

Use-Dilution Test and Newcastle Disease Virus

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ABSTRACT

LORENZ, DOUGLAS E. (University of California, Los Angeles), AND GREGORY J. JANN. Use-dilution test and Newcastle disease virus. *Appl. Microbiol.* **12**:24-26. 1964.—The use-dilution test for evaluating the effectiveness of disinfectants against bacteria was modified to determine the effectiveness of disinfectants against a group of viruses. Modifications were kept to a minimum to retain the general principles of the test and thereby retain the test's familiarity among testing laboratory personnel. Modifications included the use of a standard allantoic fluid suspension of Newcastle disease virus instead of a standard bacterial culture. The only other modification was the inoculation of six embryonated chicken eggs (10 to 12 days old) with 0.1 ml of nutrient broth into which a carrier ring was transferred after a standard period in diluted disinfectant. The death or survival of 60 embryos, then, is the criterion by which a disinfectant can be judged effective at use-dilution. Experiments are described which establish the validity of the modified test procedure. The effectiveness of nine common disinfectants against Newcastle disease virus as judged by this test procedure is reported.

Although several tests to determine the effectiveness of a disinfectant against bacteria have been standardized to the extent that they are commonly used, no such test has been presented for viruses. In general, viruses are probably as sensitive to common disinfectants as are bacteria. The assumption that a disinfectant which is effective against bacteria will be effective against viruses is probably the most practical approach testing laboratories can take toward evaluating disinfectants for their viricidal action. It must be emphasized, however, that some index of effectiveness for a disinfectant is needed if that disinfectant is to be used with confidence in areas of high viral contamination, such as in some hospital wards, animal quarters, and virus laboratories.

The purpose of this paper is to describe a modification of the use-dilution test for bacteria which can be used to evaluate the effectiveness of disinfectants against one group of viruses, the myxovirus group. The use-dilution test was modified only to the extent that the activity of viruses surviving a given disinfectant dilution must be determined in embryonated eggs. Therefore, the materials and methods vary only slightly from the standard bac-

terial test, and the modified test can easily be incorporated into routine disinfectant-testing programs.

Two terms will be used repeatedly in this description: *bacterial use-dilution test* which refers to the procedure described by the Association of Official Agricultural Chemists (1960) and *viral use-dilution test* which refers to the procedure described in this paper.

MATERIALS AND METHODS

The bacterial use-dilution test procedure is outlined here to orient the description of the modifications. In the bacterial use-dilution test 20 sterile carrier rings were placed in a standard, liquid culture of bacteria. After 15 min, ten rings were removed and dried at 37 C for 20 to 60 min. Subsequently, one ring was placed in a tube of disinfectant. The disinfectant was diluted to that concentration at which the manufacturer specifies its practical application. After 10 min, a ring was transferred to a tube containing 10 ml of standard broth, and this tube was incubated in a standard manner. Ten carrier rings manipulated in this manner constituted a bacterial use-dilution test. If the ten tubes showed no evidence of bacterial growth, the disinfectant was judged effective at use-dilution, i.e., the dilution at which it is to be used as prescribed by the manufacturer.

This procedure was modified for testing the activity of disinfectants against viruses in the following way. Instead of using a standard bacterial culture, a standard allantoic fluid suspension of Newcastle disease virus (NDV) was used. The carrier rings were placed in the virus suspension. Then they were dried and transferred to the disinfectant tubes in the exact manner described in the bacterial use-dilution test. But, instead of transferring a ring from the disinfectant to 10 ml of broth, it was transferred to 1 ml of broth. Subsequently, 0.1 ml of this broth was inoculated into the allantoic sac of each of six 10-day embryonated chicken eggs. If none of the inoculated embryos was killed by viral infection within 5 days after inoculation, the disinfectant was judged effective at the dilution tested.

The details of these modifications are now described.

Reagents and apparatus. The reagents and equipment required for the viral use-dilution test are the same as those listed for the bacterial use-dilution test described by the Association of Official Agricultural Chemists (1960). Additional material required includes: screw-capped, Pyrex tubes (150 by 20 mm) for storing virus suspensions; 100 by 15-mm tubes; 1-ml syringes, graduated in 0.1 ml;

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25-gauge hypodermic needles ($\frac{5}{8}$ in. long); an egg shell punch or electric drill with carborundum burr; egg racks; flexible collodion; normal saline; tincture of iodine; and fertile chicken eggs.

Fertile eggs may be purchased from commercial hatcheries, and arranged in the papier-mache holders in which they are obtained from the hatcheries. The eggs were incubated at 37 C, and were turned once a day during incubation.

Standard suspension of virus. An allantoic fluid suspension of NDV, California strain 11914, obtained from R. A. Bankowski, Davis, Calif., was used in these tests. Stock suspensions of the virus were prepared in the following manner. A stock allantoic fluid suspension of the virus was diluted 1:1,000 in normal physiological saline. Then 0.1 ml of this diluted suspension was inoculated into the allantoic sac of each of 50 or more 10-day embryonated eggs. At 1 day after inoculation, the eggs were candled, and all nonviable embryos were discarded. On the second day after inoculation, all embryos were usually dead from NDV infection. The eggs were then chilled by placing them in a refrigerator for several hours.

The allantoic fluid was harvested first by removing the shell above the air space of the egg. Then, by use of a sterile blunt instrument, such as forceps, the membranes were depressed and held away from the pipette which was used to withdraw the clear allantoic fluid. If the yolk sac was broken or excessive bleeding occurred, the contents of such an egg were discarded. The amount of infectious allantoic fluid from individual eggs varied from 2 to 10 ml, depending on the size of the egg and the success of avoiding the various membranes which tend to clog the pipette. All allantoic fluid was pooled in a single sterile flask. Subsequently, 22-ml samples were transferred to screw-capped tubes and stored at -20 C. Before use in a viral use-dilution test, a tube of virus suspension was melted and centrifuged at $1,500 \times g$ for 10 min to remove insoluble precipitates which formed when the fluid was

frozen. Only the clear supernatant fluid was used in the test.

The LD_{50} values of NDV suspensions were determined by the method described in *Methods for the Examination of Poultry Biologicals* (National Academy of Sciences, 1959). The LD_{50} of an average pool of NDV was normally greater than 10^{-9} , and no change was encountered in storage for 6 months at -20 C.

The method of inoculating eggs was as follows. The site of inoculation was marked with a small pencil mark at the time of candling the eggs. The site was at the junction of the air space and the chorioallantoic membrane, in an area of minimal vasculature. The site was swabbed with tincture of iodine, and a small hole was made in the shell without damaging the shell membrane underneath. The material to be inoculated was drawn into a syringe and then, holding the syringe vertically, the needle was plunged blindly into the allantoic sac. After 0.1 ml of material had been inoculated and the needle removed, the hole in the shell was sealed with collodion and the eggs were returned to the incubator.

Test proper. Carrier rings (20) were placed in 20 ml of clear allantoic fluid suspension of NDV which was incubated at 20 C. After 15 min, the rings were transferred to a Petri dish and dried for 20 to 60 min at 37 C. Then a ring was transferred to a tube containing 10 ml of diluted disinfectant which was also incubated at 20 C. After 1 min, the second ring was transferred to a second tube of disinfectant. This procedure was continued until ten rings had been transferred to disinfectant. At 10 min after the first ring had been transferred, it was removed and placed in 1 ml of nutrient broth contained in a tube (100 by 15 mm). This procedure was repeated until the ten rings were transferred to 1-ml portions of broth. These tubes of broth were also incubated at 20 C. Immediately after the last ring had been transferred, 0.1 ml of each broth suspension was inoculated into each of six 10- to 12-day embryonated eggs as described above. A different syringe was used for

TABLE 1. Effectiveness of nine disinfectants against Newcastle disease virus as determined by the viral use-dilution test

Disinfectant	Highest use-dilution prescribed	Highest dilution tested	Percentage of embryos killed by infection		Highest dilution at which all embryos survived
			No dilution	Use dilution	
Amphyl	1:400	1:1,000,000	None	None	1:1000
Ethanol	None	10%	None ^a	None ^b	20%
HetraCIDE	1:200	1:2,000,000	None	None	1:20,000
HetraPHYL	1:100	1:1,000,000	None	None	1:10,000
Iodine solution (N.F. X)	None	1:1,000	None	None ^b	1:1,000
Lysol	1:100	1:1,000,000	17%	33%	<1:10
Phenol, 5%	None	1:1,000	None ^c	None ^d	1:100
Roccal	1:5,000	1:1,000,000	None	None	1:10,000
Wescodyne	1:640	1:10,000	None	None	>1:10,000

^a Ethanol (70%) used.

^b Use-dilution equivalent to no dilution.

^c Phenol (5%) used.

^d Use-dilution considered 1:100.

each broth suspension. The eggs were made ready for inoculation before the test was begun. With a minimum of experience, the 60 eggs could be inoculated in less than 15 min. All procedures, except those involving the inoculations of eggs, were conducted as prescribed by the Association of Official Agricultural Chemists (1960).

On the day after inoculation, the eggs were candled, and all dead embryos were discarded from the test. Such deaths occurred from the trauma of inoculation. If more than one embryo in a set of six was killed nonspecifically, the test was considered invalid because only 40% or less of the 1-ml broth suspension were being sampled for active virus. The eggs were candled each day for an additional 4 days. If any embryos died during the period from the second to the fifth postinoculation day, the disinfectant was judged ineffective at the dilution tested. If all embryos, except those killed nonspecifically, survived, the disinfectant was judged effective at the dilution tested.

RESULTS AND DISCUSSION

It was necessary first to determine what proportion of virus was carried on the rings and survived the drying period. The LD_{50} of the virus stock suspension used in this series of experiments was $10^{-9.7}$ ml. The LD_{50} values of 10-ml portions of saline into which rings were placed after 20, 40, and 60 min of drying time were $10^{-5.5}$, $10^{-5.0}$, and $10^{-5.6}$ ml, respectively. These results indicate (i) that sufficient virus was carried on the rings into the disinfectant tubes and (ii) that the drying period of 20 to 60 min specified for the bacterial use-dilution test is satisfactory for the viral use-dilution test. Because the virus is not appreciably inactivated during the 60 min drying time, the drying period can be stated as a range of 20 to 60 min, as it is in the bacterial use-dilution test.

The second set of experiments was designed to determine (i) whether enough virus was inoculated into the embryonated eggs to establish infection, (ii) whether the disinfectant carried on the rings and eventually into the eggs affected the viability of the embryos, and (iii) whether a series of common disinfectants are effective against NDV at use-dilution by the viral use-dilution test.

Various disinfectants (Table 1) were used either undiluted, or diluted to use-dilution, or to a dilution at which the disinfectant had no effect on the virus. By not diluting the disinfectant, the embryos would be exposed to maximal amounts of disinfectant which could be carried on the rings. Thus, if these embryos survived, it could be assumed that they can tolerate the maximal amount of disinfectant available by this test procedure. By diluting the disinfectant to a very high value, the embryos would be exposed to maximal amounts of virus which could be inoculated by this procedure. Thus, if these embryos were killed, it could be assumed that sufficient virus was being inoculated to initiate infection, and the test would be valid.

It can be seen from the data presented in Table 1 that most of commercial disinfectants tested were effective at

use-dilution. Only one had a deleterious effect on the embryos. This particular disinfectant was not deleterious to the embryos when diluted 1:100 or more, however. At a dilution of 1:100, one-third of the embryos died of NDV infection as determined by hemagglutination tests on the allantoic fluids of the killed embryos.

The use of the hemagglutinating capacity of NDV to confirm the cause of death of an embryo demonstrates one advantage of using NDV in this test procedure. The hemagglutination test was conducted simply by mixing a drop of suspected, allantoic fluid with a drop of blood cells from a normal embryo. The blood cells clumped together within a few minutes after mixing the two materials. Normal or bacterial contaminated allantoic fluid did not agglutinate the blood cells.

In those tests using iodine-containing disinfectants, both nutrient broth and fluid Thioglycollate Medium (BBL) were used as the inoculating medium. No difference in the results of the tests was demonstrable.

Embryos of various ages were compared in an attempt to determine the maximal age range, and in this way make the test less restrictive. It was found, however, that embryos older than 12 days gave variable results. Thus, the age of the embryos must be standardized at 10 to 12 days.

NDV appears to be an excellent virus for use in a test such as the one described here. This virus is readily available, easily propagated, easily detected, and not highly contagious for laboratory personnel. This virus also is representative of a large group of viruses, including influenza, mumps, parainfluenza, and many other viruses associated with respiratory diseases of both man and animals.

There is one obvious short coming to this test procedure which must be mentioned. That is, the procedure is restricted to viruses which cause the death of chicken embryos. This limitation eliminates the use of enteroviruses and other more resistant viruses in this test procedure. This disadvantage could be circumvented by adapting the test to tissue-culture methods. Instead of using chick embryos as test hosts, or any laboratory animal susceptible to virus infection, the rings could be transferred to tubes of viable cells and the death or survival of these cells then would be the criterion of the effectiveness of the disinfectant. The bacterial use-dilution test was not modified to this extent simply because it was assumed that tissue culture methods are probably not yet commonplace in most testing laboratories. Egg inoculation procedures are simple enough to require only minor modifications of the use dilution test.

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