A COMPARATIVE STUDY OF THE USE OF VARYING CONCENTRATIONS OF AGAR IN THE TEST MEDIUM USED TO DETECT CONTAMINANTS IN BIOLOGIC PRODUCTS[1]

CAROLYN R. FALK, HELEN BIENIASZ BUCCA, AND MARGARET P. SIMMONS

Bureau of Laboratories of the Department of Health of the City of New York

Received for publication August 1, 1938

INTRODUCTION

Previous reports on the bactericidal action of antiseptics used in biologic products (Rosenstein and Levin, 1935; Falk and Aplington, 1936) showed that, when a given organism was inoculated into both broth and agar media, growth sometimes appeared in the agar and not in the broth and vice-versa. These findings could not be attributed to bacteriostatic action caused by insufficient dilution of the antiseptic present, since identical results were at times obtained in the control tubes containing no antiseptic. It was therefore indicated that neither broth nor agar used alone could be relied upon to detect organisms which may be found as contaminants (staphylococci, diphtheroids, and *Pseudomonas pyocyaneus*) in biologic products. These observations were later corroborated by Eldering and Kendrick (1936).

A semifluid medium, 0.1 per cent agar, was recommended by Hitchens (1921) for the detection of bacterial contaminants, either aerobic or anaerobic, particularly in substances containing a high percentage of antiseptic. While this medium has not attained the universal use that 0.03 per cent glucose broth has for testing the sterility of biologic products, it has been used in conjunction with this broth by Wadsworth (1927) and others.

[1] Presented at the Thirty-Ninth General Meeting of the Society of American Bacteriologists, Washington, D. C. December 30, 1937. Journ. Bact. 35: 1, 75.

More recently, Spray (1936) reported that anaerobes previously considered difficult to cultivate, grew well on a semisolid medium without a seal. We have not found, however, any extended experimental or statistical studies which demonstrated that a semifluid, semisolid, or solid medium would be preferable to broth for the detection of the more common forms of bacterial growth, or as a substitute for the regular 0.03 per cent glucose broth for testing the sterility of biologic products. It seemed, therefore, advisable to make such a study, particularly since the use of two very different types of medium is obviously undesirable in any laboratory concerned with large scale production of biologic products.

EXPERIMENTAL METHOD

The efficiency of different concentrations of agar in the medium used for the detection of bacterial growth was evaluated by (1) adding organisms directly to the test media; (2) adding organisms to typical biologic products, containing preservative, and subculturing to the various test media; and (3) adding a biologic product known to have a bacterial contaminant to the various test media.

Concentrations of agar in the test media varied from 2.0 to 0.001 parts of agar per hundred (or one to 2000 parts of agar per 100,000). The media containing from one to 0.001 parts of agar per hundred were prepared by diluting 2 per cent biologic-products agar with sufficient biologic-products broth (containing 0.03 per cent glucose) so that the desired concentrations of agar were obtained. The method of preparing these basic media has been described in previous papers (Rosenstein and Levin, 1935; Falk and Appington, 1936). The media, made up in 300 cc. batches, were tubed aseptically in 6″ x $\frac{5}{8}$″ tubes, 10 cc. being delivered into each tube. Immediately before use the test medium was heated at 100°C. in the Arnold sterilizer for one hour, followed by quick cooling to 45°C. As in our previous studies, the test organisms were types which may be encountered as contaminants in biologic products. In fact the strains used were actually isolated from such products over a period of years.

In the experiments to be described here, the staphylococci, diphtheroids, and *P. pyocyaneus* previously used were supplemented by hay bacilli, streptococci and *Escherichia coli*. Eighteen-hour broth cultures, containing approximately 100,000,000 organisms per cubic centimeter (colony counts varied from 60-000,000 to 250,000,000), of the above-mentioned organisms were diluted with broth so that the 10^{-4} dilution contained approximately 10,000 organisms per cubic centimeter, the 10^{-5} dilution 1000 organisms per cubic centimeter, the 10^{-6} dilution 100 organisms per cubic centimeter and the 10^{-7} dilution 10 organisms per cubic centimeter. A plate count was made to determine the exact number of organisms present in every experiment. One-tenth of a cubic centimeter of the appropriate dilution was then added to each of the various test media directly or to typical biologic products, which were subcultured to the test media. These tests were then incubated at 36°C. for seven days, during which time observations were made daily. All tests were carried out in duplicate, each complete series of tests being repeated at least once. The identity of organisms in tubes showing growth was checked by smears stained by Gram's method.

EXPERIMENTAL RESULTS

Typical results are given in tables 1 to 3. In the first series of experiments, in which organisms diluted with broth were used, there was a striking variation in the rate and character of the growth of the organisms in the various test media. These differences were apparently dependent on the percentage of agar present. Growth was inhibited when the percentage of agar was too high as well as when the medium was too fluid (table 1). In all cases, when the concentration of agar in the medium was between 0.06 and 0.50 parts per hundred, growth was most luxuriant. This fact became increasingly evident as the size of the inoculum decreased. In fact, when the medium contained concentrations of agar between 1.0 and 0.12 per cent, the presence of very few organisms could be demonstrated, whereas negative results were obtained in the media containing either

TABLE 1

The effect of different concentrations of agar on the growth of organisms when varying inoculums are used

7 day readings

AGAR	HAY BACILLI			STAPHYLOCOCCI			P. PYOCYANEUS			DIPHTHEROIDS		
	DILUTED			DILUTED			DILUTED			DILUTED		
	10^{-5}	10^{-6}	10^{-7}	10^{-5}	10^{-6}	10^{-7}	10^{-5}	10^{-6}	10^{-7}	10^{-5}	10^{-6}	10^{-7}
per cent												
2.0	++	++	++	++	++	+	+	+	−	−	−	−
1.0	++	++	++	++	++	+	++	+	+	+	+	+
0.50	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	+	+
0.25	++++	++++	++++	++++	++++	+++	++++	++++	+	+++	+++	++
0.12	++++	++++	++++	++++	++++	−	++	++	+	++	++	+
0.06	++++	++++	++++	++++	++	−	++	++	−	++	++	+
0.03	+++	+++	++	++	++	−	+	+	−	+	+	+
0.015	++	++	+	++	++	−	+	+	−	+	+	+
0.008	++	++	+	++	++	−	+	+	−	+	+	+
0.004	++	+	+	++	++	−	+	+	−	+	+	+
0.002	++	+	+	++	+	−	+	+	−	+	+	+
0.001	+	+	+	+	+	−	+	+	−	+	+	+
0.000	+	+	+	+	−	−	+	+	−	+	+	+

+, scant growth; ++, moderate growth; +++, good growth; ++++, luxuriant growth; −, no growth.

more or less agar in many cases. As might be expected, individual preferences for definite concentrations of agar in the medium were shown by the different test organisms. For example, staphylococci inoculated in small numbers did not grow in a medium containing less than 0.25 per cent agar, diphtheroids if the medium contained more than 1 per cent agar, *P. pyocyaneus* if more than 1 per cent or less than 0.25 per cent agar was present; hay bacilli on the other hand, showed growth in all the test media, but this growth was most luxuriant when the medium contained between 0.06 and 0.25 per cent agar.

To determine the practical application of these results, another series of experiments was carried out in which actual routine working conditions were more nearly approximated. Results obtained when test organisms were added to typical biologic products are shown in table 2. Typical products included a bacterial vaccine, an antitoxic globulin, and an antibacterial serum. Eighteen-hour broth cultures of each of the organisms were diluted with broth so that each cubic centimeter contained 10,000 organisms; 0.5 cc. of this dilution was then added to sterile 5 cc. samples of the above named products. Subcultures were made to the various test media after the culture had been in contact with the product for thirty minutes, since in most cases the preservative present might be expected to destroy the test organisms on continued standing. Again as in our first experiments, maximum growth was obtained when the concentration of agar in the medium varied from 0.06 to 0.50 per cent. The exact optimum concentration of agar needed, as before, depended on the type organisms present. The intermediate environment to which the organisms had been subjected, also, seemed to influence the concentration of agar in the medium which produced the most luxuriant growth on subculture. Organisms added to the bacterial vaccine corresponded most closely to the broth control, most luxuriant growth occurring in media containing from 0.12 to 0.5 per cent agar. On the other hand, organisms added to the antitoxic-globulin needed a more fluid medium, that is, one containing from 0.12 to 0.06 per cent of agar; and organisms added to the bacterial serum

TABLE 2

The effect of different concentrations of agar on the growth of organisms

Readings made after incubation at 36°C. for 96 hours

AGAR	HAY BACILLI				STAPHYLOCOCCI				P. PYOCYANEUS			
	Broth control	Bacterial vaccine	Antibacterial serum	Antitoxic-globulin	Broth control	Bacterial vaccine	Antibacterial serum	Antitoxic-globulin	Broth control	Bacterial vaccine	Antibacterial serum	Antitoxic-globulin
per cent												
2.0	++	++	++	+	+	+	−	+	++	+	+	+
1.0	+++	++	+++	++	++	++	+	++	++	+	+	+
0.5	++++	+++	+++	+++	++	+++	++	+++	+++	+++	++	+++
0.25	++++	++++	++	+++	+++	+++	+++	+++	++++	++++	+++	+++
0.12	++++	++++	−	++++	++++	++++	++++	++++	++++	++++	++++	++++
0.06	+++	+++	−	++++	+++	+++	++	++++	+++	+++	+++	++++
0.03	+++	++	−	+++	++	++	−	+++	++	++	+	+++
0.000	++	+	−	++	++	+	−	++	++	+	+	++
	DIPHTHEROIDS				STREPTOCOCCI				E. COLI			
2.0	++	+	+	+	+	+	+	+	+	+	+	+
1.0	++	++	+	++	++	++	++	++	++	++	++	+
0.5	++++	+++	++	+++	+++	+++	+++	++	+++	+++	++	+++
0.25	++++	++++	+++	+++	++++	+++	+++	++	++++	++++	+++	+++
0.12	++++	++++	−	++++	++++	+++	++++	++++	++++	++++	++++	++++
0.06	++	+++	−	++++	+++	++	++	++++	+++	+++	+	++++
0.03	++	++	−	+++	++	++	−	+++	++	++	−	+++
0.00	++	+	−	++	+	+	−	++	++	+	−	++

+, scant growth; ++, moderate growth; +++, good growth; ++++, luxuriant growth; −, no growth.

TABLE 3

The comparative efficiency of different concentrations of agar in the sterility test medium when gram-positive aerobic bacilli were present in a known contaminated product

AGAR	DAYS INCUBATED AT 36°C.														
	2	4	7	2	4	7	2	4	7	2	4	7	2	4	7
per cent															
2.0	−	++	++	−	++	++	−	++	++	−	++	++	−	++	++
1.0	+	+++	+++	+	+++	+++	+	+++	+++	+	+++	+++	+	+++	+++
0.5	++	+++	+++	++	+++	+++	++	+++	+++	++	++++	++++	−	++++	++++
0.25	++	++++	++++	++	++++	++++	++	++++	++++	++	++++	++++	+++	++++	++++
0.12	++	++++	++++	+++	++++	++++	−	+++	++++	+++	++++	++++	+++	++++	++++
0.06	++	+++	+++	++	+++	+++	++	++	+++	++	+++	+++	++	+++	+++
0.03	++	++	+++	++	+++	+++	−	++	+++	+	+++	+++	++	+++	+++
0.00	−	−	+*	−	−	−	−	−	−	−	−	−	−	−	−
	Vial 1			Vial 2			Vial 3			Vial 4			Vial 5		

+, scant growth; ++, moderate growth; +++, good growth; ++++, luxuriant growth; −, no growth.

* Growth observed in only one of two similarly inoculated tubes.

seemed to favor the media with concentrations of agar varying from 0.12 to 0.5 per cent, often giving negative results if less than 0.1 per cent agar was present. However, irrespective of the type of organism or its environment, optimum growth was regularly obtained in the media containing from 0.12 to 0.25 per cent of agar.

Soon after the completion of these experiments, we were able to secure several vials of a product which had become contaminated with a gram-positive aerobic bacillus in the process of manufacture. This particular product had shown growth in only one broth fermentation tube out of the twenty-five inoculated in routine sterility tests. The poured agar plates also showed no growth. For the experimental series of tests, 0.1 cc. portions from each vial were added to each of two tubes containing 10 cc. of test medium. Results of these tests are given in table 3. Again, only one of the ten broth tubes inoculated showed growth, while good growth was observed in every tube in which the medium contained between 1.0 and 0.1 per cent of agar. This experiment also demonstrated that the time at which growth was first observed in any one tube was influenced by the concentration of agar present. Good growth was first noticed in 48 hours in media containing concentrations of agar varying from 0.12 to 0.25 per cent, but it required at least 96 hours to show the same type of growth in media containing more than 0.25 per cent agar or less than 0.12 per cent agar, and as much as seven days when broth alone was used.

PRACTICAL APPLICATION

The experiments which have been described all showed that bacterial growth could be readily detected in media containing between 0.1 and 0.2 per cent agar. It therefore seemed advisable to use this medium in conjunction with the regular biologic-products broth for the routine testing of sterility of biologic products. All sterility tests were carried out following the recommendations of the National Institute of Health, with the exception that one half of the volume of product to be tested was added to the broth in fermentation tubes, while the re-

mainder was added to the 0.1 per cent agar medium. For routine purposes, the medium was dispensed in 7″ x 1″ metal capped tubes, each tube containing 35 cc. of medium. This medium received the same preparatory treatment as the biologic-products broth in fermentation tubes, that is, heating at 100°C. for one hour in the Arnold sterilizer, within two hours before use, followed by rapid cooling to 45°C. As has been pointed out by Wadsworth (1927), it is of the utmost importance that the 0.1 per cent agar medium be clear, so that individual colonies may be observed throughout the medium as

TABLE 4

The comparative efficiency of biologic-products broth and 0.1 per cent agar for the detection of contaminants in biologic products

LOT	B.P. BROTH			0.1 PER CENT AGAR		
	Tubes inoculated	Tubes contaminated	Contamination	Tubes inoculated	Tubes contaminated	Contamination
			per cent			*per cent*
a	42	42	100	70	53	76
b	30	3	10	30	24	80
c	60	17	28	100	76	76
d	60	38	63	100	14	14
e	72	8	11	120	71	59
f	92	1	0.9	130	34	26
g	36	0	0.0	60	9	15
h	100	2	2.0	120	19	16
i	36	1	2.8	60	5	8.3
Total.....	528	112	21.2	790	305	38.6

well as growth on the surface. Cloudy media necessitate subculture or the preparation of smears, in which case, any dead organisms present may cause confusion. To facilitate the inoculation and reading of tests, whenever possible, the broth and 0.1 per cent agar tubes were arranged in parallel rows in monel metal racks constructed for this purpose. Careful statistics were kept on the results obtained with these two media in the routine testing of the sterility of biologic products for a period of eight months. The comparative efficiency of the biologic-products broth and 0.1 per cent agar in the tests which showed bacterial contamination are given in table 4. Each lot showing

bacterial growth was retested at least once. The contaminating organisms in these lots were Gram-positive aerobic bacilli. The adoption of the mixture of phenol and merthiolate, as a preservative in most biologic products has markedly reduced the contaminations caused by *P. pyocyaneus*, diphtheroids and staphylococci, but this mixture is not effective against sporebearing organisms of the hay bacillus group. In general, growth was demonstrated in approximately twice as many of the 0.1 per cent agar tubes as in the biologic-products broth tubes. If only a light contamination was present, few if any broth tubes showed growth, in contrast to from 8 to 26 per cent of the tubes containing 0.1 per cent agar. In fact, in one of these nine lots showing growth, the product would have been released as sterile had only broth fermentation tubes been inoculated. On the other hand, where the contamination was extensive, growth was observed in both types of medium, but in most cases growth appeared earlier and was detected in more of the 0.1 per cent agar tubes. This was, however, reversed in two cases, one in which slightly more of the broth tubes showed growth, and another in which the apparent difference was more significant, only 14 of the 0.1 per cent agar tubes showing growth in contrast to 63 per cent of the broth tubes. We have also observed that the inclusion of the 0.1 per cent agar medium offers a great advantage over the exclusive use of biologic-products broth in fermentation tubes in that this medium contains just enough agar to permit the formation of separate colonies, thus permitting a quantitative estimate of the extent of the contamination present.

SUMMARY AND CONCLUSIONS

These experiments demonstrate the value of using a medium containing small percentages of agar (0.06 to 0.25 per cent) for the detection of bacterial growth. This applies even to such common forms as hay bacilli and staphylococci, which are ordinarily considered easy to cultivate. Since little difference in growth could be detected in media containing between 0.1 and 0.25 per cent agar, 0.1 per cent agar was chosen for com-

parative tests with 0.03 per cent glucose biologic-products broth for the routine testing of biologic products for sterility. (It is of interest as has been mentioned in the introduction to note that this medium was suggested by Hitchens in 1921, but did not obtain the universal acceptance that biologic-products broth did.) The results of our routine tests with 0.1 per cent agar have confirmed our experimental findings, as well as confirming and extending those of Hitchens (1921) and Spray (1936). The adoption of a semifluid medium as a standard means of detecting and giving a quantitative estimate of the extent of bacterial growth in biologic products can therefore be recommended. Applications to other materials and procedures involving bacterial growth are also indicated.

REFERENCES

ELDERING, G., AND KENDRICK, P. L. 1936 Some practical considerations in B. pertussis vaccine preparation. Am. Jour. Pub. Health **26**: 506–511.

FALK, C. R., AND APLINGTON, S. 1936 Studies on the bactericidal action of phenol and merthiolate used alone and in mixtures. Am. Jour. Hyg., **24**: 285–308.

HITCHENS, A. P. 1921 Advantages of culture mediums containing small percentages of agar. Jour. Inf. Dis., **29**: 390–407.

ROSENSTEIN, C., AND LEVIN, I. 1935 The bactericidal and antiseptic action of preservatives frequently used in biological products, and the effect of these preservatives on the potencies of the products. Am. Jour. Hyg. **21**: 260–279.

SPRAY, R. S. 1936 Semisolid media for cultivating and identification of sporulating anaerobes. Jour. Bact. **32**: 135–155.

WADSWORTH, A. 1927 Standard Methods. Williams & Wilkins, **93**, 544.