+	+	+	+	−	−	NT	NT
4	+	+	+	+	+	+	NT	NT
5	+	+	+	+	+	−	−	−
6	+	+	+	+	−	−	−	−
7	+	+	+	+	−	−	−	−
8	+	+	+	−	−	−	−	−
% Positive	100	100	100	75	29	14	0	0
No. positive/total no. of trials	8/8	8/8	8/8	6/8	2/7	1/7	0/5	0/5

[a] The initial concentration of poliovirus was 1.64×10^2 PFU/ml. PCR results based on seminested PCR (30 cycles of normal PCR plus 30 cycles of seminested PCR). No viruses were detected in the cell culture after 5-min exposure to HCl.
[b] NT, no sample taken.

experiments, an additional 30 cycles of seminested PCR was performed by using the methods described by Straub et al. (13), with the exception that the Mg and deoxynucleoside triphosphate concentrations were lowered to 1 and 0.2 mM, respectively, and the annealing temperature was increased to 60°C. For high-input-titer experiments, only 30 cycles of PCR was performed, and estimates of the viral titer by PCR were made by making serial 10-fold dilutions of the sample in HPLC-grade distilled water and subjecting them to PCR. The endpoint was the most-dilute sample resulting in positive amplification. Hence, the approximate titer was the inverse of that dilution. PCR products were visualized by agarose gel electrophoresis as described previously (1, 13). Cell culture assays were conducted by using a presence or absence test for low-titer experiments and the plaque assay method for high-titer experiments (2).

The sensitivity of the PCR for detection of PV1 in distilled water was conducted by making 10-fold dilutions of the virus in HPLC-grade distilled water and subjecting these dilutions to reverse transcriptase (RT)-PCR and seminested PCR. Sensitivities of 1.4 and 0.02 PFU were obtained for RT-PCR (30 cycles) and seminested PCR (additional 30 cycles), respectively (data not shown).

Results of experiments in which poliovirus was exposed to either 1 N HCl or 1 N NaOH revealed several interesting findings. A minimum of 45 min was required to render poliovirus nucleic acid undetectable by seminested PCR (Table 1) when the virus was exposed to 1 N HCl. However, cell culture results from the same experiment revealed that total loss of infectivity occurred after 5 min of exposure to 1 N HCl. Exposure to 1 N NaOH destroyed nucleic acids more efficiently than did exposure to 1 N HCl (Table 2). Here, only a minimum of 3 min was needed to render the viruses undetectable by seminested PCR. Loss of cell culture infectivity occurred 30 s after exposure to 1 N NaOH.

Results similar to those in the experiments using 1 N NaOH were obtained when viruses were exposed to free chlorine (Table 3). After 5 min of exposure to 0.5 mg of free chlorine per liter, no viruses were detected after seminested PCR. Chlorine demand ranged from 0.02 to 0.15 mg/liter after 10 min. Loss of PCR detectability occurred twice as fast when the initial free chlorine concentration was doubled to 1 mg/liter (Table 3). A minimum of 3 min was required for loss of detectability by seminested PCR. Chlorine demand was similar to that in the experiments using 0.5 mg/liter. No virus was detected by cell culture after 1 min of exposure to either 0.5 or 1 mg of initial free chlorine per liter.

Chlorine and UV light disinfection studies correlating loss of cell culture infectivity with loss of PCR detectability are shown in Fig. 1 and 2, respectively. Because of the higher initial input of virus (1.64×10^5 and 1.66×10^6 PFU/mL for chlorine and UV, respectively), only 30 cycles of PCR was required to determine the $\log_{10}$ reduction of the virus. Good correlation was observed between the $\log_{10}$ decrease in virus titers in RT-PCR and cell culture when the viruses were exposed to 1 mg of free chlorine per liter. However, this was not the case for UV disinfection experiments. The titer in RT-PCR decreased by only 1 $\log_{10}$ after an applied dose of 2,500 $\mu W/s/cm^2$, and no further decrease in the titer in RT-PCR was observed with

TABLE 2. Degradation of PV1 nucleic acid by 1 N NaOH[a]

Trial no.	Result for contact time (min) of:						
	0	0.5	1	3	5	10	15
1	+	NT[b]	+	NT	−	−	−
2	+	NT	+	NT	−	−	−
3	+	+	+	−	−	−	−
4	+	+	+	−	−	−	−
% Positive	100	100	100	0	0	0	0
No. positive/total no. of trials	4/4	2/2	4/4	0/2	0/4	0/4	0/4

[a] The initial concentration of PV1 was 1.64×10^2 PFU/ml. PCR results were based on seminested PCR (30 cycles of normal PCR plus 30 cycles of seminested PCR). No viruses were detected in cell culture after 30-s exposure to 1 N NaOH.
[b] NT, no sample taken.

TABLE 3. Degradation of PV1 nucleic acid by free chlorine[a]

Initial chlorine concn (mg/liter)	Result for contact time (min) of:										Residual chlorine (mg/liter)[b]
	0	1	2	3	4	5	6	7	8	9	
1	+	NT[c]	NT	−	−	−	−	−	−	−	0.90
	+	+	+	−	−	−	−	−	−	−	0.94
	+	+	+	−	−	−	−	−	−	−	0.98
0.5	+	NT	NT	NT	NT	+	−	−	−	−	0.35
	+	+	+	+	+	+	−	−	−	−	0.44
	+	+	+	+	+	−	−	−	−	−	0.48

[a] The initial concentration of PV1 was 1.64×10^2 PFU/ml. PCR results were based on seminested PCR (30 cycles of normal PCR plus 30 cycles of seminested PCR). No viruses were detected in cell culture after 1-min exposure to either 1 or 0.5 mg of free chlorine per liter.

[b] Final residual concentration after 9-min exposure to free chlorine.

[c] NT, no sample taken.

prolonged dosages. In contrast, the titer in cell culture consistently decreased with increasing UV doses.

PCR is becoming an increasingly popular method to detect viruses in environmental samples. False positives can arise from the lack of quality control during the method, and this has been reviewed extensively. However, no study has adequately addressed the possibility of false positives arising from contaminated equipment used to collect and process samples. Assumptions that the equipment, after adequate disinfection, is free of viruses have been based on cell culture assays (2) but require reevaluation when PCR is also used as a method to detect viruses in a sample. The purpose of this work was to determine if previous assumptions concerning disinfection of field and laboratory equipment used to process environmental samples for virus detection held true for PCR-based detection as well as detection by cell culture.

In all experiments, the initial concentration of poliovirus was 1.64×10^2 or 1.64×10^5 PFU/mL. These concentrations of virus are well above the expected concentration of enteroviruses typically found in water or wastewater samples. For example, the maximum concentration of virus isolated from surface water that has been reported is 620 PFU/liter (9). Assuming that 400 liters of this sample was concentrated to 30 ml, as is the usual practice (2), this would represent a titer of approximately 8.3×10^3 PFU/ml. Undigested sewage sludge typically contains between 10^2 to 10^4 viruses per g (dry weight) of sludge (14). However, only 10 to 20 g (dry weight) is typically analyzed (12).

Environmental virology laboratories frequently collect multiple field samples of water and wastewater on the same day by using the same hoses, pumps, filter housings, reservoirs, and other equipment. Disinfection of the equipment in the field, between samples, is necessary to prevent cross contamination. In the field, equipment is disinfected with chlorine at an initial concentration of approximately 1 to 5 mg of free chlorine per liter, with a contact time of 15 to 30 min (2). This protocol has been proven to be effective in inactivating viruses that may be present in the sample on the basis of cell culture assays. The current study demonstrated that degradation of contaminating nucleic acids also occurs and can be accomplished within the time required in the protocol, provided that a minimum of 0.35 to 0.50 mg of free chlorine residual per liter remains after 10 min of disinfection. In addition, degradation of nucleic acid sequences correlated to loss of cell culture infectivity based on high-titer experiments.

The filter elution and reconcentration procedures used for processing water and wastewater samples require several pH adjustment steps. Sensitive equipment, such as pH probes, must be disinfected between these steps, especially when multiple samples are processed on the same day. The current protocol for probe disinfection suggests that glass probes be soaked or washed in a 1 N HCl solution for 5 min (2). Our studies indicated that cell culture infectivity was lost within 5 min of exposure to 1 N HCl. However, after seminested PCR, a minimum of 45 min was required to completely degrade nucleic acid sequences. Therefore, the current procedures for probe disinfection must be reevaluated, since contamination of the probes with inactivated viruses still containing intact viral nucleic acid sequences could also be a source of false-positive results by PCR.

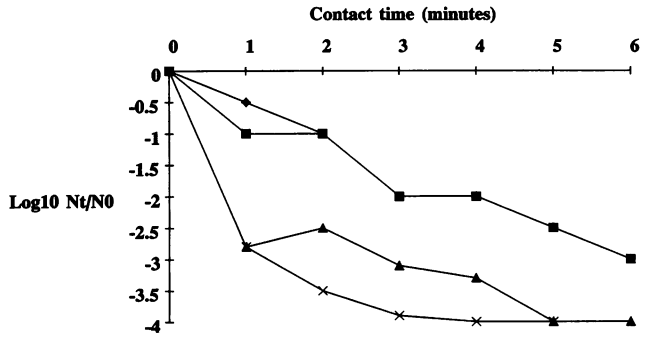

FIG. 1. Comparison of poliovirus inactivation by 1 mg of free chlorine per liter in cell culture and PCR. The initial concentration of PV1 was 1.64×10^5 PFU/ml. PCR results are based on 30 cycles of PCR. ◆, PCR trial 1; ■, PCR trial 2; ▲, plaque assay trial 1; ×, plaque assay trial 2.

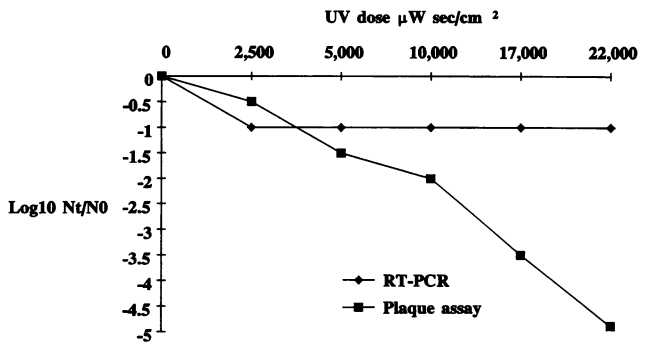

FIG. 2. Comparison of poliovirus inactivation by UV light in cell culture and PCR. The initial concentration of PV1 was 1.66×10^5 PFU/ml. PCR results are based on 30 cycles of PCR.

As an alternative to HCl, we evaluated the efficacy of 1 N NaOH to degrade viral nucleic acid sequences, with great success. These results are not surprising since RNA is extremely sensitive to degradation by dilute alkali (7). The major disadvantage of using sodium hydroxide to disinfect glass probes is that, over time, the glass becomes etched, thereby damaging the probe.

We also evaluated the efficacy of UV light to degrade nucleic acid sequences. Several molecular biology techniques recommend the use of UV irradiation to prevent carryover contamination from PCRs (3). In the environmental virology laboratory, we commonly use UV light to decontaminate surfaces in laminar flow hoods. In terms of cell culture assays, UV effectively decontaminated a high titer of poliovirus (1.66×10^5 PFU/ml) in water. However, corresponding degradation of nucleic acid sequences was not realized. Similar results have been observed for bacterial inactivation by UV light (5).

In conclusion, degradation of nucleic acid sequences is possible in both the field and laboratory applications. Of the methods tested, we concluded that chlorine at a concentration of at least 0.5 to 1 mg/liter is the most effective disinfectant for equipment used in the field, for laboratory surfaces, and for pH probes. This was judged in terms of the time required to decontaminate nucleic acid sequences, potential corrosion problems from high- or low-pH solutions, and ability to easily neutralize its action. In contrast, UV light should not be relied on as a method of decontamination since it does not degrade viral nucleic acid and can result in false positives by PCR.

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