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This paper is dedicated to Professor Dr. h. c. mult. Kurt Mothes, Emeritus Director, Institute for the Biochemistry of Plants, German Academy of Science of Berlin, on his 70th birthday.

Collaborative Study of Aerobic Media for Sterility Testing by Membrane Filtration

FRANCES W. BOWMAN, MACK WHITE, and MIRIAM P. CALHOUN

Abstract □ The USP XVIII and NF XIII (first supplement) have replaced the sterility test medium, fluid Sabouraud, with a soybean-casein digest medium. A collaborative study performed by 12 laboratories showed that soybean-casein digest medium is superior to fluid Sabouraud medium for sterility testing by membrane filtration.

Keyphrases □ Aerobic media for membrane filtration sterility testing—collaborative study □ Soybean-casein digest medium—collaborative study of use in membrane filtration sterility tests □ Membrane filtration sterility testing—collaborative study on soybean-casein digest medium

The Food and Drug Administration has been using Sabouraud liquid medium USP (pH 5.7) for testing the sterility of antibiotics since 1964 (1). Prior to that time, a modified Sabouraud medium (also pH 5.7) was used (2). The primary reason for using this medium in the sterility test was to detect the presence of fungi rather than bacteria. A few bacteria found as contaminants of antibiotics did not grow at pH 5.7 but were recovered in thioglycollate medium (pH 7.1). Since the sterility test is intended to detect as many microorganisms as possible, it is patently undesirable to use a medium, such as fluid Sabouraud at pH 5.7, that inhibits certain bacteria. To eliminate the use of a selective medium for the sterility test, the USP XVIII and the first supplement to NF XIII replaced fluid Sabouraud USP XVII with a soybean-casein digest (SBCD) medium which has a pH of 7.3 ± 0.2. However, before implementing any change in the sterility tests for antibiotics as required in the *Antibiotic Regulations*, a collaborative study was performed to compare the growth-promoting qualities of SBCD medium to those of fluid Sabouraud. Twelve laboratories participated in this study[1].

Since preliminary work indicated that fluid Sabouraud medium at pH 7.0 supports the growth of fungi without inhibiting bacteria, this medium was also included in the study. All three media were manufactured by each of two companies (designated as Manufacturers A and B) for use in this study. A protocol containing detailed instructions and the media to be used in the study were supplied to each collaborator (3).

[1] Biological Safety Control, Becton, Dickinson and Co., Raleigh, N. C.; Bristol Laboratories, Syracuse, N. Y.; Difco Laboratories, Detroit, Mich.; Food and Drug Administration, National Center for Antibiotic Analysis, Sterility Testing Branch; Food and Drug Directorate Laboratories, Ottawa, Canada; Lederle Laboratories, Pearl River, N. Y.; Eli Lilly and Co., Indianapolis, Ind.; Laboratory of Control Activity, Division of Biological Standards, National Institutes of Health, Bethesda, Md.; Parke, Davis & Co., Detroit, Mich.; Chas. Pfizer & Co., Inc., Brooklyn, N. Y.; E. R. Squibb & Sons, Inc., New Brunswick, N. J.; and Wyeth Laboratories Inc., West Chester, Pa.

EXPERIMENTAL

The procedure used was essentially the same as that used for testing the sterility of antibiotics (4). The design of the study was similar to that used in *Reference 5*.

Organisms—Cultures or spore suspensions of the nine microorganisms used in the study were supplied by the authors. Dilutions of inocula were selected to simulate low levels of contamination that might possibly be encountered in a contaminated pharmaceutical preparation being tested for sterility. The following microorganisms were employed: *Bacillus subtilis* ATCC 6633 (spores), *Staphylococcus aureus* ATCC 6538 P; *Aspergillus niger* ATCC 6275 (spores), *Bacillus circulans* PCI 260 (spores), *Bacillus* sp. PCI 208 (spores), *Saccharomyces cerevisiae* ATCC 9763, *Candida albicans* ATCC 10231, *Escherichia coli* ATCC 10536, and *Bacillus* sp. PCI 258 (spores).

Media—Instructions for reconstitution and sterilization of the media were as follows: Weigh 120 g. of the dehydrated medium and rehydrate by adding 4 l. of distilled water. If necessary, warm the mixture to complete solution. When the medium is in solution, dispense it in 100-ml. portions to each of 36 (38 × 200-mm.) screw-capped tubes. Label each tube as to contents. Sterilize the tubes of media in an autoclave at 121° for 20 min. The autoclave temperature should be reached within 10 min.

Each collaborator prepared a total of 216 labeled tubes, comprising 36 tubes each of six media (SBCD medium A and B, Sabouraud medium pH 5.7 A and B, and Sabouraud medium pH 7.0 A and B).

Incubation Time—Incubation time was limited to 2, 5, and 7 days since the collaborative study of insulin (4), in which samples were incubated 7, 10, and 14 days, had shown that 7 was the maximum number of days required for the recovery of microorganisms where the membrane filtration procedure was used.

The following protocol was supplied to and followed by the collaborators: For each of the nine microorganisms, prepare about 150 ml. of a stock organism suspension containing approximately 100 colony-producing units (CPU) per 20 ml. of sterile 0.1% peptone solution and dispense 20 ml. into seven sterile bottles. From the remaining stock, prepare a low-level inoculum of approximately 5 CPU/20 ml. by adding 1 ml. to 19 ml. of sterile 0.1% peptone solution in each of seven bottles. Immediately perform the membrane filtration test on the contents of each of the 14 bottles as follows: Filter the entire contents of one bottle through a 0.45-μ membrane filter. Rinse the filter by filtering 100 ml. of sterile 0.1% peptone solution through it. Cut a circular disk approximately 17.5

Table I—Number of Collaborators Reporting Positive Tests in the Recovery of Microorganisms in Several Aerobic Media

Microorganism	Inoculum Expressed[a] CPU's	Sabouraud pH 5.7 Manufacturer A—Days of Incubation 2	5	7	Sabouraud pH 5.7 Manufacturer B—Days of Incubation 2	5	7	Sabouraud pH 7.0 Manufacturer A—Days of Incubation 2	5	7	Sabouraud pH 7.0 Manufacturer B—Days of Incubation 2	5	7	Soybean-Casein Digest Medium Manufacturer A—Days of Incubation 2	5	7	Soybean-Casein Digest Medium Manufacturer B—Days of Incubation 2	5	7
Bacillus subtilis	1–5	1	4	6	1	2	7	4	5	5	6	10	11	8	11	11	12	12	12
ATCC 6633 (spores)	50–100	5	7	8	4	10	11	8	11	12	9	11	11	12	12	12	12	12	12
Staphylococcus aureus	1–5	0	0	0	12	12	12	0	0	1	8	12	12	5	10	10	9	11	12
ATCC 6538	50–100	0	1	2	1	6	10	1	4	4	9	11	12	9	12	12	10	11	11
Aspergillus niger	1–5	6	9	9	6	10	11	6	10	10	6	10	10	6	12	12	6	10	10
ATCC 6275 (spores)	50–100	10	12	12	10	11	11	9	12	12	10	12	12	11	12	12	11	12	12
Bacillus circulans	1–5	0	1	2	1	3	4	0	0	1	3	8	10	1	2	4	3	5	7
PCI 260 (spores)	50–100	0	0	1	0	2	4	1	2	3	7	9	10	4	6	7	6	9	10
Bacillus sp.	1–5	1	3	7	1	3	8	0	4	7	1	4	7	1	2	4	2	5	7
PCI 208 (spores)	50–100	1	4	8	1	5	7	0	6	9	0	8	8	1	3	5	2	7	8
Saccharomyces cerevisiae	1–5	8	12	12	8	10	10	4	9	10	7	10	10	6	12	12	9	12	12
ATCC 9763	50–100	12	12	12	10	11	11	11	12	12	10	11	11	10	11	12	12	12	12
Candida albicans	1–5	7	11	11	7	10	12	7	10	10	7	11	12	7	11	11	8	11	11
ATCC 10231	50–100	10	11	11	9	10	10	10	11	11	11	12	12	10	12	12	12	12	12
Escherichia coli	1–5	9	11	11	6	10	11	10	12	12	5	7	7	9	9	9	10	11	11
ATCC 10536	50–100	11	12	12	8	11	11	11	12	12	11	12	12	10	10	10	11	11	11
Bacillus sp.	1–5	0	0	1	1	1	1	1	1	1	1	3	6	4	11	11	5	11	11
PCI 258	50–100	0	3	3	0	0	2	1	1	1	0	3	10	6	10	10	8	11	11

[a] Number of colony-producing units.

mm. in diameter from the center of the membrane, and transfer it to a 38 × 200-mm. tube containing 100 ml. of SBCD broth from Manufacturer A. Using sterile forceps, transfer the outer portion of the membrane into a second similar tube also containing SBCD broth from Manufacturer A. Repeat the above test for the low and high microorganism populations for the other five media.

As a control on each inoculum for each microorganism, filter the contents of the seventh bottle but do not cut the membrane. Instead, place the entire membrane on the surface of a peptone-casein agar plate. Incubate the plates in an inverted position at 22–25° until colonies are visible. Count the number of colonies and record the number of CPU's. This confirms whether the expected inoculum is obtained. If the inoculum is found to be incorrect for any one of the microorganisms, repeat the test. Incubate all of the tubes at 22–25° and read them at 2, 5, and 7 days. Record the presence or absence of growth of each microorganism in each of the 36 tubes of each medium containing either the periphery or the disk portion of the membrane. (Growth in a tube containing either the periphery or the disk or both will be reported as a positive test in the final analysis.) Confirm all isolates from observable growth as being the same organisms as the original inoculum.

RESULTS AND CONCLUSIONS

The average inocula, as confirmed by plate counts, were 4.7 CPU/20 ml. for the low level and 83 CPU/20 ml. for the high level. As shown in Table I, the test results from 12 laboratories verified among the collaborators that, for the recovery of the nine microorganisms, SBCD medium is the best of the media tested when a membrane filtration sterility test is performed. A 7-day incubation period gives the highest recovery of organisms. On statistical analysis, the collaborator times media interaction suggested that the results with a given medium may be dependent on the experience of the collaborator with the particular method. This is in agreement with the assessment by Streeter and Robertson (6) that a laboratory needs at least 3 months of experience with the filtration technique before reproducible and certain results can be expected.

The effect of the individual collaborator's experience or lack of it was borne out by the results reported on *B. circulans* PCI 260 and *Bacillus* sp. PCI 258 in Sabouraud medium pH 5.7. These organisms were selected for the study because they do not grow at pH 5.7 but do grow at approximately pH 7.0; nevertheless, 18 recoveries were reported at pH 5.7 out of a possible 48. Also, several collaborators commented that Manufacturer B's SBCD medium presented a problem in reading when the growth was sparse. The difficulty was apparently due to a precipitate in the medium which macroscopically simulated sparse growth. This problem could be solved in an actual sterility test by subculture. However, in this study a subculture in pH 7.0 medium would merely have shown growth of the dormant spores which had been added to the samples being tested.

As a result of this collaborative study, the *Antibiotic Regulations* will be amended to change Medium E from fluid Sabouraud medium USP to soybean-casein digest medium USP, thus assuring a high recovery of microorganisms and improving the sensitivity of the antibiotic sterility test.

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