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Comparative Study of Seven Media for Sterility Testing

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Abstract □ A comparative study of the efficacy of sterility testing of pharmaceuticals according to two systems is described. The culture media used in the first system were: dithionite–thioglycollate (HS-T) broth, recommended by the Nordic Pharmacopoeia Board, and a peptone liver digest medium or a peptone liver digest agar. The media used in the second system were: fluid thioglycollate medium and soybean–casein digest medium, both prescribed by the USP XVIII, and Sabouraud liquid medium or Sabouraud dextrose agar. The supplemental use of one of the two latter media has been recommended, because the USP XVIII media were considered inadequate. A total of 180 cultures of fastidious bacteria, yeasts, and molds was investigated. The first system was found superior to the second.

Keyphrases □ Sterility testing—comparison between seven media divided into two systems, 180 cultures of bacteria, yeasts, and molds □ Culture media for sterility testing—comparison between seven media divided into two systems, 180 cultures of bacteria, yeasts, and molds □ Medium for sterility testing—two systems compared using 180 cultures of bacteria, yeasts, and molds

For sterility testing of pharmaceutical products, the FDA, USP XVI, and USP XVII prescribed fluid thioglycollate medium and Sabouraud liquid medium. A collaborative study performed by 12 laboratories in the United States and Canada showed that soybean–casein digest medium is superior to Sabouraud liquid medium (1). Accordingly, USP XVIII and the First Supplement to NF XIII replaced Sabouraud liquid medium with soybean–casein digest medium. In Germany, the Subcommittee for Microbiology, a Division of the Pharmaceutical Committee of the Confederation of the Pharmaceutical Industry, recommended the use of three media: fluid thioglycollate medium, soybean–casein digest medium, and Sabouraud liquid medium for sterility testing. The Nordic Pharmacopoeia Board recommended dithionite–thioglycollate (HS-T) broth[1].

In 1972, the author developed peptone liver digest medium and peptone liver digest agar to replace Sabouraud liquid medium and Sabouraud dextrose agar. Dithionite–thioglycollate (HS-T) broth and both peptone liver digest medium and peptone liver digest agar contain neutralizing constituents and supplementary mineral salts. To find out which media are most reliable for sterility testing of pharmaceuticals, the efficacy of these seven media was investigated.

MATERIALS AND METHODS

Microorganisms[2]—To simulate low levels of contamination which might possibly occur in a contaminated pharmaceutical preparation being tested for sterility, the inoculum was diluted so that every vessel (test tube or petri dish) of medium contained about 50–200 colony-producing units as determined by a plate count. When petri dishes were used, the inoculum was suspended in buffer solution and 0.1 ml was spread out on the surface of the solidified medium which had been poured the day before.

The following microorganisms were employed:

I. 18 strains belonging to 15 species of the genus *Bacillus*.
II. 22 strains belonging to 12 species of the genus *Clostridium*.
III. 48 strains belonging to 40 species of the following 22 genera: *Bacteroides, Comamonas, Corynebacterium, Desulfovibrio,*

[1] This medium was developed recently by Dr. Clausen, University of Oslo, Oslo, Norway.

[2] The 180 cultures used included the following: 78 from the American Type Culture Collection, 31 from Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; 14 from the National Collection of Industrial Bacteria, Aberdeen, Scotland; 10 from the National Collection of Type Cultures, London, England; four from the Institute of Food Microbiology, Chiba University, Chiba, Japan; one from the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Ill.; one from the Commonwealth Mycological Institute, Kew, England; and 41 cultures from other sources.

Table I—Growth Ability of Vegetative Cells and Spores of 18 *Bacillus* Strains[a] in Soybean–Casein Digest Medium, Fluid Thioglycollate Medium, and Dithionite–Thioglycollate (HS-T) Broth at 32°

Growth Intensity	Number of Strains in Soybean–Casein Digest Medium						Fluid Thioglycollate Medium						Dithionite–Thioglycollate (HS-T) Broth					
	Days of Incubation						Days of Incubation						Days of Incubation					
	2		7		10		2		7		10		2		7		10	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
−	5	3	2	2	2	2	5	4	2	1	2	1	3	3	1	1	1	1
±	4	2	1	0	0	0	7	4	1	1	1	1	1	0	0	0	0	0
+	4	5	2	2	2	1	6	7	7	6	3	2	0	0	0	0	0	0
++	5	8	11	8	8	4	0	3	8	8	11	9	4	6	2	2	1	1
+++	0	0	2	6	6	10	0	0	0	2	1	5	10	9	13	11	14	12
++++	0	0	0	0	0	1	0	0	0	0	0	0	0	0	2	4	2	4
Evaluation[b]	16	22	30.5	36	36	43	9.5	15	23.5	28.5	28.5	35.5	38.5	39	51	53	52	54

[a] Ten National Collection of Industrial Bacteria strains, seven American Type Culture Collection strains, and one National Collection of Type Cultures strain, representing 15 saprophytic species. A, only vegetative cells were tested; and B, only spores were tested. [b] Evaluation according to the following point system: − = no point, ± = 0.5 point, + = 1 point, ++ = 2 points, +++ = 3 points, and ++++ = 4 points.

Table II—Growth Ability of Vegetative Cells and Spores of 22 *Clostridium* Strains[a] in Soybean–Casein Digest Medium, Fluid Thioglycollate Medium, and Dithionite–Thioglycollate (HS-T) Broth at 32°

Growth Intensity	Number of Strains in Soybean–Casein Digest Medium						Fluid Thioglycollate Medium						Dithionite–Thioglycollate (HS-T) Broth					
	Days of Incubation						Days of Incubation						Days of Incubation					
	2		7		10		2		7		10		2		7		10	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
−	22	22	22	22	22	22	21	21	21	21	21	21	1	1	0	0	0	0
±	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0
+	0	0	0	0	0	0	1	1	0	0	0	0	2	4	0	0	0	0
++	0	0	0	0	0	0	0	0	1	1	1	1	14	4	2	2	0	1
+++	0	0	0	0	0	0	0	0	0	0	0	0	5	7	17	10	13	8
++++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	10	9	13
Evaluation[b]	0	0	0	0	0	0	1	1	2	2	2	2	45	36	67	74	75	78

[a] Nine National Collection of Type Cultures strains, seven American Type Culture Collection strains, one National Collection of Industrial Bacteria strain, and five other strains, representing 12 saprophytic and pathogenic species. A, only vegetative cells were tested, and B, only spores were tested. [b] Evaluation according to the following point system: − = no point, ± = 0.5 point, + = 1 point, ++ = 2 points, +++ = 3 points, and ++++ = 4 points.

Diplococcus, Haemophilus, Halobacterium, Lactobacillus, Leuconostoc, Micrococcus, Mima, Moraxella, Mycobacterium, Nocardia, Pasteurella, Pediococcus, Pseudomonas, Sarcina, Staphylococcus, Streptococcus, Streptomyces, and *Thiobacillus.*

IV. 38 strains of yeasts including: (*a*) 21 strains belonging to 19 species of the genera *Candida, Pichia, Rhodotorula, Saccharomyces, Schizosaccharomyces, Torulopsis,* and *Trichosporon*; and (*b*) 17 strains not identified which were recovered from pharmaceutical products and production plants.

V. 54 strains of molds belonging to 48 species of the following 32 genera: *Allescheria, Alternaria, Aspergillus, Aureobasidium, Beauveria, Byssochlamys, Cercosporella, Chrysosporium, Cladosporium, Claviceps, Coccidioides, Colletotrichum, Cunninghamella, Curvularia, Epidermophyton, Fusarium, Gaeumannomyces, Geotrichum, Gliocladium, Hemispora, Keratinomyces, Marasmius, Monosporium, Mucor, Neurospora, Penicillium, Phoma, Rhinocladiella, Rhizopus, Trichoderma, Trichophyton,* and *Verticillium.*

Media—The following fluid and semisolid media were used.

Soybean-Casein Digest Medium—pH after sterilization 7.3 ± 0.2.

Fluid Thioglycollate Medium—pH after sterilization 7.1 ± 0.2.

Dithionite-Thioglycollate (HS-T) Broth[3]—pH after sterilization 7.1 ± 0.2.

Sabouraud Liquid Medium—Contained 2% dextrose, pH after sterilization 5.7 ± 0.1.

Peptone Liver Digest Medium—This medium contained the following: mycological peptone[4], 15.0 g; liver digest[5], 5.0 g; dextrose, 19.8 g; yeast extract[6], 6.0 g; malt extract[7], 4.0 g; sodium chloride, 2.92 g; sodium glycerophosphate, 5.2 g; potassium phosphate, monobasic, 1.0 g; polysorbate 80, 3.0 g; lecithin, 0.3 g; magnesium sulfate (hexahydrate), 0.25 g; cobaltous sulfate (hexahydrate), 0.001 g; manganese chloride ($MnCl_2 \cdot 4H_2O$), 0.001 g; agar[8] No. 1, 0.75 g; and distilled water, 1000 ml. The pH after sterilization was 5.8 ± 0.1.

Eight-milliliter amounts of each medium were transferred to 15 × 160-mm test tubes.

The following solid media were used.

Sabouraud Dextrose Agar—Contained 4% dextrose, pH after sterilization 5.7 ± 0.1.

Peptone Liver Digest Agar—This medium was prepared by adding 19.8 g of dextrose and 20.0 g of agar to 1 liter of peptone liver digest medium. The pH after sterilization was 5.8 ± 0.1.

Twenty-milliliter quantities of each of the two solid media were transferred to petri dishes (90 mm diameter). All media were prepared 1 or 2 days before they were required.

Incubation—Vessels inoculated with bacteria were incubated at 32 ± 2° for 14 days. Those inoculated with yeasts or molds were incubated at 26 ± 2° for 2 weeks. Each vessel was examined visually for growth on Days 2, 7, 10, and 14.

[3] Available from Oxoid Limited, London SE 1, England.

[4] Oxoid, code L 40.
[5] Merck, No. 5402.
[6] Merck, No. 3753.
[7] Merck, No. 5391.
[8] Oxoid, code L 11.

Table III—Growth Ability of 48 Strains of Nonspore-Forming Bacteria[a] in Soybean–Casein Digest Medium, Fluid Thioglycollate Medium, and Dithionite–Thioglycollate (HS-T) Broth at 32°

Growth Intensity	Number of Strains in Soybean–Casein Digest Medium, Days of Incubation			Number of Strains in Fluid Thioglycollate Medium, Days of Incubation			Number of Strains in Dithionite–Thioglycollate (HS-T) Broth, Days of Incubation		
	2	7	10	2	7	10	2	7	10
−	23	13	11	21	10	10	13	5	5
±	12	5	3	11	3	1	3	3	1
+	5	12	12	7	12	13	12	4	4
++	6	12	12	8	18	10	12	16	7
+++	2	6	10	1	5	14	8	16	25
++++	0	0	0	0	0	0	0	4	6
Evaluation[b]	29	56.5	67.5	31.5	64.5	75.5	61.5	101.5	117.5

[a] Thirty-eight American Type Culture Collection strains, four Institute of Food Microbiology strains, three National Collection of Industrial Bacteria strains, and three other strains, representing 40 saprophytic and pathogenic species (22 genera). [b] Evaluation according to the following point system: − = no point, ± = 0.5 point, + = 1 point, ++ = 2 points, +++ = 3 points, and ++++ = 4 points.

Table IV—Growth Ability of 38 Yeast Strains[a] in/on Soybean–Casein Digest Medium, Fluid Thioglycollate Medium, Dithionite–Thioglycollate (HS-T) Broth, Sabouraud Liquid Medium, Peptone Liver Digest Medium, Sabouraud Dextrose Agar, and Peptone Liver Digest Agar at 26°

Growth Intensity	Number of Strains in/on Soybean–Casein Digest Medium, Days of Incubation			Fluid Thioglycollate Medium, Days of Incubation			Dithionite–Thioglycollate (HS-T) Broth, Days of Incubation			Sabouraud Liquid Medium, Days of Incubation			Peptone Liver Digest Medium, Days of Incubation			Sabouraud Dextrose Agar, Days of Incubation			Peptone Liver Digest Agar, Days of Incubation		
	2	7	10	2	7	10	2	7	10	2	7	10	2	7	10	2	7	10	2	7	10
−	25	11	5	24	11	6	15	3	3	15	0	0	6	0	0	4	0	0	1	0	0
±	4	9	5	6	6	5	8	7	0	3	5	0	9	1	0	3	0	0	5	0	0
+	9	7	9	4	4	6	4	2	5	12	9	2	3	1	1	8	1	0	6	1	0
++	0	7	10	4	10	5	11	11	3	8	13	10	15	10	2	18	9	3	16	4	1
+++	0	4	9	0	7	16	0	15	27	0	11	26	5	20	14	5	28	34	10	28	12
++++	0	0	0	0	0	0	0	0	0	0	0	0	0	6	21	0	0	1	0	5	25
Evaluation[b]	11	37.5	58.5	15	48	66.5	30	72.5	92	29.5	70.5	100	52.5	105.5	131	60.5	103	112	70.5	116	138

[a] Seven American Type Culture Collection and 13 Centraalbureau voor Schimmelcultures strains representing 19 saprophytic and pathogenic species (seven genera), and 18 nonclassified strains isolated from pharmaceutical products and production plants. [b] Evaluation according to the following point system: − = no point, ± = 0.5 point, + = 1 point, ++ = 2 points, +++ = 3 points, and ++++ = 4 points.

RESULTS AND CONCLUSIONS

As shown in Table I, dithionite-thioglycollate (HS-T) broth is undoubtedly the best of the three media for the recovery (in respect to rate and yield) of the 18 *Bacillus* strains tested. Just one strain, namely *B. pasteurii* NCIB 8841, did not grow either in dithionite-thioglycollate (HS-T) broth or in soybean-casein digest medium or fluid thioglycollate medium. At least a 7-day incubation was required to obtain the best recovery of the species of the genus *Bacillus*. It did not make any difference whether the inoculum was composed of vegetative cells or spores. Fluid thioglycollate medium contains mercaptoacetate (thioglycollate) which was reported (2) to be definitely inhibitory to the growth of some *Bacillus* species; this inhibition was confirmed. However, the degree of inhibition or noninhibition appears to be influenced by other constituents of the medium; *e.g.*, in dithionite-thioglycollate (HS-T) broth, mercaptoacetate seems to be nontoxic. In view of the facts that not only *B. pasteurii* NCIB 8841 but also *B. coagulans* NCIB 9365 did not grow in soybean-casein digest medium and that some fastidious species (with respect to nutritive requirements) showed only poor and slow growth in both soybean-casein digest medium and fluid thioglycollate medium, it would seem that the two latter media are definitely inferior to dithionite-thioglycollate (HS-T) broth for the recovery of the *Bacillus* species.

Table II makes it clear that dithionite-thioglycollate (HS-T) broth is far better than the two other media with regard to the recovery of the 22 *Clostridium* strains tested. From the quantitative point of view, optimal recovery of all 12 species of the genus *Clostridium* tested was attained after 7-10 days of incubation. As expected, soybean-casein digest medium was found to be not at all suitable as a recovery medium for strictly anaerobic microorganisms, especially *Clostridia*.

Fluid thioglycollate medium inhibited the growth of almost all of the *Clostridium* species tested. These findings are somewhat analogous to previous results (3), where sodium thioglycollate was inhibitory to the germination of spores of several *Clostridium* species on solid agar medium. However, thioglycollate proved to be noninhibitory on the growth of *Clostridia* in dithionite-thioglycollate (HS-T) broth, as demonstrated in Table II.

Table III shows that dithionite-thioglycollate (HS-T) broth is by far the best of the three tested media with respect to the recovery of nonspore-forming bacteria, especially fastidious species. Five strains will not grow in dithionite-thioglycollate (HS-T) broth, but these are chemolithotrophic and halophilic bacteria which will grow neither in soybean-casein digest medium nor in fluid thioglycollate medium. Furthermore, it was not possible with fluid thioglycollate medium to recover five other strains which also belong to five species of bacteria. The recovery with soybean-casein digest medium was poorer than with fluid thioglycollate medium. Hence, it follows that dithionite-thioglycollate (HS-T) broth will give a better recovery than soybean-casein digest medium and fluid thioglycollate medium together.

A 10-day incubation is required to obtain optimal recovery of nonspore-forming bacteria. Prolonging the incubation to 14 days did not increase the recovery for dithionite-thioglycollate (HS-T)

Table V—Growth Ability of 54 Mold Strains[a] in/on Soybean–Casein Digest Medium, Fluid Thioglycollate Medium, Dithionite–Thioglycollate (HS-T) Broth, Sabouraud Liquid Medium, Peptone Liver Digest Medium, Sabouraud Dextrose Agar, and Peptone Liver Digest Agar at 26°

Growth Intensity	Number of Strains in/on																				
	Soybean–Casein Digest Medium			Fluid Thioglycollate Medium			Dithionite–Thioglycollate (HS-T) Broth			Sabouraud Liquid Medium			Peptone Liver Digest Medium			Sabouraud Dextrose Agar			Peptone Liver Digest Agar		
	Days of Incubation			Days of Incubation			Days of Incubation			Days of Incubation			Days of Incubation			Days of Incubation			Days of Incubation		
	2	7	10	2	7	10	2	7	10	2	7	10	2	7	10	2	7	10	2	7	10
−	11	3	3	14	10	5	12	7	7	8	2	1	12	2	1	17	1	1	17	3	1
±	28	2	1	20	4	9	15	2	1	11	0	1	8	1	0	10	1	0	8	1	2
+	11	20	7	16	11	8	14	5	3	25	10	5	22	4	2	19	4	2	20	3	1
++	4	24	19	4	23	7	13	21	4	9	22	12	10	8	5	4	15	5	4	10	2
+++	0	5	24	0	6	25	0	19	39	1	20	35	2	27	16	4	32	43	4	24	19
++++	0	0	0	0	0	0	0	0	0	0	0	0	0	12	30	0	1	3	1	13	29
Evaluation[b]	33	84	117.5	34	77	101.5	47.5	105	128.5	51.5	114	134.5	52	149.5	180	44	134.5	153	48	147.5	179

[a] Nineteen American Type Culture Collection strains, 18 Centraalbureau voor Schimmelcultures strains, one Northern Utilization Research and Development strain, one Commonwealth Mycological Institute strain, and 15 other strains, representing 48 saprophytic, pathogenic, and phytopathogenic species (32 genera). [b] Evaluation according to the following point system: − = no point, ± = 0.5 point, + = 1 point, ++ = 2 points, +++ = 3 points, and ++++ = 4 points.

broth, soybean-casein digest medium, or fluid thioglycollate medium. As shown in Table IV, the best recovery of yeasts was attained with peptone liver digest agar and peptone liver digest medium. Although all strains of yeasts tested were able to grow in Sabouraud liquid medium and on Sabouraud dextrose agar, these two media have a drawback insofar as they contain no constituents that will neutralize preservatives and enhance the growth of impaired cells. Furthermore, both growth rate and abundance in peptone liver digest medium and on peptone liver digest agar are greater than in Sabouraud liquid medium and on Sabouraud dextrose agar, respectively. In none of the other three media was it possible to recover all of the tested yeast strains. Growth was not observed in dithionite-thioglycollate (HS-T) broth (three strains, two osmophilic and one nonidentified), in soybean-casein digest medium (five strains, three osmophilic and two nonidentified), and in fluid thioglycollate medium (six strains, two osmophilic, three nonidentified, and one pathogenic). Therefore, the number of strains of yeasts recovered with peptone liver digest medium or peptone liver digest agar was higher than that recovered with both soybean-casein digest medium and fluid thioglycollate medium. A 10-day incubation was necessary to obtain the best recovery of yeasts when using peptone liver digest medium or peptone liver digest agar.

Of the seven media evaluated in Table V, peptone liver digest medium and peptone liver digest agar proved to be superior to the other five. Just one rare species of mold, namely *Marasmius foetidus* CBS 20847, did not grow either in peptone liver digest medium or on peptone liver digest agar. Although in Sabouraud liquid medium and on Sabouraud dextrose agar, 53 out of 54 strains of molds were able to grow, both of these media have the disadvantage mentioned previously. Like yeasts, molds also were able to grow in peptone liver digest medium and on peptone liver digest agar more quickly and more abundantly than in Sabouraud liquid medium or on Sabouraud dextrose agar. In soybean-casein digest medium, three strains belonging to three species of molds did not grow: eight other strains belonging to eight species showed slow and poor or very poor growth. In dithionite-thioglycollate (HS-T) broth, seven strains belonging to six species did not grow; four other strains belonging to four species showed slow and faint or very faint growth. Generally, the growth of molds in fluid thioglycollate medium was not as abundant as that in peptone liver digest medium. Furthermore, in fluid thioglycollate medium, five strains belonging to four species did not grow; 17 other strains belonging to 13 species showed slow and poor growth (Table V). Accordingly, neither soybean-casein digest medium nor fluid thioglycollate medium, nor both together, can replace either peptone liver digest medium or peptone liver digest agar. The heaviest growth of molds was obtained after a 10-day incubation when using the two latter media.

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