A NOTE ON THE USE OF MEMBRANE FILTERS IN STERILITY TESTING

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Received May 20, 1959

Filtration techniques have been applied advantageously in testing antibiotics and certain other medicaments, including oily solutions, for sterility, on the one hand by eliminating any residual bacteriostatic activity and on the other by eliminating interference through precipitation or the development of cloudiness. Membrane filters have been shown to be satisfactory for this purpose, and in some respects they are superior to asbestos pad filters.

Since their introduction a few years ago in the bacteriological examination of water supplies[1,2], membrane filters have become established tools for assessing small numbers of micro-organisms. They have been used for counting bacteria in the air[3], for counting surviving bacteria in the air[3], for counting surviving bacteria in certain disinfection tests[4] and have also been advocated in the sterility testing of antibiotics[5]. This communication records some of our experiences and findings in the testing of antibiotics and of oily solutions over about the last two years. Suitable membranes are made in this country by Courtaulds Ltd. and marketed by Oxo Ltd.; they are of such a porosity as will retain all, or very nearly all, of the bacteria present in a given fluid sample.

The technique of membrane filtration consists simply of filtering a known volume of the sample to be examined through a single membrane, suitably mounted in a modified Seitz-type filter holder (Gallenkamp), and then culturing the membrane either on the surface of a prepared nutrient agar plate or by immersion in a liquid nutrient medium. In surface culture, a quantitative assessment can be made, usually by a direct colony count after incubation (it can also be done microscopically after staining[6]), but with liquid media the result is qualitative only; this, however, is normally adequate for sterility testing purposes.

Experimental

The organisms used in the experimental work here reported were a Gram-positive spore former of the *Bacillus subtilis* type, *Staphylococcus aureus* (F.D.A. disinfectant testing strain), and *Chromobacterium prodigiosum* (*Serratia marcescens*).

Efficacy of Membrane Filters

Preliminary tests with membrane filters showed that they could not always be relied upon to retain all of the organisms in a given sample. For this reason, membrane filters were discarded for several years in favour of asbestos pad filters, but more recent tests showed that membranes would allow organisms to pass through only when the inoculum

was numerically high; with small inocula of between 10 and 500 viable cells (the level of contamination most likely to be found in sterility testing) the filtrate was always sterile. Experiments on the recoveries of different test organisms on the membranes themselves confirmed this, and typical recoveries of *Chr. prodigiosum* are given in Table I. These

TABLE I

RECOVERY OF *Chr. prodigiosum* BY MEMBRANE FILTRATION

Numbers of organisms in 100 ml. sample		
Estimated inoculum	Colonies recovered on membrance	Growth in filtrate
9	7	Nil
10	11	"
11	8	"
17	18	"
44	42	"
50	48	"
70	75	"

results were obtained by filtering 100 ml. amounts of the dilute suspensions into sterile bottles each containing 100 ml. of nutrient broth, washing twice with 100 ml. of sterile water or saline, disconnecting each filter from its bottle and then incubating, at 37°, the filtrates for 7 days and the membranes by the agar surface culture method for 2–3 days.

Removal of Antibiotics by Washing

One advantage of the filtration technique is that it allows residual inhibitory substances to be effectively removed before the final culturing, and this applies particularly to the antibiotics other than penicillin. To

TABLE II

REMOVAL OF NEOMYCIN FROM FILTERS BY WASHING

Sample	Neomycin in washings (u./ml.) from			
	Membrane filter		Asbestos pad filter	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Original solution ..	c13,000	c13,000	c13,000	c13,000
1st 100 ml. wash ..	140	107	430	360
2nd " " " ..	12	<10	17	15
3rd " " " ..	<10	<10	<10	<10

illustrate this, 100 ml. amounts of a 2 per cent solution of Neomycin Sulphate (i.e., containing about 13,000 u./ml.) were filtered through membrane or asbestos pad filters. The filters were then washed with three portions of 100 ml. of sterile saline and the neomycin content of each of the washings assayed by the standard biological method. The results are given in Table II; they illustrate the superiority of the membrane filter in this respect.

Recovery of Organisms from Antibiotic Solutions

One hundred ml. amounts of solutions of Streptomycin Sulphate (20 per cent) and of Neomycin Sulphate (2 per cent) were inoculated with

small numbers of spores of *B. subtilis* and filtered through separate membranes. Each membrane was washed with two 100 ml. amounts of sterile saline and then either incubated on an agar plate surface or cut in two and each portion incubated in 250 ml. of nutrient broth. The results, summarised in Table III, show a high recovery of organisms from the streptomycin solution, particularly in the qualitative tests, when an estimated inoculum even as low as 5 cells gave a positive response. The results with neomycin were not as encouraging, but these were presumably due to the lethal action of the neomycin, because when the liquid media showing negative responses were inoculated lightly at the end of the incubation period there was an immediate vigorous growth.

TABLE III

RECOVERY OF ORGANISMS FROM ANTIBIOTIC SOLUTIONS

Antibiotic solution (per cent)	Estimated inoculum in 100 ml.	Colonies recovered on filter	Growths in 250 ml. broths
Streptomycin Sulphate 20	9	4	
	25	21	
	28	20	
	5		++
	25		++
	28		++
	33		++
Neomycin Sulphate 2 ..	18	3	
	28	0	
	7		++
	9		++
	28		−−
	28		+−

Test organism—spore-forming Gram-positive bacillus.

Moreover, in one test with the agar plate method, there was a recovery of 60 colonies from an estimated inoculum of 67 spores when the test spores were inoculated into the last washing water.

The absence of bacteriostatic carry-over was further confirmed in a series of experiments in which, after filtering different lots of the antibiotic solutions and washing as before, the membranes were transferred to 250 ml. of culture medium, which was then inoculated lightly (5 to 180 viable cells) with either *Staph. aureus* or *Chr. prodigiosum*. In each of 13 experiments, there was no apparent inhibition of growth.

Recovery of Organisms from Oils

Fifty ml. amounts of arachis oil and of stilboestrol solution in oil were inoculated each with one small loopful of a dilute aqueous suspension of *B. subtilis* spores, and shaken to disperse the organisms. Each of the 50 ml. amounts was then filtered through a dry membrane and the membrane incubated in the usual way. Ten such experiments were made, and with inocula even as low as 8 to 10 spores there was a positive response in every case, growths occurring in periods ranging from 24 hours to 4 days.

In further experiments, the introduction of an aqueous phase was avoided by using a small inoculum (10 to 100 spores) of a freeze-dried

culture of *B. subtilis*. The culture was first suspended in oil and the appropriate amount added to samples of arachis oil and oily solutions of progesterone and stilboestrol; these were then filtered through membranes as before. In each case the test organism was recovered, and the filtrate was sterile.

Discussion

The principal advantage of the membrane filtration method in sterility testing is that having filtered the sample, the membrane can be washed free from any inhibitory or other interfering substances in the original solution; thus the amount of culture medium normally required to dilute out any bacteriostatic effect in the medicament can be much reduced. It is, therefore, particularly useful in testing the antibiotics such as streptomycin and neomycin, for which there is no known inactivator. The method also allows the strongly adsorbed bacteriostats such as the organic mercurials and the quaternary ammonium compounds to be "inactivated" on the filter by washing with a suitable inactivating solution rather than by the more cumbrous and less desirable method of adding the inactivator to the final culture medium. In this respect, membranes are superior to asbestos pad filters because the latter, being fibrous and relatively thick, absorb and retain sufficient of the active material to exert some bacteriostatic action in the final test; moreover the filtration time is rather lengthy.

A further advantage of the method is that it often eliminates the necessity for subsequent subculturing at the end of the normal incubation period, and thus economises in testing time, when the preparation under test produces a cloudiness or precipitate. It can, therefore, be applied to the testing of drugs such as oils and oily solutions (but not oily suspensions), thiopentone sodium and certain of the insulin preparations, all of which cause some cloudiness and precipitation in the medium and so mask any bacterial growth which may have taken place.

The testing of oils for sterility by the normal methods has never been considered satisfactory, largely because of the difficulty of recovering contaminating organisms present in the non-aqueous phase. Here again membrane filters have proved useful, largely because they allow nearly all of the oil to be filtered away and so permit any organisms present more easily to enter the aqueous phase and develop normally. It also helps in subsequent cultural manipulations by eliminating any tendency towards emulsification and the development of opalescence, thus avoiding the need for subsequent subculturing.

A disadvantage of the method is that it requires skill in aseptic handling, otherwise any contaminating organism gaining access during the filtration or subsequent transfer of the membrane to its culture medium will show itself as a false-positive. For this reason, a sterile room is needed with a suitable aseptic screen, or better still the sealed screen technique should be employed[7]. Low recoveries have also been reported in some instances with phenol-damaged organisms[8].

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After Mr. Sykes presented the paper there was a DISCUSSION. The following points were made.

The membranes were unsatisfactory with suspensions but had been used regularly for other injectable preparations. Volumes as small as 1 ml. could be used for sterility testing. Recovery had only been studied with a small inoculation of anaerobic organisms. It was not possible to obtain a uniform dispersion in oil of freeze-dried oil suspensions of organisms. Organisms recovered from oils could not be grown on the filters, and suspension of the membrane in a culture medium was necessary. The viscosity of solutions had little influence on the time of filtration, and membranes were much faster than Seitz pads as filters. The amount of any antibacterial agent that might be concentrated in the membrane after filtration of the small quantities of aqueous solutions used, should not be sufficient to influence the result. The membrane method had been used for testing for sterility ox and horse sera in quantities up to 50 ml., but it cannot be used for preparations containing waxes, because of the effects of the organic solvents on the organisms.