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Testing of disinfectants

Disinfectants used in hospitals and laboratories must be tested periodically to ascertain its potency and efficacy. As certain disinfectants lose potency on standing and addition of organic matter, their efficacy must be tested. While certain methods help in selecting the right dilution of disinfectant for use others test the efficacy of disinfectant already in use. Some methods compare the performance with that of phenol whereas other methods simply state if the disinfectant is effective or not. There are several methods of testing disinfectants, with their own advantages and disadvantages. All these tests can be allocated to one of the following disinfectant tests: carrier test, suspension test, capacity test, practical test, field test or in-use test.

Disinfection process validation is defined as "establishing documented evidence that a disinfection process will consistently remove or inactivate known or possible pathogens from inanimate objects."

Robert Koch described a disinfectant test in the article *Uber Desinfektion*, in 1881. A silk thread was contaminated by submersion in a liquid culture of *Bacillus anthracis*. After drying, the contaminated thread was immersed in several disinfectant solutions for a given exposure time. The thread was then cultured in a nutrient broth and no growth after incubation indicated activity of the disinfectant. He concluded from the comparisons of disinfectant solutions that mercuric chloride was the most active disinfectant. The results were erroneous as the disinfectants residues were also carried over to the subculture medium. The problem was overcome by Geppert in 1890 by neutralising the disinfectant at the end of exposure period.

Carrier tests:

These tests are the oldest tests. The test described by Robert Koch was a carrier test. In these tests, the carrier such as a silk or catgut thread or a penicylinder (a little stick) is contaminated by submersion in a liquid culture of the test organism. The carrier is then dried and is brought in contact with the disinfectant for a given exposure time. After the exposure, it is cultured in a nutrient broth; no growth indicates activity of the disinfectant tested whereas growth indicates a failing. By multiplying the number of test concentrations of the disinfectant and the contact times, a potentially active concentration-time relationships of the disinfectant is obtained. Example of a carrier test is the former use-dilution test of the American Association of Official Analytical Chemists (AOAC, 1990). Limitation of the carrier tests are: a) the number of bacteria dried on a carrier is hard to standardize and b) the survival of the bacteria on the carrier during drying is not constant.

The AOAC Use-dilution test is a carrier-based test. The organisms used are *Salmonella cholerasuis, S. aureus* and *P. aeruginosa*. Carriers (stainless steel cylinders) are meticulously cleaned, sterilized by autoclaving in a solution of aspargine, cooled and inoculated with a test organism by immersing in one of the culture suspensions. The cylinders are drained on filter paper, dried at 37^{o}C for 40 minutes, exposed to the use-dilution of the disinfectant for 10 minutes, and cultured to assess the survival of the bacteria. A single test involves the evaluation of 60 inoculated carriers (one organism) against one product sample. In addition to the 60 carriers, 6

carriers are required to estimate carrier bacterial load and 6 more are included as extras. Thus, a total of 72 seeded carriers are required to perform a single test. A result showing no growth in all ten tubes confirms the result of phenol coefficient test. If any carrier produces growth, the test must be repeated using a lower dilution of the disinfectant. Use-dilution test is performed to confirm the efficiency of disinfectant dilution derived from phenol coefficient test.

Suspension tests:

In these tests, a sample of the bacterial culture is suspended into the disinfectant solution and after exposure it is verified by subculture whether this inoculum is killed or not. Suspension tests are preferred to carrier tests as the bacteria are uniformly exposed to the disinfectant. There are different kinds of suspension tests: the qualitative suspension tests, the test for the determination of the phenol coefficient (Rideal and Walker, 1903) and the quantitative suspension tests. Initially this was done in a qualitative way. A loopful of bacterial suspension was brought into contact with the disinfectant and again a loopful of this mixture was cultured for surviving organisms. Results were expressed as 'growth' or 'no growth'. In quantitative methods, the number of surviving organisms is counted and compared to the original inoculum size. By subtracting the logarithm of the former from the logarithm of the latter, the decimal log reduction or microbicidal effect (ME) is obtained. An ME of 1 equals to a killing of 90% of the initial number of bacteria, an ME of 2 means 99% killed. A generally accepted requirement is an ME that equals or is greater than 5: at least 99.999% of the germs are killed. Even though these tests are generally well standardized, their approach is less practical.

Determination of phenol coefficient:

Phenol coefficient of a disinfectant is calculated by dividing the dilution of test disinfectant by the dilution of phenol that disinfects under predetermined conditions.

Rideal Walker method:

Phenol is diluted from 1:400 to 1:800 and the test disinfectant is diluted from 1:95 to 1:115. Their bactericidal activity is determined against *Salmonella typhi* suspension. Subcultures are performed from both the test and phenol at intervals of 2.5, 5, 7.5 and 10 minutes. The plates are incubated for 48-72 hours at 37°C. That dilution of disinfectant which disinfects the suspension in a given time is divided by that dilution of phenol which disinfects the suspension in same time gives its phenol coefficient.

Disinfectant	Dilution	Growth of test organism in subculture after exposure for:			
		2.5 mins	5 mins	7.5 mins	10 mins
Test disinfectant	1:400	NG	NG	NG	NG
	1:500	G	NG	NG	NG
	1:600	G	G	NG	NG
	1:700	G	G	G	NG
	1:800	G	G	G	G
Phenol	1:95	G	NG	NG	NG
	1:100	G	G	NG	NG
	1:105	G	G	G	NG
	1:110	G	G	G	NG
	1:115	G	G	G	G

For example, after 7.5 minutes, the test organism was killed by the test disinfectant at a dilution of 1;600. In the same period the test organism was killed by phenol at a dilution of 1:100.

$$\text{Phenol coefficient} = \frac{600}{100} = 6$$

This result indicates that the test disinfectant can be diluted six times as much as phenol and still possess equivalent killing power for the test organism.
Disadvantages of the Rideal-Walker test are: No organic matter is included; the microorganism *Salmonella typhi* may not be appropriate; the time allowed for disinfection is short; it should be used to evaluate phenolic type disinfectants only.

Chick Martin test: This test also determines the phenol coefficient of the test disinfectant. Unlike in Rideal Walker method where the test is carried out in water, the disinfectants are made to act in the presence of yeast suspension (or 3% dried human feces) to simulate the presence or organic matter. Time for subculture is fixed at 30 minutes and the organism used to test efficacy is *S.typhi* as well as *S.aureus*. The phenol coefficient is lower than that given by Rideal Walker method.

	Rideal -Walker	**Chick-Martin**
Volume medium	5.0 ml	10.0 ml
Diluent for test disinfectant	Distilled water	Water with yeast suspension or feces
Reaction temperature	17.5±0.5ºC	30ºC
Organism	*Salmonella typhi*	*Salmonella typhi, Staphylococcus aureus*
Sampling times	2.5, 5.0, 7.5, 10.0 min.	30.0 min.
Calculation of coefficient	Dilution test killing in 7.5 mins divided by same for phenol	Mean concentration of phenol showing no growth after 30 min. divided by same for test

The phenol coefficient test recommended by AOAC included two test organisms (*S. aureus* and *P. aeruginosa*) and included the disinfectant inactivators in the recovery medium. The recovery medium Letheen broth contains the inactivator Lecithin and Polysorbate 80. In separate tests, the bacterial suspensions are added to standard dilutions of pure phenol and several dilutions of the test disinfectant. After contact time of 5, 10 and 15 minutes, samples are transferred to the recovery medium by a standard wire loop. When the positive and negative cultures have been recorded, the result of the test is expressed as phenol coefficient. It is calculated by dividing the highest dilution of the disinfectant that kills the test inoculum in ten minutes but not in five minutes by the dilution of phenol that gives the same result.

Disinfectant kill time test
This test was designed to demonstrate log reduction values over time for a disinfectant against selected bacteria, fungi, and/or mold. The most common organisms tested include: *Bacillus subtilis, Bacillus atrophaeus, Bacillus thuringiensis, Staphylococcus aureus, Salmonella cholerasuis, Pseudomonas aeruginosa, Aspergillus niger*, and *Trichophyton mentagrophytes*. A tube of disinfectant is placed into a waterbath for temperature control and allowed to equilibrate. Once the tube has reached temperature, it is inoculated to achieve a concentration of approximately 10^6 CFU/mL. At selected time points (generally five points are used including zero) aliquots are removed and placed into a neutralizer blank. Dilutions of the neutralizer are made and selected dilutions plated onto agar. Colonies are enumerated and log reductions are calculated.

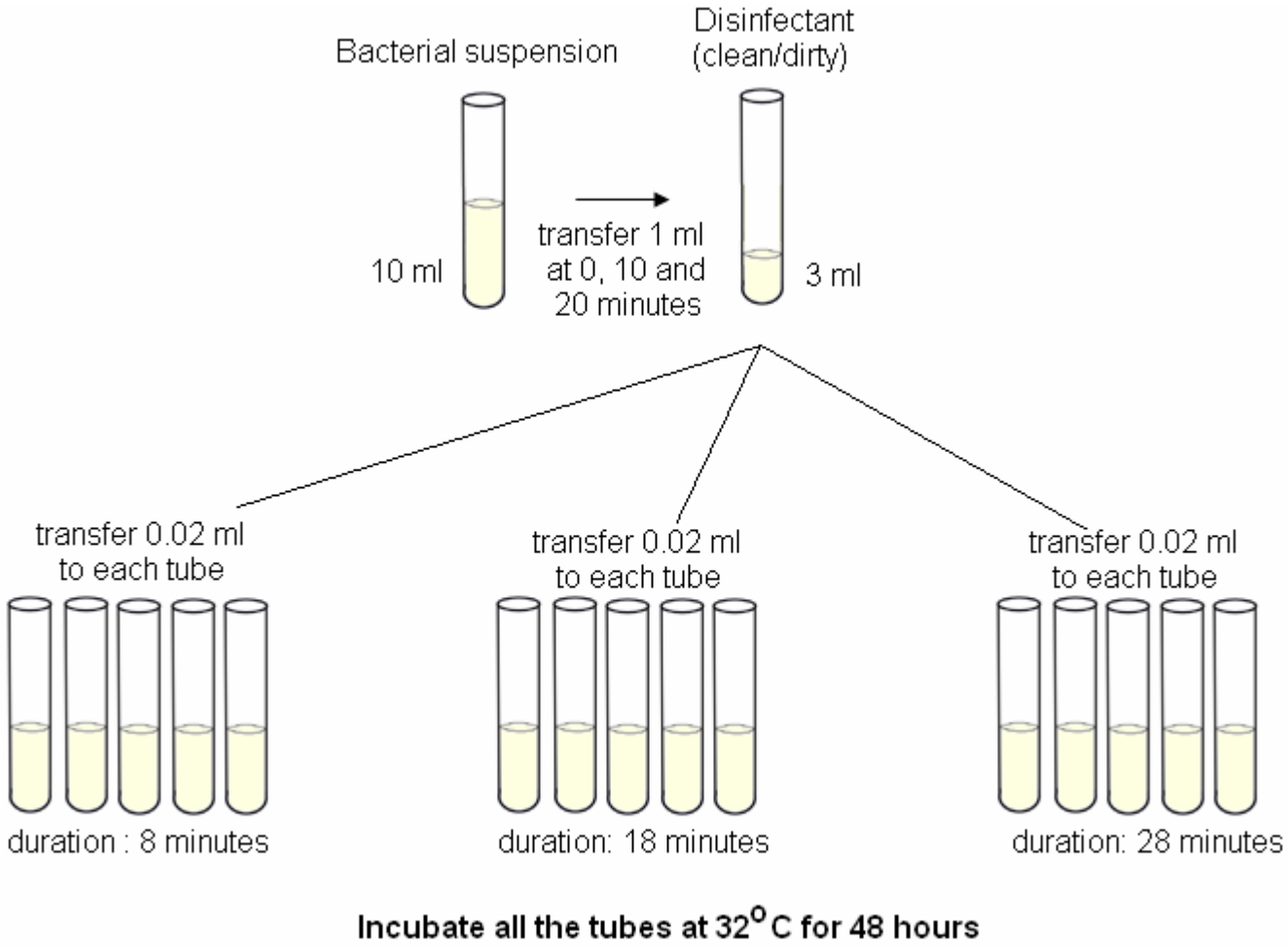
Capacity tests:

Each time a soiled instrument is placed into a container with disinfectant, a certain quantity of dirt and bacteria is added to the solution. The ability to retain activity in the presence of an increasing

load is the capacity of the disinfectant. In a capacity test, the disinfectant is challenged repeatedly by successive additions of bacterial suspension until its capacity to kill has been exhausted. Capacity tests simulate the practical situations of housekeeping and instrument disinfection. The best known capacity test is the Kelsey-Sykes test (Kelsey and Sykes, 1969).

Kelsey-Sykes test is a triple challenge test, designed to determine concentrations of disinfectant that will be effective in clean and dirty conditions. The disinfectant is challenged by three successive additions of a bacterial suspension during the course of the test. The duration of test takes over 30 minutes to perform. The concentration of the disinfectant is reduced by half by the addition of organic matter (autoclaved yeast cells), which builds up to a final concentration of 0.5%. Depending on the type of disinfectant, a single test organism is selected from *S. aureus, P. aeruginosa, P. vulgaris* and *E. coli*. The method can be carried out under 'clean' or 'dirty' conditions. The dilutions of the disinfectant are made in hard water for clean conditions and in yeast suspension for dirty conditions. Test organism alone or with yeast is added at 0, 10 and 20 minutes interval. The contact time of disinfectant and test organism is 8 min.

The three sets of five replicate cultures corresponding to each challenge are incubated at 32^{o}C for 48 hours and growth is assessed by turbidity. The disinfectant is evaluated on its ability to kill microorganisms or lack of it and the result is reported as a pass or a fail and not as a coefficient. Sets that contain two or more negative cultures are recorded as a negative result. The disinfectant passes at the dilution tested if negative results are obtained after the first and second challenges. The third challenge is not included in the pass/fail criterion but positive cultures serve as inbuilt controls. If there are no positive cultures after the third challenge, a lower concentration of the disinfectant may be tested.

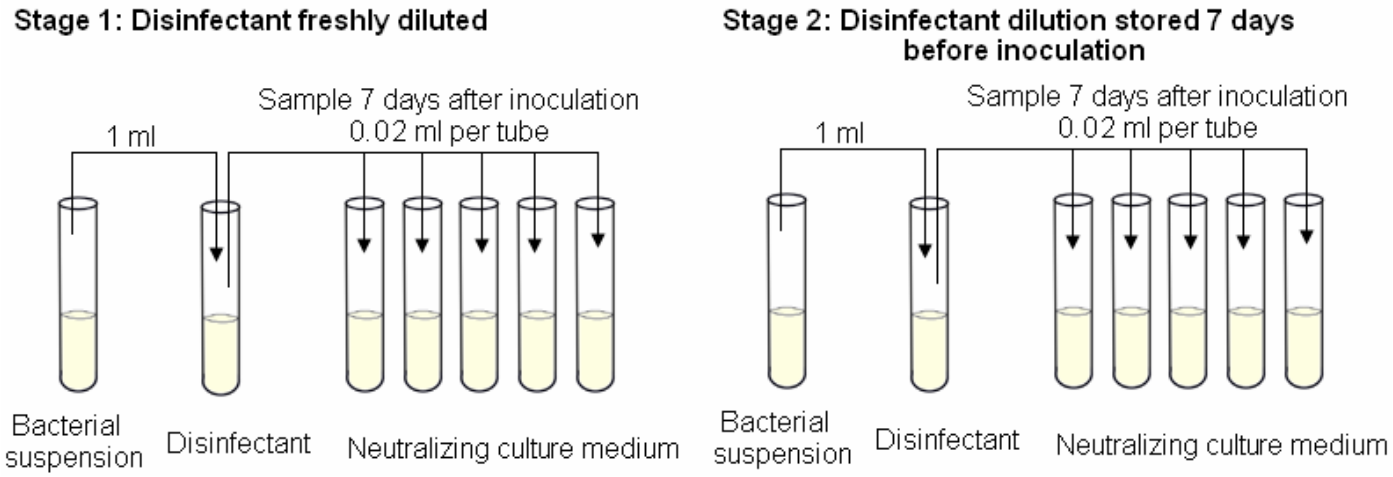


Specimen result of a test

Concentration	Inoculum count	Challenge number			Result
		1	2	3	
1.0	2×10^9	+++++	+++++	+++++	Fail
1.5	2×10^9	- - - -+	- -+++	+++++	Pass
2.0	2×10^9	- - - - -	- - - - -	- - - - +	Pass

The capacity test of Kelsey and Sykes gives a good guideline for the dilution of the preparation to be used. Disadvantage of this test is the fact that it is rather complicated.

Test for stability and long-term effectiveness:
Recommended concentrations based on Kelsey Sykes test apply only to freshly prepared solutions but if the solutions are likely to be kept for more than 24 hours, the effectiveness of these concentrations must be confirmed by a supplementary test for stability of unused solution and for the ability of freshly prepared and stale solutions to prevent multiplication of a small number of bacteria that may have survived the short term exposure. *P. aeruginosa* is used a test organism. Sufficient disinfectant solution is prepared for two tests. One portion is inoculated immediately and tested for growth after holding for seven days at room temperature. The other portion is kept at room temperature for seven days and then inoculated with a freshly prepared suspension of test organism. It is also tested for growth seven days after inoculation. If growth is detected, a higher concentration of disinfectant must be tested in the same way.



Practical tests:

The practical tests under real-life conditions are performed after measuring the time-concentration relationship of the disinfectant in a quantitative suspension test. The objective is to verify whether the proposed use dilution is still adequate in the conditions under which it would be used. The best known practical tests are the **surface disinfection tests**. Surface tests assess the effectiveness of the selected sanitizer against surface-adhered microorganisms. The test surface (a small tile, a microscopic slide, a piece of PVC, a stainless steel disc, etc.) is contaminated with a standardized inoculum of the test bacteria and dried: then a definite volume of the disinfectant solution is distributed over the carrier; after the given exposure time the number of survivors is determined by impression on a contact plate or by a rinsing technique, in which the carrier is rinsed in a diluent, and the number of bacteria is determined in the rinsing fluid. In order to determine the spontaneous dying rate of the organisms caused by drying on the carrier, a control series is included in which the disinfectant is substituted by distilled water; from the comparison of the survivors in this control series with the test series, the reduction is determined quantitatively.

There is an essential difference between a carrier test and a surface disinfectant test: in the former case the carrier is submerged in the disinfectant solution during the whole exposure time, whereas in the latter case the disinfectant is applied on the carrier for the application time and thereafter the carrier continues to dry during the exposure. Surface tests can reflect in-use conditions like contact times, temperatures, use-dilutions, and surface properties.

Surface Time kill Test

A 24 hour culture in nutrient broth culture is prepared. A volume of microbial culture (usually 0.010 mL to 0.020 mL) is placed onto the center of each of a number of sterile test surfaces. This inoculum can be spread over the sterile test surface in a circular pattern to achieve a thin, uniform coverage with the test microorganism if desired. To measure initial microbial concentrations, one or more untreated, inoculated test surfaces are harvested and microorganisms are enumerated. The remaining inoculated test surfaces are treated with the disinfectant, each for a different length of time. Immediately after the treatment times have elapsed, the test surfaces are placed into a solution that neutralizes the disinfecting action of the product, and microorganisms surviving treatment with the disinfectant or sanitizer are cultured and enumerated. Results of the timekill study are tabulated and reported, usually by charting microbial concentrations on the test surfaces as a function of treatment time with the disinfectant or sanitizer.

In-use test:

A simple to use test was described by Maurer in 1985 that can be used in hospitals and laboratories to detect contamination of disinfectants. A 1 ml sample of the disinfectant is added to 9 ml diluent which also contains an inactivator. Ten drops, each of 0.02 ml volume of the diluted sample are placed on each of two nutrient agar plates. One is incubated at $37^{o}C$ for three days and the other at room temperature for seven days. Five or more colonies on either plate indicate contamination.

British standard tests for quaternary ammonium compounds:

This test was initially described in 1960 to distinguish bactericidal action from the high level of bacteriostatic activity, which is characteristic of QACs. This test is also applicable to other bactericides such as chlorhexidine and synthetic phenols. The inactivator used contains 2% lecithin and 3% non-ionic detergent (polysorbate 80). The test is performed using suspensions of gram negative and gram positive bacteria with or without the inclusion of organic matter. If a series of samples are taken from a dilution of the disinfectant containing 5×10^{8} to 5×10^{9} bacteria per ml at the start of the test, a death curve may be prepared from the colony counts on the agar containing recovery medium and reduction factors up to 10^{6} (99.99% kill) can be verified.

In order to determine the antimicrobial value of QAC, it was revised as the highest dilution of the disinfectant that, under the test conditions, will reduce the microbial population to a colony count not greater than 0.01% of that in the control. In this revision, E. coli was used and the contact time was 10 minutes. The challenge medium is *E. coli* culture suspension with equal amount of horse serum. One ml of the challenge is added to nine ml of each dilution of the disinfectant along with two control tubes containing diluent alone. At the end of exposure period, one ml each of the mixture is added to 9 ml of inactivator and the surviving bacteria are counted as colony forming units on agar plates.

Testing schemes:

The antimicrobial efficiency of a disinfectant is examined at three stages of testing. The first phase concerns laboratory tests in which it is verified whether a chemical compound or a preparation possesses antimicrobial activity: for these preliminary screening tests essentially quantitative suspension tests are considered. The second stage is still carried out in the laboratory but in conditions simulating real-life conditions. Not disinfectants, but disinfection procedures are

examined. It is determined in the practical tests in which conditions and at which use-dilution after a given contact time the preparation is active. The third phase comprises the field tests or pilot studies, and the variant of in-use tests. In these tests it is verified whether, after a normal period of use, germs in the disinfectant solution are still killed.

Most studied are the bactericidal tests in which the activity towards vegetative bacteria is examined. AOAC has schedules that are applicable for fungi and yeasts too (fungicidal tests), for mycobacteria (tuberculocidal tests), for viruses (virucidal tests) and for spores of bacteria (sporicidal tests).

Bactericidal tests:
A bactericidal test must include the following sequence of steps:
1. The test organism is exposed to a suitable concentration of the disinfectant
2. Samples are taken at specified times and added immediately to a diluent or culture medium containing the appropriate disinfectant inactivator
3. The treated samples are cultured for surviving microorganisms.

Test organisms:
Specified strains (usually ATCC) of S*. aureus, P. aeruginosa, P. vulgaris* and *E. coli* are usually recommended. A synthetic broth is recommended for preparing a series of subcultures to be used in the tests. The 24-hour broth culture may be used without further treatment; however, it is usually filtered (to remove slime) and centrifuged. The washed bacteria are resuspended in hard water to which autoclaved yeast or serum may be added to simulate dirty conditions. Finally, the suspension is shaken with glass beads on a vortex mixer and a viable count is set up immediately before performing the test.

The disinfectant:
The concentration or dilution of the disinfectant to be tested may be based on manufacturer's recommendations. The solutions should be prepared on the day of test. Distilled water or standard hard water is used to make dilutions. Tap water is unsuitable because it may contain chemicals that may precipitate with some disinfectants.

References:
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Joan F.Gardner, Margaret M Peel. 1991. Introduction to sterilization and disinfection control, 2nd edition, Churchill Livingstone