The Inhibitory Action of Agar on Certain Strains of Pleuropneumonia-like Organisms[1]

RAYMOND J. LYNN AND HARRY E. MORTON

Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

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The pleuropneumonia-like organisms (PPLO) are recognized as a group of rather fastidious microorganisms. Various peptones, infusions, and serum supplements have been examined by many investigators relating to the development of a medium satisfactory for the *in vitro* cultivation of these organisms (Morton *et al.*, 1951; Edward, 1947; and others). However, few reports of examinations of the agar incorporated into the medium are available. While we were encountering difficulties with agar, Liebermeister (1954) encountered somewhat similar experiences in Germany. The present investigation was prompted by the sudden occurrence of severe retardation of growth of some strains of PPLO during routine cultivation of stock strains on culture medium previously found satisfactory. In reviewing the ingredients incorporated into the medium in question, it was found that agar from a different manufacturer had been used. These observations are presented to call attention to the unsuitability of some lots of agar as an ingredient in culture media to be used for the cultivation of PPLO.

EXPERIMENTAL METHODS

Growth tests were carried out on solid medium to determine if the agar incorporated was the inhibitory factor. PPLO medium (Morton *et al.*, 1951) was prepared and divided into three lots. Difco agar lot number 0140-01, control 425232, was added to the first lot. In the second, BBL agar, lot number 4868, was utilized. This was the agar present in the culture medium initially found unsatisfactory. BBL agar, lot number 5966, was added to the third lot of medium. The final concentration of agar incorporated in all instances was 1.5 per cent. The pH of the medium was adjusted to 7.8 before autoclaving. Bacto PPLO serum fraction (Smith and Morton, 1951) was added as the supplement immediately before use in an amount so that the final concentration was 1 per cent. Seven strains of PPLO isolated from human sources and one strain isolated from a calf served as the testing microorganisms. All of these strains had been subcultured at 5-day intervals on laboratory medium for periods exceeding a year. The original inoculations were made from the laboratory medium which contained the Difco agar. A small block of medium approximately 1 cm square showing optimal growth was removed, inverted, and streaked once over the surface of the test medium. The block was discarded after the streaking procedure. The plates were inverted and incubated at 37 C. Examination of the plates with a binocular microscope at a magnification of 100× was made at 3-, 5-, and 7-day intervals. Subsequent transfers were made from areas showing maximum growth on the 5-day-old incubated plates according to the procedure described above. Where no growth was apparent the initial procedure was repeated. Three such transfers were conducted in determining whether the test medium would support the growth of a specific strain of PPLO. The size of the colonies, number of colonies, and characteristic colony morphology, based upon previous observations made during the course ot routine subculturing, were used as criteria of growth.

The ash of the inhibitory agar was examined to determine if an inhibitory factor was present in this fraction of the agar. A 15-g portion of the agar which showed maximum inhibition, BBL agar lot number 4868, was ashed in a muffle furnace. The ash was dissolved in mineral acid and made up to 10 ml with distilled water. Aliquots of this solution were added to a complete medium previously shown to be satisfactory. The pH of the medium was readjusted to pH 7.8 with 1.0 N NaOH and 1 per cent Bacto PPLO serum fraction added.

An attempt was made to remove the inhibitory effect by boiling the complete medium with 2 per cent packed horse red blood cells according to the method described by Dienes (1939).

To another lot of the medium, 0.15 per cent soluble starch (Merck) was added before autoclaving in an attempt to relieve the inhibition.

Subsequent attempts to remove the inhibition were made by extracting the dry agar with 3 volumes of ether and methanol.

RESULTS

The results of each series of experiments are compiled in table 1. Each series is made up of at least 3 subtransfers as described in the section on experimental methods.

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TABLE 1. *The inhibitory effect of agar as measured by the relative growth of certain strains of pleuropneumonia-like organisms on solid medium*

Basal Medium (Morton *et al.* 1951) Plus	Strains of PPLO										
	Campo	07	39	110	Lomax	60	48	SF-29*	V-73†	101†	468†
Difco agar lot no. 0140-01	4+‡	4+	4+	4+	4+	3+	4+	4+	4+	4+	4+
BBL agar lot no. 5966	4+	4+	2+	+	+	0	0	2+	—	—	—
BBL agar lot no. 4868	4+	3+	+	0	0	0	0	+	—	—	—
	4+	4+	2+	2+	0	0	0	3+	—	—	—
	4+	3+	3+	2+	0	0	0	3+	+	0	—
Difco agar lot no. 0140-01 with ash of BBL agar lot no. 4868	4+	4+	4+	4+	+	2+	2+	3+	—	—	—
	4+	4+	4+	3+	2+	3+	2+	4+	3+	2+	—
BBL agar lot no. 4868 heated with 2 per cent packed horse red blood cells	4+	4+	3+	2+	—	—	+	4+	+	0	0
BBL agar lot no. 4868 with 0.15 per cent soluble starch	4+	4+	3+	2+	—	—	+	3+	2+	+	0
Methanol-ether extracted, BBL agar lot no. no. 4868	4+	4+	3+	2+	0	0	0	3+	0	0	—

* Calf strain.

† Recent isolates from humans which required blood for initial isolation.

‡ 0, no growth; +, poor growth; 2+, fair growth; 3+, moderate growth; 4+, good growth; —, not tested.

Optimal growth was obtained with all strains of PPLO in each series with the control medium which contained Difco agar. Near-optimal or optimal growth was observed with 3 human strains and the 1 calf strain on the medium containing BBL agar, lot number 5966. Sparse growth was obtained with 2 other strains from human sources, whereas 2 human strains failed to grow on this medium. Three series of tests conducted at various periods during the course of the experiment incorporated BBL agar, lot number 4868, into the basal medium. As shown in table 1, three strains of PPLO from human sources failed to grow in any of the test series. Another human strain, 110, failed to grow in the first series of tests but showed fair to moderate growth in the last two test series. Two recently isolated strains from human sources, designated as V-73 and 101, which had required hemoglobin for initial isolation (Feo *et al.*, 1956), were inhibited by this agar. Strain V-73 showed only sparse growth, whereas strain 101 failed to grow.

It was further noted that, with the exception of the Campo L strain, very few colonies possessed the "fried-egg" appearance usually associated with growth of PPLO. On those plates rated poor or fair in the table, the colonies were large and vacuolated with uneven borders.

The incorporation of aliquots of the ash obtained from lot number 4868 of BBL agar seemed to produce a slight inhibitory effect. However, the inhibition appeared somewhat less than that encountered in the medium containing agar of lot number 4868.

There was no appreciable lessening of the inhibitory effect by heating the medium with the 2 per cent packed horse red blood cells or by adding 0.15 per cent starch to the medium. Extracting the agar with ether and methanol did not alter the inhibition.

DISCUSSION

The above results indicate that certain lots of agar are inhibitory for some strains of pleuropneumonia-like organisms. It was reported by Gould *et al.* (1944) that growth of certain strains of *Neisseria gonorrhoeae* were inhibited by agar or some substance associated with agar. In this instance the inhibitory effect could be relieved by starch, gastric mucin, or the insoluble fraction of whole yeast autolysate. Dienes (1939) found that a more satisfactory medium for the L-organisms could be attained by adding 2 per cent packed horse blood sediment to the medium and heating it just to the boiling point. Neither of these procedures—adding starch or heating with red blood sediment—produced any appreciable effect in this study.

Recently, Liebermeister (1954) observed that one type of pulverized agar of a German manufacturing firm inhibited significantly the growth of PPLO and *Streptobacillus moniliformis*. No differences were apparent in the physical properties of the agar gel of the inhibitory agar when compared to a suitable one.

In the present study no procedure was found that

would relieve the inhibitory effect of the agar. It should be pointed out that the particular strains which showed maximal inhibition are those which grow poorly, if at all, in liquid medium. Also, three of these strains were recent isolates which had shown a requirement for blood on initial isolation.

SUMMARY

Certain lots of agar provided for bacteriological use have been found to be unsatisfactory for the cultivation of some strains of pleuropneumonia-like organisms.

Those strains of pleuropneumonia-like organisms which were more fastidious were observed to be the most likely to be inhibited by the unsatisfactory lots of agar.

The inhibitory action of certain lots of agar for pleuropneumonia-like organisms was not removed to any great extent by the addition of starch to the medium, by heating the medium with 2 per cent packed red blood cells, or by extracting the agar with ether and methanol.

Agar becomes another ingredient of bacteriological culture medium which should be checked for its growth-promoting properties before being marketed as being suitable for the cultivation of pleuropneumonia-like organisms.

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New Enrichment and Plating Media for the Isolation of *Salmonella* and *Shigella* Organisms[1]

A. A. HAJNA AND S. R. DAMON

Bureau of Laboratories, Indiana State Board of Health, Indianapolis, Indiana

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In a recent publication (Hajna, 1955) a new specimen preservative, "SP," devised for use in the collection, transportation, and preservation of stool specimens, rectal swabs, and sputum for the isolation of salmonellae, shigellae, klebsiellae, and other gram negative bacteria, was described. In shipped specimens employing this preservative, a comparison with buffered glycerol saline showed that more shigellae and salmonellae were recovered. This recovery was due to suppression of gram positive cocci and spore-bearing organisms by the sodium desoxycholate included in the formula.

Using various combinations of the enrichment broths and plating media (Hajna, 1955) it was noted that there was considerable variation in productivity among the tetrathionate broths (Muller, 1923; Kauffman, 1930; Schaeffer, 1935; Knox *et al.*, 1942; Ruys, 1934) when specimens were transferred to the enrichment broths from the "SP" transporting fluid. An effort was made, therefore, to develop a better tetrathionate broth which would include the best features of the broths. At the same time a new base was introduced by the authors, and is herein described.

The plating media used in the present study were: BBL[2] desoxycholate citrate lactose-sucrose agar, Bacto[3] MacConkey agar, Bacto SS agar, and Hajna's modification of the bismuth sulfite agar of Wilson and Blair. In addition, two new media, brom cresol purple-desoxycholate agar (BCP-D) and brom cresol purple-desoxycholate citrate lactose sucrose agar (BCP-DCLS), were employed.

The present report is based on the use of the new media, but retaining Leifson's selenite "F" broth (Leifson, 1936) for comparison with the new "TT" broth.

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[2] Baltimore Biological Laboratory, Baltimore, Maryland.

[3] Difco Laboratories, Inc., Detroit, Michigan.