Application of Membrane Filtration to Antibiotic Quality Control Sterility Testing

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Membrane filtration procedures for antibiotic sterility testing as described in the recently amended Antibiotic Regulations of the Food and Drug Administration (FDA) offer an improved approach for determining the sterility of antibiotics.

FOR ALMOST 20 years the Antibiotic Regulations[1] of the Food and Drug Administration for the sterility testing of antibiotics required 10 containers from each batch to be tested, six in thioglycollate broth for bacteria and four in Sabouraud fluid medium for molds and yeasts (1). In testing penicillin preparations, the antibiotic was inactivated by the addition of penicillinase to the thioglycollate medium. Since there were no suitable inactivators for the nonpenicillin antibiotics, no inactivator was added to the medium used for testing them, and for the most part any organisms detected were those highly resistant to the inhibitory action of the particular antibiotic.

DISCUSSION

To improve the sterility test, several possibilities were investigated. Serial dilution techniques to reduce the antibiotic to a subminimal inhibitory concentration were explored. This procedure is unsatisfactory since it also dilutes the contaminating organisms. Dilution of the antibiotic sample by large initial volumes of culture media was found to be both costly and impractical. Finally, a filtration procedure was developed employing a membrane filter composed of cellulose esters (2). The antibiotic preparations were first solubilized and the solution was filtered through a circular membrane filter approximately 47 mm. in diameter, with a porosity of 0.43–0.47 μ. Any organisms contaminating the preparation were trapped on the membrane, which was then washed with sterile water to remove any residual antibiotic without affecting the organism. Portions of the washed filter disk were placed in sterile fluid thioglycollate for 5 days at 32°.

Research on the application of the membrane filtration technique to the sterility testing of the antibiotic drugs produced practical methods for solubilizing and filtering each antibiotic preparation. Modifications were made to accommodate antibiotic powders and various formulations in which they are incorporated.

Use of the filtration technique for testing the tetracycline antibiotics eliminates two major problems. The antibiotic which would inhibit the growth of bacterial contamination, if present, is removed and degradation is avoided. When tetracyclines are added to thioglycollate medium, the breakdown products produce acidity, turbidity, and discoloration of the medium. The acidity prevents growth of most contaminating microorganisms, and the turbidity and dark color prevent visual observation of contamination.

Sodium novobiocin and amphotericin B also presented problems in that the slight acidity of Sabouraud broth rendered these antibiotics insoluble, again interfering with visual detection of growth. Filtration eliminates this difficulty. The antibiotics are dissolved in 0.1% peptone solution, filtered, and washed three times. For practical purposes no antibiotic is transferred to the Sabouraud broth because none remains on the membrane.

The procedures for the sterility testing for antibiotics published March 28, 1964 (3), gave membrane filtration procedures official status and culminated years of evaluating their merit. In addition to increasing the sensitivity of the test by the use of filtration procedures, the amended regulations increased the sample size to 20 representative units from each "filling operation" to be tested in both of the media previously mentioned. The term "filling operation" is defined as that period of time not longer than 24 consecutive hr. during which a homogeneous quantity of a drug is being filled continuously into market-size containers and during which no changes are made in the equipment used for filling. Both the direct method and the membrane filtration procedure are described in detail in the regulations. The direct method is used for insoluble preparations and the membrane filtration for soluble preparations. The membrane filtration procedure specifies that 300 mg. of a solid drug or 1 ml. by volume of a liquid drug from each of 20 immediate containers be aseptically transferred to 200 ml. of 0.1% (w/v) peptone solution. Since bacterial contamination is not related to the dosage of the antibiotic tested, a constant amount (weight or volume) of the antibiotic was selected for each sterility test. After complete solubilization, the antibiotic solution is filtered through a bacteriological membrane filter. All air entering the system is passed through air filters capable of removing microorganisms. Three 100-ml. portions of 0.1% peptone water are then filtered through the membrane to remove as much as possible of the residual antibiotic. A 17.5-mm. diameter disk is aseptically cut from the center of the filtering area and transferred to a sterile 38 × 200-mm. (outside dimensions) test tube containing approximately 90 ml. of sterile thioglycollate medium. The remaining portion of the membrane is transferred to a second similar tube containing approximately 90

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[1] The Antibiotic Regulations are issued under Section 507 of the Federal Food, Drug, and Cosmetic Act.

ml. of fluid Sabouraud medium. The tubes are incubated for 7 days at 32° and 25°, respectively, and then examined for visible growth. The batch meets the requirements of the test if no tube shows growth. If growth is observed in any tube, the test is repeated with twice the number of containers. The batch meets the requirements if no tube on the repeat test shows growth.

The regulations require that specific environmental tests to assess the suitability of the testing conditions be made frequently enough to assure the validity of the test results. The responsible head of each sterility testing laboratory must determine which methods are to be used for air analysis and what levels of microbial contamination of the air are significant and tolerable. Special devices for sampling air to determine its microbial content are helpful in evaluating the conditions of the areas in which the tests are performed. Some of the devices and techniques commonly used for the microbiological analysis of air are the settling plate technique, the sieve and slit-type samplers, and liquid impingement devices. Although settling plates are easy to use, they yield a limited amount of information, since only particles of certain dimensions will settle onto the plates. Another disadvantage is that the volume of air sampled cannot be measured. The liquid impingement samplers, which force a definite quantity of air through an impingement fluid, recover all of the airborne contamination in the volume of air sampled, thus giving more quantitative information. In addition to requiring tests to assess the area in which the sterility tests are performed, the regulations specify that tubes of thioglycollate to which penicillinase has been added must be tested for sterility, either prior to use or at the time of the test. If the environmental or other tests show sufficient evidence that the results obtained in the first or second official sterility tests are not valid, additional tests may be performed.

As previously noted, 0.1% peptone solution was chosen as the diluting fluid for the filtration procedure. Sterile distilled water or physiological saline had been used for many years to dissolve antibiotics prior to transferring them to the media in the sterility tests (4). The deleterious effect of distilled water on some microorganisms has been well documented in the literature (5). (The "British Pharmacopoeia" specifies sterile physiological saline for dissolving antibiotic powders, and U.S.P. XVII requires that a sterile diluent be used to dissolve solids for the general sterility test.) A diluting fluid which would minimize the destruction of small populations of vegetative cells during the pooling, solubilizing, and filtering of antibiotics was sought. A diluting fluid affording protection to vegetative cells is a necessity when a large number of filtration sterility tests are performed on the same day because each test cannot be carried to completion before another test is started. Therefore, the diluent must protect the vulnerable vegetative cells in an antibiotic milieu until they are safely separated from the antibiotic and transferred to the growth medium.

Straka and Stokes (6) reported that destruction of vegetative bacteria can be minimized by the use of 0.1% peptone solution. Jane-Williams (7) studied the survival of bacteria in six different diluents and found that 0.1% peptone solution was the only one that did not kill one or more of the test organisms.

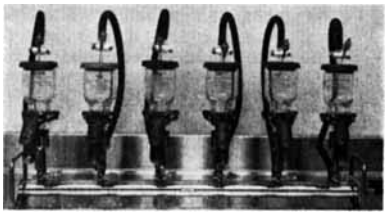

Fig. 1.—Sterility test unit.

EXPERIMENTAL

Equipment.—To accommodate the large number of samples tested daily for quality control by filtration techniques, new equipment had to be designed. The filtering apparatus used in developing the technique has been assembled by a manufacturing firm into a compact "sterility test unit" (as shown in Fig. 1) consisting of six separate filtering devices on a manifold. This unit was designed expressly for sterility testing of antibiotics and was produced to meet the specific requirements described in the Antibiotic Regulations.

The completely assembled unit is steam sterilized at 121° for 20 min., with stopcocks open and membranes in position in the funnels. It is brought into the filtering area where, after all stopcocks have been closed, the metal intake tube of the manifold is aseptically attached to an overhead bottle of sterile 0.1% peptone solution. The metal exhaust tube of the manifold is attached by means of a connecting hose to an empty bottle. The latter, in turn, is connected to a vacuum outlet. All exhaust valves are turned to an "off" position. The vacuum is then turned on, and the unit is ready for use.

Tests on Diluting Fluids.—The survival of organisms in antibiotics dissolved in 0.1% peptone solution, distilled water, and 0.85% sodium chloride solution was compared. No attempt was made to adjust the pH of the distilled water or saline. However, the pH ranged between 6.5 and 6.9 before the addition of the antibiotic, and the pH of the 0.1% peptone solution was 7.0 ± 1. Bacteriological peptone (Difco B 118) was used. The seed organisms were added to two flasks each containing 200 ml. of the given diluent in which the antibiotic powder was dissolved. One flask of each diluent was filtered immediately and the second flask was filtered after 1 hr. After the solutions containing the organisms were filtered through a 47-mm. diameter membrane having a porosity of 0.45 μ, the membranes were washed three times with 100 ml. of the individual diluent. Each membrane was then aseptically moved to the surface of sterile nutrient agar in sterile Petri dishes (20 × 100 mm.). The plates were covered with sterile porcelain tops, glazed on the outside, and incubated at 32° for 5 days. The colonies were counted and the survival was noted for each contact period, initially and after 1 hr. The amount of each antibiotic used was the amount required for the sterility test of that particular antibiotic in the Antibiotic Regulations. For example, 300 mg. of sodium penicillin G from 20 immediate containers is the amount pooled for a

TABLE I.—VIABILITY OF ORGANISMS RECOVERED FROM ANTIBIOTIC SOLUTIONS FILTERED IMMEDIATELY[a] AND AFTER 1 hr. AT 25°C.

Organism	Antibiotic	Amt., Gm.	Estimated No. Cells	Recovery After 5 Days at 32°C. Peptone, 0.1% Ini.	Peptone, 0.1% 1 hr.	Saline, 0.85% Ini.	Saline, 0.85% 1 hr.	Distilled Water Ini.	Distilled Water 1 hr.	Medium
S. aureus ATCC 6538P	Tetracycline hydrochloride	1.0	40	36	28	28	2	34	0	Peptone–casein agar
Serratia marcescens ATCC 14756	Tetracycline hydrochloride	1.0	20	18	16	16	10	18	14	Peptone–casein agar
Streptococcus pyogenes ATCC 8668	Tetracycline hydrochloride	1.0	50	34	22	2	0	6	0	Peptone–casein agar
Bacillus subtilis (spores)[b]	Dihydrostreptomycin sulfate	6.0	55	50	48	42	50	52	48	Peptone–casein agar
Aspergillus sp. (spores)	Polymyxin B′ sulfate	6.0	42	38	37	40	38	35	42	Sabouraud dextrose agar
S. aureus ATCC 6538P	Sodium penicillin G	6.0	30	20	28	18	0	12	4	Peptone–casein agar
Escherichia coli ATCC 11699	Sodium penicillin G	6.0	64	50	44	44	0	52	0	Bacto E.M.B. agar

[a] As soon as the organisms were added. [b] ATCC 6633 strain made resistant to dihydrostreptomycin.

test. Therefore, 6-Gm. portions were dissolved in each of two flasks containing 200 ml. of each of the three diluents. As shown in Table I, the survival of spores of either bacteria or fungi was not affected either by the nature of the diluent or by the time of exposure to the antibiotic solution. However, the vegetative cells added to the antibiotic solutions and immediately filtered survived to approximately the same extent in each diluent. When the filtration was delayed for 60 min., the survival rate was higher in 0.1% peptone solution.

Sodium Thioglycollate.—The fluids for solubilizing insoluble antibiotics should be nontoxic to microorganisms. Although no sterile zinc bacitracin products are in use, an aerosol spray can containing zinc bacitracin for topical use is tested. The Antibiotic Regulations require this product to contain no more than 10 microorganisms per container. To prepare it for the test it must be filtered through a membrane filter in the same way as samples tested for absolute sterility. Early attempts to solubilize zinc bacitracin (for sterility testing) were unsuccessful. For potency assay zinc bacitracin is dissolved in 0.1 *N* hydrochloric acid, but for sterility testing the acidic solution cannot be used. It was noted that zinc bacitracin is soluble in thioglycollate medium but not in Sabouraud medium. Investigation disclosed that the chelating properties of sodium thioglycollate were responsible for the solubilization. Consequently, 0.05% sodium thioglycollate is added to the 0.1% peptone solution to solubilize zinc bacitracin prior to filtration. Since the required concentration of sodium thioglycollate is the same as that in thioglycollate medium, there is no problem of toxicity to microorganisms when it is used as a component of the diluting fluid.

Polysorbate.—To overcome filtration difficulties with a tetracycline aerosol containing isopropyl myristate, 0.5% polysorbate 80 is added to the 0.1% peptone water. (Aerosols to be tested for sterility by using filtration procedures are sprayed into an empty sterile flask, and after the propellant evaporates, the residue is dissolved.)

Penicillinase.—To solubilize procaine penicillin G alone or in combination with streptomycin, the enzyme penicillinase is aseptically added to the peptone diluting fluid. Bowman (8) reported that procaine penicillin G could be solubilized rapidly by using a high-titer penicillinase. It has been found that approximately 337,000 Levy units of penicillinase will solubilize 6 Gm. of procaine penicillin G for the membrane filtration sterility test.

Isopropyl Myristate.—The use of isopropyl myristate as a diluent for petrolatum-based ointments was first suggested by Sokolski (9), who improved the recovery of viable cells from ointments by a filtration technique. Isopropyl myristate dissolves certain sterile petrolatum-based antibiotic ointments so that these preparations can also be tested by membrane filtration. The Antibiotic Regulations have been amended (10) to include a sterility test for sterile ophthalmic ointments containing bacitracin, neomycin, and polymyxin. Portions of 0.1 Gm. from each of 10 immediate ointment containers are aseptically transferred to 100 ml. of isopropyl myristate at 47°. The process is then repeated with another 10 ointment containers. After the ointment has dissolved, the solutions are filtered as described for the membrane filtration sterility test. Since it is difficult to wash residual antibiotics, especially neomycin, from membranes through which oleaginous preparations have been filtered, a rinse medium has been devised to help overcome this problem. The rinse medium contains bacteriological peptone and beef extract to protect the vegetative cells during the rinsing period. (Each filter is rinsed five times with 100 ml. of the rinse medium.) Polysorbate 80 is included in the rinse medium to decrease the filtration time, and sodium chloride 3% (w/v) is added to reverse the action of the neomycin which adheres to the membrane.

RESULTS AND CONCLUSIONS

As previously mentioned, the Antibiotic Regulations contain instructions for performing sterility tests by both membrane filtration and the direct method. Some antibiotics, sodium penicillin G for example, may be tested by either method. However, if the direct method is used, the ability of the penicillinase to inactivate all the penicillin in the

sample under test is checked by adding the proper amount of penicillin to another tube of thioglycollate medium containing the penicillinase and inoculating it with 1 ml. of a 1:1000 dilution of an 18–24 hr. culture of *Staphylococcus aureus* ATCC 6538P. Typical microbial growth must be observable after 24 hr.

The regulations also include any exceptions required for testing specific antibiotics. When 200 ml. of a 3% solution of an antibiotic cannot be filtered, as in the case of tetracyclines or chloramphenicol, 50-mg. portions are used from each of 20 containers instead of the 300 mg. required by the general method.

Investigational studies and experience have shown that the filtration procedure offers better assurance of the sterility of antibiotics. Bowman and Holdowsky (11), in a study of the survival of bacteria in dihydrostreptomycin sulfate solutions, showed the superiority of the membrane filter method over the direct method in recovering organisms sensitive to dihydrostreptomycin. Dihydrostreptomycin solutions were artificially contaminated with 50,000 spores/ml. of six different bacteria whose vegetative cells were sensitive to dihydrostreptomycin. When membrane filtration was used, organisms were consistently recovered at intervals up to 3 months (the duration of the testing period). The same solutions gave negative results when tested by the direct method.

Lightbown (12) reported on dihydrostreptomycin solution contaminated with up to 4000 viable particles per vial of a relatively resistant strain of *Klebsiella pneumoniae* or a sensitive strain of *S. aureus* and examined by the "British Pharmacopoeia" dilution method and by membrane filtration. With the resistant strain of *K. pneumoniae*, 34 samples out of 87 were found to be contaminated by the filtration test, while the dilution test detected no contamination; with *S. aureus* 54 samples out of 93 were found contaminated when tested by filtration, while the dilution test detected no contamination. He also reported that 7-year-old batches of dihydrostreptomycin sulfate tested by filtration procedures revealed the presence of contaminating organisms which had survived in the dry powder. The contamination had not been revealed by earlier dilution tests.

Twenty vials, each containing 10 ml. of a suspension of procaine penicillin G in dihydrostreptomycin sulfate solution, were tested in this laboratory and found contaminated by the filtration procedure. An additional 40 vials were retested by both the filtration method and the direct method. The membrane filtration method recovered the same contaminant, a Gram-positive bacillus. The direct method showed no growth in 40 tubes of thioglycollate or 40 tubes of Sabouraud medium after incubation for 7 days at 32° and 25°, respectively.

In the first year after the new sterility regulations became effective, a total of 8,190 sterility tests were performed. The membrane method was used for 4,743 and the direct method for 3,447. The filtration procedures were found to reduce the cost of sterility testing, the reduction being due primarily to significant savings of media and of manhours involved in the preparation of media. When a preparation was submitted for certification with 25 "filling operations" from one batch, the better coverage and the economy of the filtration method were apparent. Under the former regulations, 10 of these single-dose containers would have been tested, whereas under the new regulations, 1,000 were tested. Had this been a multiple dose container, only 500 would have been required, since each container could be used to furnish a test dose for both a thioglycollate and a Sabouraud tube. In any event, the direct method would have required 1,000 tubes of media, while the filtration method required only 50.

The membrane filtration sterility test has been officially accepted by the United Kingdom and the United States for antibiotic sterility testing. The "British Pharmacopoeia," 1963, requires filtration tests for testing the sterility of parenteral preparations of bacitracin, oxytetracycline, tetracycline, polymyxin, streptomycin, vancomycin, viomycin, and methicillin. For sterility tests of all antibiotics, U.S.P. XVII and N. F. XII refer to the Antibiotic Regulations of the Food and Drug Administration.

REFERENCES

(1) 21 CFR, Section 141a.2, *Federal Register*, **27**, 13002 (December 29, 1962).
(2) Holdowsky, S., *Antibiot. Chemotherapy*, **2**, 49(1957).
(3) 21 CFR, Section 141.2, *Federal Register*, **29**, 4119 (March 28, 1964).
(4) 21 CFR, Section 141a.2, *ibid.*, **15**, 7178(October 26, 1950).
(5) Topley and Wilson, "Principles of Bacteriology and Immunity," revised by Wilson, G. S., and Miles, A. A., Williams and Wilkins Co., Baltimore, Md., 1946, p. 117.
(6) Straka, E. P., and Stokes, J. L., *Appl. Microbiol.*, **5**, 21(1957).
(7) Jane-Williams, D. J., *J. Appl. Bacteriol.*, **26**, 398(1963).
(8) Bowman, F. W., *J. Pharm. Sci.*, **52**, 705(1963).
(9) Sokolski, W. T., and Chidester, C. G., *ibid.*, **53**, 103 (1964).
(10) 21 CFR, Section 141e.433, *Federal Register*, **30**, 14254 (November 1965).
(11) Bowman, F. W., and Holdowsky, S., *Antibiot. Chemotherapy*, **8**, 406(1958).
(12) Lightbown, J. W., *Proc. Round Table Conf. Sterility Testing*, London, 1963.