Test Method for Determining the Viricidal Activity of Disinfectants Against Vesicular Stomatitis Virus

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Received for publication 12 September 1969

A test method using embryonating chicken eggs for the evaluation of several types of disinfectants as viricides against vesicular stomatitis virus was developed which gave specific, reproducible results.

Standard methods are available for evaluating bactericides and fungicides (2), but there are no existing standard techniques available for evaluating viricides. Klein (5) reported a marked difference in susceptibility of viruses to different viricides. The difference depended primarily on whether the virus was lipophilic or hydrophilic and on the chemical nature of the disinfectant.

Procedures have been described previously for testing general viricides. Lorenz and Jann (6) described a carrier procedure similar to existing bactericidal testing methods (2). Another technique has been described in which the virus was injected into susceptible animal hosts after disinfectant treatment (1). Klein and DeForest (Fed. Proc., p. 319, 1965) reported a procedure in which virus and disinfectants were mixed, allowed to react, diluted, and then injected into susceptible hosts.

Techniques for measuring the resistance of vesicular stomatitis virus (VSV) to disinfectants have been reported. Patterson et al. (8) stated that 3% lye did not inactivate VSV in 2 hr, although other disinfectants were shown to be effective. Death of 9-day embryonating chicken eggs was used to indicate presence of infectious virus. Fong and Madin (3) studied the effect of hydrogen ion concentration on VSV and found the virus stable in a pH range of 3.0 to 11.6 using embryonating eggs inoculated by the allantoic route. Galloway and Elford (4) determined that a pH of 3.0 or less was viricidal within 2 hr. The test suspension was injected intradermally into the foot pad of guinea pigs to determine viral infectivity. Pyl (9) used guinea pigs as host animals for studying viral infectivity after disinfectant treatment of the virus. Vesicular stomatitis virus was stable in a pH range of 4.0 to 10.7 for 33 days and for 1 day at pH 3.0, but a pH of 1.5 was immediately lethal.

The objective of this study was to develop an easily reproducible method for evaluating the effect of disinfectants on the VSV.

MATERIALS AND METHODS

Embryonating eggs (chicken). The eggs for these experiments were purchased from a commercial hatchery and incubated at 39 C until the day of use. After injection with virus or virus-disinfectant mixtures, the eggs were incubated at 35.5 C by the method of Sigurdsson (11), who previously reported that a temperature of 35 to 36 C was more suitable for VSV cultivation in eggs than 39 to 40 C.

Virus. The virus was kindly supplied by Diagnostic Virology, Diagnostic Services, Animal Health Division, National Animal Disease Laboratory, Ames, Iowa. The ninth egg passage of the VSV New Jersey strain was propagated in 8-day-old embryonating eggs, inoculated in the allantoic cavity. The eggs were candled after 24 hr and dead embryos were considered to be viable. From 36 to 48 hr after inoculation, the eggs were candled at 2-hr intervals. Eggs containing dead embryos were stored at 4 C until all dead embryos were collected. These eggs were used as the source of virus.

Disinfectants. The disinfectants selected varied from pure chemicals to those containing two or three active ingredients and in some cases a surfactant. The disinfectants were diluted with sterile distilled water to 1.11 times the final concentration used. A 9-ml amount of the diluted disinfectant was placed in a 10-ml glass serum bottle closed with rubber stoppers and metal closures at 80 C in a refrigerator.

For use, the virus suspension was thawed at room temperature and held at 0 C. Penicillin (5,000 μg/ml) and streptomycin (9 mg/ml) were added to the suspension to control bacterial contamination.

Virus assay. The virus concentration was assayed by using the 50% lethal dose (LD50) calculated by the method of Reed and Muench (10). The LD50 titer of virus varied in these experiments from 10-6.9 to 10-3 in each 0.1 ml of virus suspension. The titer of the virus was determined by making serial dilutions and injecting 0.1 ml of each dilution into five 8-day-old em-
bryonating chicken eggs. Separate needles and syringes were used for each dilution.

**Egg inoculation procedure.** The eggs were inoculated by using the dropped membrane technique. A dilution of virus-disinfectant mixture was placed on the CAM in the artificial air cell with a syringe and needle. The needle holes were sealed with a cellulose nitrate base adhesive.

**Disinfectant toxicity controls.** Five embryonating eggs were injected with 0.1 ml of the greatest concentration of each disinfectant used, diluted 1:9 with 0.05 M phosphate-buffered saline (PBS), pH 7.0, to determine whether the chemicals were toxic to the embryos.

**Liquid dilution test procedure.** One vial with 9 ml of disinfectant at 1.11 times the concentration to be tested was placed in a water bath at 20 C. A 1-ml amount of virus suspension was added to each vial of disinfectant with a syringe and hypodermic needle. The suspension was mixed by hand shaking for 15 sec when virus was introduced and just before each portion of virus disinfectant was removed. At 5, 10 and 30 min after introducing the virus into the disinfectant, 1.0 ml was removed with a 1-ml syringe and placed in a serum bottle with 9 ml of PBS, which was kept at 0 C. When the above procedure was complete, five eggs were inoculated with 0.1 ml per egg (on the CAM) from each contact time interval. The eggs were candled daily for 3 days and on the 6th day. Embryos that died within 24 hr were considered injury deaths. All embryos in which there was doubt as to the cause of death were refrigerated. A 0.1-ml amount of allantoic fluid from the egg suspected to contain virus was placed on the CAM of an 8-day embryonating egg and incubated for 6 days. If the embryo did not die, the previous embryo death was attributed to injury.

**Carrier disinfectant test method.** A test method using porcelain Penicylinders (Fisher Scientific Co., Chicago, Ill.) as vehicles for carrying virus to the disinfectant proved unsuccessful. This method was similar to the Association of Official Agricultural Chemists (2) bactericide test procedure and the virus dilution technique of Lorenz and Jann (6). The cylinders were soaked in the virus suspension having an LD₅₀ titer of 10⁻⁵ per 0.1 ml for 20 min to absorb the virus and allowed to dry for 20 min. The carrier was placed into the disinfectant at the use concentration at 20 C for 10 min. The carrier was removed from the disinfectant and the excess was allowed to drain back into the container. The carrier was placed in 2.0 ml of PBS at 0 C and hand-shaken for 1 min to remove the virus. Five 0.1 ml portions were inoculated into 8-day embryonating eggs. As a control, the LD₅₀ of the virus per cylinder before disinfectant treatment was determined by shaking the cylinders in 10 ml of PBS for 1 min, making several dilutions in PBS and inoculating 0.1 ml of each dilution into 8-day-old embryonating chicken eggs.

**RESULTS**

The dilution method for testing disinfectants was developed after determination of the best route of virus introduction into eggs, means of virus and disinfectant contact, and concentration of virus to be used for measuring viricidal efficiency.

Higher virus titers were obtained when the virus suspension was placed on the surface of the CAM than when the virus suspension was injected into the allantoic cavity, with the use of the same virus suspension. An LD₅₀ of 10⁻⁴.₄ was obtained by the allantoic cavity route, and greater than 10⁻⁵.₃ by the CAM route.

The method of using the porcelain carrier procedure was not useful. The LD₅₀ titers of virus recovered from the carriers without disinfectant treatment were 10⁻⁶.₄, 10⁻⁻⁵, and 10⁻⁻¹₄ per 0.1 ml for three separate experiments. The LD₅₀ titer of virus on a comparable carrier not dried for 20 min was greater than 10⁻⁴.₄.

Twelve disinfectants were tested by using the dilution procedure, of which seven inactivated the virus within 10 min, whereas five failed to do so in 30 min. The disinfectants used, concentrations, and test results are listed in Table 1. The results were based on a minimum of four tests in which the LD₅₀ virus titer was 10⁻⁵ or greater (Table 2).

The virus titer had considerable effect on the results obtained. When the LD₅₀ titers were lower

**TABLE 1. Action of disinfectants as viricides after a 10 min contact time at 20 C.**

| Disinfectant type                | Concen (%) | Viricidal |
|----------------------------------|------------|-----------|
| Cresylic acid                   | 1.0        | Yes       |
| Substituted phenolic            | 0.5        | Yes       |
| Phenol                          | 2.5        | Yes       |
| Chlorinated phenol              | 0.2        | Yes       |
| Quaternary ammonium compound   | 5.0        | No        |
| HCl (pH 1.9)                    | 0.4        | Yes       |
| NaOH (pH 12.2)                  | 10.0       | No        |
| KOH (pH 13.3)                   | 10.0       | No        |
| Na₂CO₃ (pH 11.1)                | 10.0       | No        |
| Ethyl alcohol                   | 70.0       | No        |
| Sodium orthophenyl-phenate      | 80.0       | No        |
| NaOCl (commercial bleach)       | 2.0        | Yes       |
|                                  | 0.645      |           |

*No detectable virus after 10 min of contact with disinfectant at 20 C.

*50 to 53% mixed phenols as saponified cresol solution.

*Orthophenylenol 15% p-tert-amylphenol 6.3%, alcohol 4.7%.

*2,4,5-Trichlorophenol 18.2%.

*Alkyl dimethylbenzylammonium chlorides (alkyl groups C₄ to C₁₂) 10%.
than $10^{7.5}$, some disinfectants inactivated all the detectable virus in 10 min, but not when the LD$_{50}$ titer was greater than $10^{7.8}$, even when the concentration was increased. The viricidal effect of all the disinfectants was not altered by an increase in virus titer (Table 3).

**Table 2. Examples of reproducibility of viricide tests**

| Disinfectant and concn | Test no. | Determination | Log LD$_{50}$ virus titer |
|------------------------|---------|--------------|--------------------------|
|                        |         | 5 min$^a$ | 10 min$^a$ | |
| Cresylic acid (1%)$^b$ | 1       | 0/5$^c$   | 0/5$^c$   | 10$^{9.3}$ |
|                        | 2       | 1/5       | 0/5       | 10$^{7.9}$ |
|                        | 3       | 1/5       | 0/5       | 10$^{7.8}$ |
|                        | 4       | 1/5       | 0/5       | 10$^{7.8}$ |
| Quaternary ammonium compound (5%)$^b$ | 1   | 5/5       | 2/5       | 10$^{7.4}$ |
|                        | 2       | 3/5       | 3/5       | 10$^{7.4}$ |
|                        | 3       | 2/5       | 2/5       | 10$^{7.4}$ |
|                        | 4       | 2/5       | 2/5       | 10$^{7.4}$ |
| NaOCl (0.645%)$^c$     | 1       | 2/5       | 0/5       | 10$^{9.3}$ |
|                        | 2       | 0/5       | 0/5       | 10$^{8.6}$ |
|                        | 3       | 2/5       | 0/5       | 10$^{8.6}$ |
|                        | 4       | 0/5       | 0/5       | 10$^{8.5}$ |
| NaOH (10%)$^c$         | 1       | 5/5       | 4/5       | 10$^{8.6}$ |
|                        | 2       | 5/5       | 4/5       | 10$^{8.6}$ |
|                        | 3       | 4/5       | 3/5       | 10$^{8.6}$ |
|                        | 4       | 5/5       | 5/5       | 10$^{8.6}$ |

$^a$ Contact time.  
$^b$ See footnotes to Table 1.  
$^c$ Values are expressed as positive/total number of tests.

**Table 3. Effect of virus concentration on the viricidal activity of disinfectants**

| Disinfectant | Conc | Viricidal activity$^a$ |
|--------------|------|------------------------|
|              |      | $10^{4.5-6}$ | $10^{3.5-4}$ | $>10^{3}$ |
| Na$_2$CO$_3$ | 10   | <10$^a$   | >30         | >30       |
|              | 3    | <30       | ND         | ND        |
| Cresylic acid | 1    | ND$^a$   | <10        | <10       |
| NaOH         | 3    | <5        | >30        | >30       |
|              | 10   | ND        | ND         | >30       |
| KOH          | 10   | <5        | <30        | >30       |
|              | 3    | <5        | ND         | >30       |
| Quaternary ammonium compound | 0.33 | ND       | >10        | >10       |

$^a$ Log LD$_{50}$ virus titer.

**DISCUSSION**

Disinfectant test methods for evaluation of viricides are much more complex than for the evaluation of bactericides. The virus recovery system requires the use of a living host which is susceptible to the toxic effect of viricides. In this study a dilution of the germicide was required to decrease the toxicity.

Methods reported previously were not adaptable in their entirety for evaluation of viricides against VSV. Lorenz and Jann (6) described a use dilution type test, a modification of the Association of Official Agricultural Chemists bactericidal test. This procedure was repeated by using porcelain carriers but was not an effective test method. Very few virus particles could be recovered from the carriers after 20 min of drying. The number of virus particles recoverable from each carrier had an LD$_{50}$ of about $10^{3.4}$ when the original number of virus particles in the suspension had an LD$_{50}$ of $10^{4.5}$ per 0.1 ml. This amount of virus on the carrier was not sufficient for a reliable viricide test.

Klein and DeForest (5) reported a viricidal test method using a suspension of virus. Their method was not applicable in its entirety, since an insufficient quantity of virus was used to test the viricide and the route of inoculation of embryonating eggs was not efficient for recovery of VSV. McClain and Madin (7) reported recovery of greater numbers of virus particles by the dropped membrane technique than by direct inoculation into the allantoic fluid. In this study it was confirmed that the dropped membrane procedure enabled the recovery of over 100 times more virus from the same sample.

The virus used in these tests was of a uniform resistance to a reference chemical, phenol. With phenol, the virus suspension was detectable after 5 min, but not after 10 min of contact, when the titer was LD$_{50}$ of $10^{7.5}$ to $10^{8.5}$. Eight-day embryonating eggs were required. The inoculum was placed on the surface of the CAM and incubated at 35.5 ± 0.5 C to obtain comparable results.

The loss in virus titer due to dilution of chemicals beyond toxic limits for embryos was 1 log. An effective germicide was one that reduced the virus titer by an LD$_{50}$ of $10^{8.5}$ virulent virus or more per 0.1 ml within 10 min. Three time intervals, 5, 10, and 30 min, were used initially for determination of viricidal action, but the majority of the disinfectants inactivated the virus within 10 min, and, therefore, the 30 min of contact time was eliminated in the final test.

The conditions for completion of the disinfectant tests were described including the best methods for recovery of VSV in eggs with the use of the dropped membrane technique and incubation at 35.5 C.
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ACKNOWLEDGMENTS

I acknowledge the helpful advice of E. M. Ellis and the technical assistance of Vera A. Valde.

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