Bull. Org. mond. Santé
Bull. World Hlth Org. } 1952, 7, 353-356

A CHOLERA MEDIUM WITH MORE THAN TENFOLD YIELD

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Manuscript received in July 1952

Vibrio cholerae is easy to cultivate, but growth is not always satisfactory. As poor growth is an obstacle in diagnostic work, Felsenfeld and his associates [1] set out to re-evaluate various culture methods. Their best results were obtained when using plain peptone; consequently these authors decided to employ a medium containing 1% peptone (Difco) and 0.5% sodium chloride, as well as agar when plating was intended. Although poor growth interferes with diagnostic work, it is a still greater hindrance in the preparation of vaccines. The value of improved media can be inferred from the fact that probably several hundred million doses of vaccine are needed yearly in countries all the world over.

We have therefore tried to devise a simple medium, yielding well and producing organisms of appropriate antigenic properties. The experiments were carried out with strains no. 41-1 (Ogawa) and no. 35A3 (Inaba).[2]

All media used for comparison contained 2.5% agar, and had a pH of about 7.6-7.8. 5 ml of the medium were dispensed into 50-ml flat-bottomed boiling-flasks; these flasks proved to be more suitable than slants, as the inoculum could be spread more evenly, and the organisms were easily harvested. The inoculum was taken from a 24-hour-old culture on agar; the volume of the inoculum was about 0.3 ml, corresponding to about 23 million organisms, as estimated by comparison with Wellcome opacity standards. In the flasks 5 ml of agar had a free surface of about 18 cm^2, hence each square centimetre was sown with approximately 23/18 (i.e., 1.3) million *V. cholerae*. After incubation for 22-24 hours, the growth was harvested by rinsing with 20 ml of saline, containing 0.01% merthiolate; the suspension was then heated in a water-bath at 56°C for half an hour, and the growth estimated turbidimetrically. Triplicates were always taken, and in the case of any suspected improvement the medium was twice

[1] Felsenfeld, O., Soman, D. W., Waters, T. & Ishihara, S. J. (1951) *J. Bact.* **62**, 175

[2] These strains were kindly provided by Professor L. E. Ranta, University of British Columbia, Vancouver, Canada.

prepared anew; the experiments were then repeated in quadruplicate. Although variations between different runs of the same medium proved to be low, a further check was afforded by flasks containing a reference standard medium. Thus the gain due to improved composition of an experimental medium could be expressed in two ways : either by the number of organisms harvested from one flask, or by the relationship :

$$\frac{\text{harvest from experimental medium}}{\text{harvest from reference standard medium}}$$

This expression, less influenced by varying strength of inoculum, temperature, and time of incubation than by the absolute number of organisms, is shown in table I, where it is designated "multiplication factor". The mean yield per flask of the reference standard medium (see below) was 19.5×10^9 organisms. The multiplication factor times 19.5×10^9 therefore represents the number of organisms obtained from one flask of the experimental medium. Obviously, the choice of such a reference standard medium had to be arbitrary. Though much of the present work had already been done when the study of Felsenfeld and his associates came to our notice, all experiments were repeated, and their peptone medium (medium I in table I) was adopted as reference standard. The results for both strains investigated proved to be somewhat similar, and were therefore pooled in table I.

The performance of the reference medium could be improved by replacing the peptone partly or wholly by casein hydrolysate (Squibb). The concentrations tried were : (*a*) 0.5% peptone + 0.5% casein hydrolysate, and (*b*) 1.0% casein hydrolysate. We found glycerol to be a very powerful nutrient for either strain; it was capable of increasing several times the yield of the reference medium as well as of media containing peptone and casein hydrolysate, or casein hydrolysate without peptone. In repeatedly-performed titrations the optimal concentration of glycerol was found to be 2.2%, i.e., 1.5 ml of glycerol per 100 ml of medium; concentrations of 1.0 ml and 2.0 ml of glycerol per 100 ml of medium were slightly less effective. This action of glycerol was greatly enhanced by the presence of phosphates; phosphate in its optimal concentration (0.25% disodium hydrogen phosphate) raised the yield of the standard medium by about 39%, as can be seen by comparing medium I with medium II in table I. Medium I, the reference standard medium, has a multiplication factor of 1.00 (by definition), while the multiplication factor of medium II was found to be 1.39. The yield of medium I could be increased 2.31 times by incorporating 2.2% glycerol, while the corresponding increase in the presence of phosphate (medium II) became 8.30/1.39 = 5.97. Thus there seems to be reason to assume that synergism is involved in the joint action of glycerol and phosphate; for if both substances were independent, the joint action would rather be expected to result in a multiplication factor of

$1.39 \times 2.31 = 3.21$, and not in a factor of 8.30 as was actually found. In the presence of Bovril and Marmite, the peptone medium did not gain from the incorporation of phosphate (media III and IV in table I, with factors 2.90 and 2.92 respectively); but here also the effect of phosphate became visible when glycerol was added (factors 8.50 and 8.82 respectively). In one medium (IX) the addition of phosphate (medium X) caused a decrease in yield. Nevertheless, the beneficial effect of phosphate was clearly shown in the presence of glycerol.

TABLE I. GROWTH OF VIBRIO CHOLERAE ON VARIOUS SOLID MEDIA AT pH 7.6-7.8, AFTER 22-24 HOURS

Composition of medium	Medium No.									
	I*	II	III	IV	V	VI	VII	VIII	IX	X
Peptone (Difco)										
0.5%	—	—	—	—	—	—	—	—	+	+
1.0%	+	+	+	+	—	—	—	—	—	—
Casein hydrolysate (Squibb)										
0.5%	—	—	—	—	—	—	—	—	+	+
1.0%	—	—	—	—	+	+	+	+	—	—
Sodium chloride 0.5%	+	+	+	+	+	+	+	+	+	+
Disodium hydrogen phosphate 0.25%	—	+	—	+	—	+	—	+	—	+
Bovril 0.15%	—	—	+	+	—	—	+	+	+	+
Marmite 0.15%	—	—	+	+	—	—	+	+	+	+
Agar 2.5%	+	+	+	+	+	+	+	+	+	+
Multiplication factor **										
without glycerol	1.00	1.39	2.90	2.92	2.89	2.39	3.90	4.15	4.10	2.94
with 2.2% glycerol	2.31	8.30	8.50	8.82	4.06	6.10	9.50	12.06	9.07	12.56

* Medium I (peptone (Difco) 1%, sodium chloride 0.5%, agar 2.5%) is the reference standard medium; its average yield is taken as unity, and equals 19.5×10^9 *V. cholerae* per flask.

** The multiplication factor is the fraction : $\frac{\text{harvest from experimental medium}}{\text{harvest from reference standard medium}}$.

The multiplication factor times 19.5×10^9 indicates the average yield per flask of experimental medium ; e.g., for medium X with glycerol : $19.5 \times 10^9 \times 12.56 = 238 \times 10^9$ *V. cholerae*.

Table I indicates that the best medium among those tested seems to be medium X with glycerol. It consists of : peptone (Difco) 0.5%, casein hydrolysate (Squibb) 0.5%, sodium chloride 0.5%, disodium hydrogen phosphate 0.25%, Bovril 0.15%, Marmite 0.15%, glycerol 2.2%, and agar 2.5%. Its yield is more than twelve times that of plain peptone agar, and *V. cholerae* grown on this medium possess an antigenic power satisfying the requirements of the United States Public Health Service.

SUMMARY

Nine different cholera media were compared with a reference standard medium (plain peptone agar), with the object of finding a simple medium with an increased yield. It was found that a high yield—more than twelve times that obtained with the plain peptone agar—was produced with a medium containing : 0.5% peptone (Difco), 0.5% casein hydrolysate (Squibb), 0.5% sodium chloride, 0.25% disodium hydrogen phosphate, 0.15% Bovril, 0.15% Marmite, 2.2% glycerol, and 2.5% agar. The good growth obtained with this medium is mainly due to the joint action of glycerol and phosphate. The organisms grown have good immunizing properties.

RÉSUMÉ

Neuf milieux différents, destinés à la culture du vibrion cholérique ont été comparés à un milieu de référence (gélose simple à la peptone). Il s'agissait de trouver une formule simple assurant un rendement meilleur que les milieux courants. L'un des milieux expérimentés a donné un rendement plus de douze fois supérieur à celui du milieu de référence; il contenait 0,5% de peptone (Difco), 0,5% d'hydrolysat de caséine (Squibb), 0,5% de chlorure de sodium, 0,25% de phosphate disodique, 0,15% de Bovril, 0,15% de Marmite, 2,2% de glycérol et 2,5% de gélose. L'excellent développement des vibrions dans ce milieu est dû principalement à l'action conjuguée du glycérol et du phosphate. Les propriétés immunisantes des germes ainsi obtenus se sont révélées très satisfaisantes.