Test Method for the Evaluation of Virucidal Efficacy of Three Common Liquid Surface Disinfectants on a Simulated Environmental Surface

J. W. GAUSTAD, C. R. McDUFF, AND H. J. HATCHER

Research and Development Department, Economics Laboratory, Inc., St. Paul, Minnesota 55102

The Association of Official Analytical Chemists bacterial use-dilution test for evaluating liquid surface disinfectants was modified to determine the efficacy of three common environmental germicide compounds against representatives of four virus groups. Modifications were made to conform to the Environmental Protection Agency guidelines on virucidal testing procedures. Alterations included: the use of a concentrated tissue culture preparation of virus instead of a standard bacterial culture; HEp-2 tissue culture cells as a visible test system in place of standard nutrient broth; and controls to measure the virus titer end point and the toxicity of the disinfectant to the cell cultures. Comparison of control end points with results from the test proper were the measure of the effectiveness of the germicides against the viruses. Results are described which agree with those based on other methods previously reported.

A standard test method for the evaluation of the antiviral activity of liquid surface disinfectants is currently unavailable. Several procedures have been reported on the virucidal efficacy of disinfectants (2, 4, 8, 9, 12, 15). However, only two instances in the literature indicated the use of an environmental surface which would simulate actual in-use conditions of the disinfectant and serve as a carrier for the test virus. Lorenz and Jann (9) used stainless-steel cylinders as specified in the Association of Official Analytical Chemists use-dilution test on an environmental surface (7) and obtained satisfactory results with Newcastle disease virus in embryonated chicken eggs. The virus recovered from the stainless-steel cyclinders was sufficient to determine the effectiveness of the disinfectants tested. Cylinder drying times of 20, 40, and 60 min resulted in the recovery of $10^{-5.5}$, $10^{-5.0}$, and $10^{-5.6}$ 50% lethal doses (LD_{50}) per ml, respectively, from cylinders exposed to $10^{-9.7}$ LD_{50} per ml of virus stock suspension. Wright (15), however, obtained disappointing results with porcelain cylinders and vesicular stomatitis virus in eggs. The porcelain cylinders apparently were not effective vehicles for simulating in-use conditions of a disinfectant. Only $10^{0.6}$, $10^{1.5}$, and $10^{1.74}$ LD_{50} per 0.1 ml were recovered from cylinders exposed to $10^{9.5}$ LD_{50} per 0.1 ml of virus stock suspension. Most procedures were similar to that of Klein (8), in which the virus and disinfectant were mixed directly together in a specified proportion and concentration.

The Environmental Protection Agency (EPA) has established guidelines for the evaluation of the virucidal efficacy of disinfectants (14). They require the use of a hard (environmental) surface as a carrier for the test virus. However, since most disinfectants are toxic to tissue culture in vitro, the germicide-treated virus should be "detoxified" either physically or chemically to reduce or eliminate the cytotoxic effect to the cells. If a detoxification step is not performed or is only partially effective, at least a 3 log reduction of the virus must be demonstrated over and above the cytotoxicity caused by the disinfectant. Furthermore, no virus should be detectible in any of the disinfectant-treated virus dilutions; thus indicating complete inactivation of the virus by the disinfectant.

The purpose of this study was to develop a relatively simple method based on EPA guidelines for making virucidal efficacy claims on commercially available liquid surface disinfectants.

MATERIALS AND METHODS

Tissue culture cells and media. The tissue culture media were obtained commercially (Associated Biomedic Systems Inc. [ABS]) and supplemented with heat-inactivated calf serum (ABS), penicillin, streptomycin (ABS), and Fungizone (Flow Laboratories Inc.). HEp-2 cells (Flow Laboratories Inc.) have been

serially cultivated in this laboratory for 2 years. Growth medium consisted of medium 199 and 10% calf serum plus antiobiotics and maintenance medium consisted of medium 199, 5% calf serum, and antibiotics.

Viruses. Herpes simplex virus, MP strain; poliovirus, type 1 Brunhilde strain; and vaccinia virus, WR strain, were kindly supplied by the Pesticides Regulation Division, Microbiology Laboratory, EPA. Adenovirus type 3 strain G.B. was purchased from the American Type Culture Collection as ATCC number VR3, lot number five.

All four viruses were grown in monolayer cultures of HEp-2 cells processed by freezing and thawing four times in a dry ice-alcohol bath. The virus cell suspensions were then centrifuged at $1,000 \times g$ for 20 min to remove cell debris, the supernatant fluid was drawn off, and the virus stock pool was concentrated by negative-pressure ultrafiltration according to Craig (5) and Smith (13) and stored at -90 C until use.

Negative-pressure ultrafiltration. The filtration apparatus (Fig. 1) was assembled as described by Craig (5). Vacuum was applied slowly to the virus stock, and the virus stock was filtered from 12 to 24 h. The volume of virus was concentrated from 250 ml to approximately 10 ml. The virus concentrate was then filtered through a 0.22 micrometer Millex filter (Millipore Corp).

Disinfectants. A quaternary ammonium compound, phenolic, and iodophor were selected as the three common types of disinfectants (Economics Laboratory, Inc.). The quaternary ammonium product (Mikro-Quat) contained as active ingredients alkyldimethylbenzyl ammonium chloride plus ethylenediaminetetraacetic acid. The phenolic (Mikro-Bac) contained three complex phenol derivatives, and the iodophor (Milroklene) was an organo-iodine complex plus phosphoric acid. Before each efficacy test, a fresh use concentration of the disinfectant to be evaluated was prepared in sterile deionized distilled water. The use concentration of the above disinfectants was 1:200 for the quaternary ammonium compound, 1:128 for the phenolic, and 75 ppm titratable iodine for the iodophor.

Virus titer assay. The titer of each test virus was determined by making serial 10-fold dilutions and inoculating 48- to 72-h-old HEp-2 cell cultures. Virus concentration was expressed as the 50% tissue culture infective dose ($TCID_{50}$) and calculated by the technique of Reed and Muench (10). The $TCID_{50}$ varied from $10^{5.9}$ for adenovirus to $10^{8.8}$ for poliovirus, after negative-pressure ultrafiltration.

Germicide detoxification. Letheen broth (Difco)

FIG. 1. *Assembled ultrafiltration apparatus.*

was utilized to help neutralize the cytotoxic effects of the germicide on the cell cultures.

Carrier disinfectant test method. The test is comprised of three parts, a cytoxicity control, a virus control, and the test proper.

(i) Cytoxicity control. Five pennicylinders of type 304 stainless steel (S & L Metal Products Corp., Maspeth, N.Y.) were placed separately into five sterile tubes (20 by 150 mm), each containing 10.0 ml of the test germicide at the use concentration. The cylinders were exposed for 10 min to the germicide, and each carrier was placed in a separate tube containing 1.8 ml of letheen broth and shaken vigorously for 5 min. Serial 10-fold dilutions (10^{-1} through 10^{-7}) were made by adding 0.2 ml of the previous dilution into 1.8 ml of maintenance medium 199 in the next dilution. Each dilution (0.2 ml) was inoculated onto a monolayer of HEp-2 cells in a tissue culture tube (16 by 125 mm; Falcon Plastics, Oxnard, Calif.) Each dilution was absorbed on the cells for the equivalent time required for adequate absorption of the virus. The equivalent absorption times were 2 h for herpes simplex, 30 min for poliovirus, 45 min for adenovirus, and 1 h for vaccinia virus. The temperature of absorption for all portions of the test method was 37 C. After the absorption time was complete, 2.0 ml of maintenance medium 199 was added to each tissue culture tube. They were then incubated at 37 C for 3 to 5 days, examined for cytoxicity end point, and calculated as the $TCID_{50}$ by the technique of Reed and Muench (10).

(ii) Virus control. Five sterile cylinders were placed in the concentrated stock pool of the test virus, exposed 15 min to the virus, placed in a sterile petri dish containing matted filter paper, and put into a 37 C incubator until dry (approximately 20 to 25 min). Each carrier was then placed in a separate tube containing 1.8 ml of letheen broth and manipulated as in the cytoxicity control. The virus titer end point was calculated as the $TCID_{50}$.

(iii) Test proper. Five sterile cylinders were exposed to the virus control and dried, and then each carrier was placed separately into a sterile tube (20 by 150 mm) containing 10 ml of a use dilution of the test disinfectant, exposed for 10 min, and placed separately in a sterile tube containing letheen broth. The remaining portion of the procedure was performed as in the cytoxicity and virus controls.

RESULTS

It was necessary first to determine the concentration of virus that survived the drying period on the cylinders and, secondly, the effectiveness of the letheen broth in neutralizing the disinfectant before inoculation into tissue culture. Based on EPA guidelines, the virus titer had to exceed the level of cytoxicity by at least 3 logs. Virus survival rate was evaluated in both concentrated and unconcentrated stock pools as log 10 after drying on the stainless-steel cylinders (Table 1). It should be noted that the drying time for the virus-contaminated cylin-
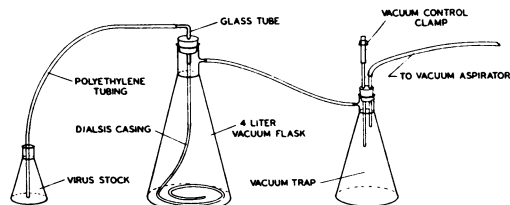
TABLE 1. *Average log 10 of virus concentration and survival rate from the stainless-steel cylinders after drying at least 20 min at 37 C[a]*

Virus	Log 10 before concentration	Log 10 after concentration	Drying time before concentration (min)	Drying time after concentration (min)	Log 10 survival before concentration	Log 10 survival after concentration
Herpes simplex	5.2	7.9	20	20	1.1	6.3
Poliovirus	5.9	8.8	20	22	3.8	5.3
Vaccinia virus	2.4	6.2	21	25	0.9	4.7
Adenovirus	3.3	5.9	23	25	1.6	4.8

[a] Based on titers obtained in duplicate studies on the efficacy of the disinfectant against the four test viruses.

ders was 20 min, and all drying times indicated in Table 1 are the minimal times in which the cylinders appeared to be dry or almost so. The data from Table 1 indicates that a method for virus concentration is necessary if a sufficient number of virus particles are to be retained on the cylinders. Furthermore, cytoxicity must be held to a minimum, and letheen broth reduces toxicity levels significantly (Table 2).

Of the three disinfectants tested (Table 3), the iodophor was the most effective as it inactivated at least the EPA minimal acceptable level of all four test viruses. The quaternary and phenolic disinfectants were comparable in virucidal activity and failed to meet minimal EPA requirements of a 3 log reduction against poliovirus.

DISCUSSION

The determination of antiviral activity of a disinfectant is much more difficult to ascertain than its effectiveness as an antibacterial agent. Virus recovery, which requires a living host, is further complicated by the fact that most germicides are toxic to the host system. In addition, like antibacterial disinfectants, virucides must meet the minimal requirements established by the EPA if a virucidal claim is to be made on a commercially available product.

Since commercially available disinfectants are widely used by health care facilities, animal research centers, etc., a standard method acceptable to the EPA is of utmost importance. Of the methods reported previously, most would probably not be acceptable for substantiating virucidal claims on products registered with the EPA. Lorenz and Jann (9), who performed a similar type of test procedure as reported here with the exception of using embryonated eggs instead of tissue culture, probably meet the EPA requirements for allowable virucidal claims on commercially available disinfectants.

TABLE 2. *Comparison of the toxicity of the disinfectants to the HEp-2 cells when no neutralizer is used and when neutralized by letheen broth[a]*

Germicide	$TCID_{50}$ without letheen broth	$TCID_{50}$ with letheen broth
Quaternary ammonium compound	3.7	1.2
Phenolic	4.8	1.6
Iodophor	4.1	1.3

[a] Based on cytoxicity end points obtained in duplicate studies on the efficacy of the disinfectants against the four test viruses.

We encountered the same difficulties as Wright (15) when the use of porcelain pennicylinders was evaluated as a simulated environmental surface (J. W. Gaustad, unpublished data); i.e., poor virus recovery was obtained. However, little difficulty was encountered when stainless-steel cylinders were used as a recovery surface.

The viruses used in the tests were maintained in only one cell line (HEp-2) to minimize the amount of reagents needed and to simplify the procedure for laboratories which would desire to equip themselves for virucidal efficacy testing. Undoubtedly higher virus titers could be obtained if the optimal cell line was used for each virus.

Another factor which probably aided in virus survival during the drying periods on the cylinders was that the protein from the calf serum in maintenance medium 199 was, like the virus, concentrated. This may have given the virus some protection when being dried. In addition, the concentrated protein acted as an excellent "soil load" when the virus-contaminated cylinders were exposed to the germicide. The simulated soil load mimics what might be encoun-

TABLE 3. *Log 10 of virus inactivation by the three test disinfectants with a 10-min exposure time at 20 C*

Germicide	Concn of active ingredients (ppm)	Virus	Virus titer from ring carriers (logs)	Toxicity of germicide (logs)	No. of logs virus inactivated
Quaternary ammonium compound	450	Herpes simplex	5.3	1.6	3.7
			5.7	2.2	3.5
		Poliovirus	5.1	1.2	1.1[a]
			5.6	1.2	2.3[a]
		Vaccinia virus	5.1	0.68	4.5
			4.8	0.62	4.2
		Adenovirus	4.4	0.83	3.6
			4.5	0.83	3.7
Phenolic	616	Herpes simplex virus	6.2	1.8	4.4
			5.6	2.2	3.4
		Poliovirus	5.9	2.1	2.1[a]
			5.8	2.3	1.7[a]
		Vaccinia virus	4.2	0.5	3.7
			4.5	1.0	3.5
		Adenovirus	4.2	1.0	3.2
			4.2	1.2	3.0
Iodophor	75	Herpes simplex virus	7.8	0.8	7.0
			7.3	1.2	6.1
		Poliovirus	4.5	0.8	3.7
			4.7	0.8	3.9
		Vaccinia virus	5.0	1.1·	3.9
			4.5	0.6	3.9
		Adenovirus	4.8	1.5	3.3
			4.8	1.2	3.6

[a] Failed to meet EPA requirements.

tered in actual in-use conditions. The use of letheen broth, whereas very useful in neutralizing most of the cytoxicity to the HEp-2 cell cultures, was not completely effective. When evaluated with more toxic types of disinfectants such as the "tamed aldehydes," letheen broth may prove to be completely inadequate as a neutralizer. Blackwell and Chen (4) reported the use of Sephadex LH-20 as a molecular sieve for the detoxification of germicides. Their results were encouraging, but have so far met with failure when applied to the test procedure presented here. The virus titer, due to dilution of the chemical disinfectants, is reduced to almost undetectable amounts (J. W. Gaustad, unpublished data).

The results in Table 3 agree with those of Klein and Deforest (8) and other investigators (1–4, 6, 12). Generally speaking, certain disinfectants, including quaternaries, phenolics, and iodophors, are effective against lipophilic viruses such as herpes simplex, vaccinia, and adenovirus, whereas, in this case, only the iodophor is effective against the hydrophilic viruses such as poliovirus, echovirus, and coxsackievirus.

Furthermore, no statement can be made as to the accuracy of the test procedure, as there is no current methodology for the efficient recovery of viruses from an environmental situation. Therefore, it cannot be determined if the EPA requirement of a 3 log reduction is a satisfactory standard.

In conclusion, whereas the methods used in this procedure are not optimal, and in some cases improvement is needed, the test at least aids in establishing what are the possible minima that should be expected from a virucidal disinfectant when tested on a simulated environmental surface.

LITERATURE CITED

1. Armstrong, J. A., and E. Froelich. 1964. Inactivation of virus by benzalkonium chloride. Appl. Microbiol. 12:132–137.
2. Avampato, J. E., S. M. Amundsen, and P. J. Vasington. 1969. Virucidal testing methods, p. 126–129. Proc. 55th Meet. Chem. Specialties Mfgs. Ass., New York.
3. Bartlett, P. G., and W. Schmidt. 1957. Surfactant-iodine complexes as germicides. Appl. Microbiol. 5:355–359.
4. Blackwell, J. H., and J. H. S. Chen. 1970. Effects of various germicidal chemicals on H.Ep.-2 cell culture and herpes simplex virus. J. Ass. Offic. Anal. Chem.

53:1229-1236.

5. Craig, L. C. 1965. Differential dialysis, p. 35-74. *In* C. N. Reilly (ed.), Advances in analytical chemistry and instrumentation, vol. 4. Interscience Publishers, New York.

6. Gershenfled, L. 1955. Iodine as a virucidal agent. J. Amer. Pharm. Ass. **44**:177-182.

7. Horwitz, W. (ed.). 1970. Disinfectants, p. 59-72. *In* Official methods of analysis of association of official analytical chemists, vol. 11. Association of Official Analytical Chemists, Washington, D.C.

8. Klein, M., and A. Deforest. 1963. The inactivation of viruses by germicides, p. 116-118. Proc. 49th Meet. Chem. Specialties Mfgs. Ass., New York.

9. Lorenz, D. E., and G. J. Jann. 1964. Use-dilution test and Newcastle disease virus. Appl. Microbiol. **12**:24-26.

10. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent end points. Amer. J. Hyg. **27**:493-497.

11. Roizmain, B., and P. G. Speur. 1968. Preparation of herpes simplex virus of high titer. J. Virol. **2**:83-84.

12. Rose, G. W., and W. Perlberg. 1970. Method of evaluation of virucidal inactivation by a chemical disinfectant, p. 111-115. Proc. 56th Meet. Chem. Specialties Mfgs. Ass., New York.

13. Smith, J. D., and E. DeHarven. 1973. Concentration of herpesviruses. J. Virol. **11**:325-328.

14. Tavano, J. M. 1972. Methods for evaluating the virucidal activity of disinfectants DIS-13. Disinfectant Control Section Pesticide Office, Environmental Protection Agency, Washington, D.C.

15. Wright, H. S. 1965. Test method for determining the virucidal activity of disinfectants against vesicular stomatitis virus. Appl. Microbiol. **19**:92-95.