A SIMPLIFIED LIQUID CULTURE MEDIUM FOR THE GROWTH OF HEMOPHILUS PERTUSSIS

W. F. VERWEY, ELIZABETH H. THIELE, DOROTHY N. SAGE, AND LEE F. SCHUCHARDT

Department of Bacteriology, Medical Research Division, Sharp and Dohme, Inc., Glenolden, Pennsylvania

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Hornibrook (1939) described a liquid medium for the propagation of *Hemophilus pertussis* having a casein hydrolyzate base to which were added various salts, starch, cysteine, and nicotinic acid and which was capable of growing recently isolated strains. Because of the potential advantages of such a medium, it has been studied by many investigators. Their work has confirmed Hornibrook's original observations but it has also revealed several disadvantages in the medium. Modifications of the original formula stated to prevent precipitation, simplify preparation, decrease the granularity of growth, and increase the final bacterial count have been suggested by Verwey and Sage (1945), Wilson (1945), Farrell and Taylor (1945), and Cohen and Wheeler (1946).

It is the purpose of this report to describe further the medium that has been developed in our laboratory and to define the cultural conditions that seem optimal for the growth of *H. pertussis* in this medium. Some observations concerning the antigenic properties of the liquid medium cultures are included.

EXPERIMENTAL METHODS AND RESULTS

Culture medium and growth studies. In table 1 the general formula of our medium has been compared with those of others. Quantities have been expressed in grams or milliliters per liter of culture medium, and the figures for casein hydrolyzate and sodium chloride have been calculated with recognition of the fact that "casamino" acids (Difco technical) contain approximately 40 per cent sodium chloride. Although, for purposes of simplicity in presentation, the phosphate and sulfur sources have not been specified, all formulae made use of sodium or potassium phosphate and cysteine, cystine, or glutathione.

These modifications of the Hornibrook formula have certain features in common. Calcium chloride has been reduced in amount or eliminated to prevent the troublesome precipitation of calcium phosphate that occurred frequently in the original medium. Except in the Wilson formula, the quantity of phosphate has been increased to buffer the medium against the strong tendency of *H. pertussis* to cause an alkaline reaction. In addition, the concentrations of casein hydrolyzate are increased somewhat except in the formula of Cohen and Wheeler. The formula in this paper is given specifically in table 2 and contains balanced changes representing all of these modifications. All ingredients may be added before sterilization. It is to be noted that the formula makes use of no accessory growth factors other than those supplied by the casein hydrolyzate itself. Furthermore, it has been found that no growth stimulation resulted from the addition of the

accessory growth factors present in blood cell extract or liver extract. This result was different from the earlier report of Verwey and Sage (1945) in which blood cell extract was shown to produce a considerable improvement in final bacterial

TABLE 1

Culture medium composition expressed in grams per liter

	HORNIBROOK	WILSON	FARRELL AND TAYLOR	COHEN AND WHEELER*	VERWEY ET AL.
Casein hydrolyzate	7.0	10.0	10.0	6.0	9.4
NaCl	5.0	5.0	5.0	6.5	4.6
KCl	0.2	0.2	0.2	—	0.2
$CaCl_2$	0.2	0.002	—	0.1	—
$MgCl_2 \cdot 6H_2O$	0.1	0.025†	—	0.4	0.1
Na_2CO_3	0.5	0.4	—	—	—
Phosphate	0.25	0.25	0.4	0.5	0.5
Starch	1.0	1.0	1.0	1.5	1.0
Nicotinic acid	0.01	—	0.001	—	0.02
Sulfur source	0.01	0.01	0.022	0.025	0.01
Accessory factors	—	Liver ext. 0.4 ml	Liver ext. 20 ml	Yeast dial. 50 ml	—

* $FeSO_4 \cdot 7H_2O$, 0.01; $CuSO_4 \cdot 5H_2O$, 0.005, additional ingredients.
† $MgCl_2 \cdot 8H_2O$.

TABLE 2

Liquid medium for the growth of Hemophilus pertussis

Distilled water	1 liter
Difco "casamino" acids technical grade	14 grams
KCl*	0.2 gram
KH_2PO_4	0.5 gram
$MgCl_2 \cdot 6H_2O$	0.1 gram
Nicotinic acid	0.02 gram
L-Cystine†	0.01 gram
Starch‡	1 gram

The pH is adjusted to 6.8 and the medium is then autoclaved 10 minutes at 15 pounds to get material into solution. The pH is rechecked and should be from pH 6.8 to 6.9. The medium is bottled and sterilized at 15 pounds for 15 minutes. This medium will keep from 4 to 8 months.

* Experiments have shown that the inclusion of KCl usually is not necessary.

† Glutathione may be substituted for L-cystine in the same amount.

‡ Starch granules are first thoroughly wet with cold water. This suspension is then added to hot water and the mixture brought to a "rolling" boil. The resulting solution is added to the rest of the medium.

density. The reason for this difference may be in the nature of the casein hydrolyzate supplied by the Digestive Ferments Company. Lots purchased in 1941, 1942, and part of 1943 were deficient in some factor that has appeared regularly in subsequent batches. This deficiency was overcome by blood cell extract. The identity of this factor is not known at present, although considerable information

tending to eliminate from consideration many of the recognized accessory growth substances has been obtained.

In addition to the ingredients of the culture medium, cultural conditions including inoculum size, time of incubation, surface-volume relationships, and agitation have a profound effect on the growth rate and final density of *H. pertussis* cultures grown in liquid media. Therefore, it is important to define these conditions in evaluating the results of any cultural procedure. Table 3 describes the cultural conditions employed in our laboratory and compares them with those of other workers. The sizes of the inocula that were used were not specified completely by Wilson or by Farrell and Taylor, so these have been estimated from the stated inoculum volumes and the reported densities usually obtained.

It is believed that the favorable results that have been obtained with our procedure are attributable to a combination of several factors. During the early

TABLE 3

Cultural conditions for the growth of H. pertussis in liquid media

	HORNIBROOK	WILSON	FARRELL AND TAYLOR	COHEN AND WHEELER	VERWEY ET AL.
Incubation temp.	37 C	37 C	37 C	35 C	37 C
Incubation time, in days	2–4	4–5	3	3	2–3
Agitation	Intermittent	Intermittent	Continuous	Intermittent	Continuous
Inoculum per liter	Unspecified	2,500 B	20 B	750–1,000 B	400–1,000 B
pH	7.4	7.0	7.1	7.2–7.3	6.8
Density per ml	15 B+*	15 B+*	15 B+*	10–15 B	20–50 B

* No maximum density or range of densities given.

work with Hornibrook's medium it became apparent that the development of an alkaline reaction in the cultures was a growth-limiting condition. When the buffering power of the medium was increased, further additions of casein hydrolyzate and nicotinic acid resulted in increased density. It was observed also that the increase in pH that occurred was related both to the bacterial density and to the time of incubation. Thus, 72-hour cultures that had grown to a specified density were always more alkaline than the 48-hour cultures at the same density. This observation suggested that efforts should be made to produce rapid growth so that maximal multiplication would take place before the medium had become strongly alkaline. It was found that, through the use of relatively large inocula of 400 to 1,000 billion organisms per liter, greater final densities could be produced and the cultures reached their maximal values more quickly. When these procedures were combined with the use of constant agitation on a shaking machine operating at 120 strokes a minute on a traverse of approximately 8 mm, the most satisfactory results were obtained. Cultures that were incubated in 250-ml quantities contained in liter Blake bottles placed horizontally on the

shaking machine grew regularly to densities between 20 and 50 billion organisms per ml in 48 hours.

Figure 1 shows the frequency of occurrence of various bacterial turbidities at 48 and 72 hours in a large series of cultures that have accumulated during the last three years. It can be seen that the 48-hour cultures show a marked peak between 20 and 30 billion, but almost half are above 30 billion. The maximal density found was 55 billion per ml at 48 hours and 77 billion at 72 hours.

This medium has been tried for the growth of 13 strains of *H. pertussis* that were demonstrated to be typically smooth. All but one of these strains grew readily to densities within the range previously mentioned. No adaptation seemed

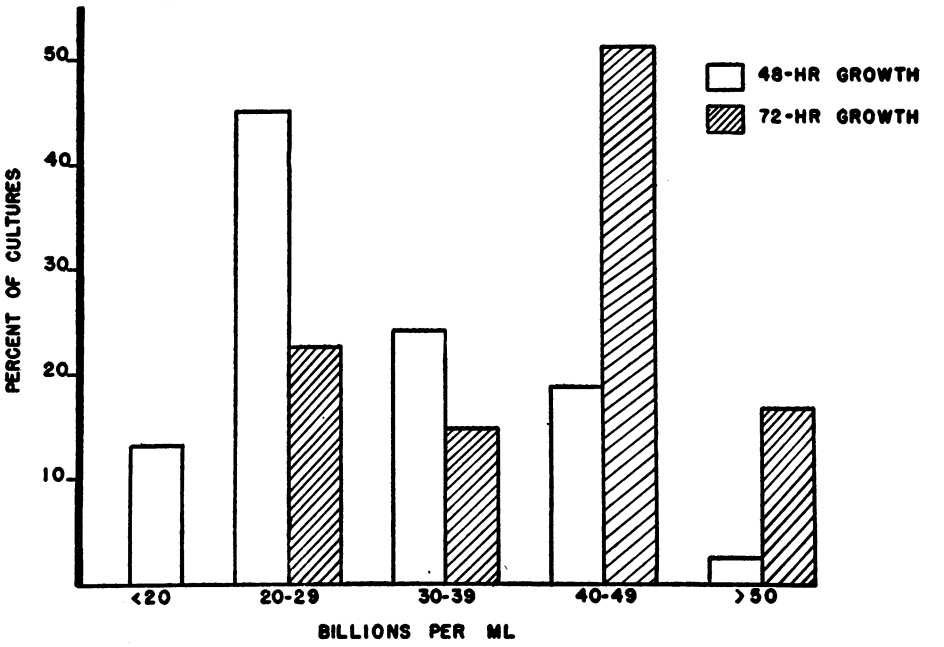

Figure 1. Density of *H. pertussis* cultures grown in liquid medium.

to be required in this medium since first subcultures from Bordet Gengou agar grow as rapidly as, and had densities equal to, subsequent subcultures.

Microscopic examinations of stained preparations indicated that organisms grown in this medium were short coccobacillary forms very similar in appearance to those harvested from Bordet Gengou agar. Filaments and clumps were relatively infrequent. Repeated consecutive subculture through as many as 19 passages in this medium caused no obvious change in cellular or colonial morphology, and the cultures remained as smooth, phase I cultures. A strain of *H. pertussis* having an intracerebral mouse virulence of 200 to 400 organisms was carried through nine successive subcultures in the liquid medium without appreciable loss of infectivity.

Recent studies have indicated that glutathione may be a considerably more

effective sulfur compound than is L-cystine when it is used in similar amounts. Although the trials of the medium containing glutathione have not been as extensive as those employing L-cystine, the results indicate that glutathione causes more rapid growth and higher densities after 48 hours of incubation. A comparison of the results obtained with these two sulfhydryl compounds is given in figure 2 in which density figures obtained with strains 2227 and 2231 have been combined. These figures were collected from a series of parallel cultures from Bordet Gengou agar inocula.

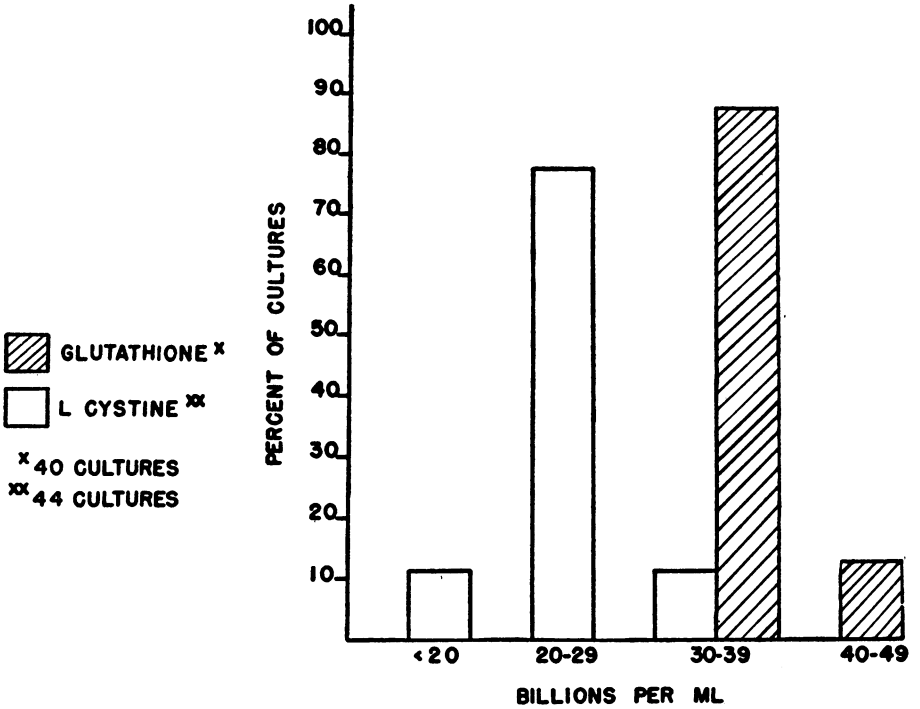

Figure 2. Density of *H. pertussis* cultures grown 48 hours in liquid medium containing L-cystine or glutathione.

Vaccine antigenicity. Pertussis cultures grown in a liquid medium and killed by the addition of sodium ethylmercuri-thiosalicylate ("merthiolate") or phenol are outstandingly suitable for the preparation of immunizing vaccines. The whole culture (properly killed and standardized) is safe for injection as a vaccine since it contains no foreign protein other than that contributed by the organisms themselves. Such a vaccine has been in use for more than four years, and over this time there has been reported no instance of sensitization that could be attributed to the medium.

Numerous vaccine preparations killed with either 0.5 per cent phenol or 1:10,000 sodium ethylmercuri-thiosalicylate have been tested for antigenicity. These vaccines when tested by the protection test methods described by Pittman (1946) or Kendrick *et al.* (1947) appear to be comparable to vaccines prepared

from organisms grown on Bordet Gengou agar. Also, they have been found to meet the standards for pertussis vaccine established by the National Institute of Health of the United States Public Health Service. Evidence accumulated in this laboratory suggests that the neutralization of the relatively high final alkalinity of liquid cultures is desirable to maintain the antigenicity of the cells during long periods of storage at refrigerator temperatures.

Solid medium studies. The liquid medium described above has been converted to a solid medium by the addition of 1.5 to 2.0 per cent agar. Only traces of growth appear following heavy inoculation. However, when human blood cells in saline equivalent to 10 to 20 per cent whole blood are added, good growth

TABLE 4

Comparative colony counts on Bordet Gengou and casein hydrolyzate blood agar

	72 HOURS			96 HOURS			120 HOURS		
	Casein hydrolyzate blood agar	BG I*	BG II†	Casein hydrolyzate blood agar	BG I*	BG II†	Casein hydrolyzate blood agar	BG I*	BG II†
Strain 2753	100	18	86	120	70	88	129	106	93
	99	23	80	115	65	95	115	115	95
	90	25	60	128	67	65	128	110	65
	96	20	83	116	47	83	116	99	83
	84	16	86	100	50	96	106	80	96
	88	10	60	120	48	73	120	110	73
Strain 2227	179	0	173	202	0	195	214	170	195
	85	43	66	124	95	86	132	107	108
Strain 2231	90	41	69	120	111	89	128	113	113
	81	45	63	129	79	83	137	101	103

Seed: 0.1 ml containing 200 organisms by turbidity estimation was spread on the surface of agar plates.

* Bordet Gengou agar prepared in the laboratory.

† Difco Bordet Gengou agar.

develops within 24 hours. This medium was compared with Bordet Gengou agar by spreading 0.1-ml quantities of dilute suspensions of *H. pertussis* on the plates. These data are summarized in table 4, and it can be seen that the casein hydrolyzate blood agar induced the growth of a larger number of colonies in a shorter period of time than did either of the two Bordet Gengou agar media that were tested.

Casein hydrolyzate blood agar containing 0.2 units of penicillin per ml has received limited trial in the isolation of *H. pertussis* from nasopharyngeal swabs. The results of this work indicated that satisfactory isolations could be obtained and that the overgrowth of contaminants was no more extensive with this medium than with Bordet Gengou agar containing comparable quantities of penicillin.

DISCUSSION

The medium that is described in this communication represents a simplification and a balancing of the original formula of Hornibrook. It has resulted in further improvement of growth-promoting properties and the elimination of all substances of indefinite chemical composition with the exception of starch and casein hydrolyzate. In addition, the medium has been found to be of considerable value both in the production of *H. pertussis* vaccines and in the study of the metabolic and antigenic constituents of the organism (Verwey and Thiele, 1949). The use of this medium and the cultural conditions described have resulted in more rapid growth and greater final bacterial counts than have been reported previously.

It is of interest that, although the medium appeared to be entirely adequate in its liquid form, it could not support the growth of *H. pertussis* when it was solidified by the addition of agar. It is not known at present whether this situation is the result of some inhibitory effect of agar or of a peculiarity of the respiratory requirements of the organism. However, even though blood cells are required for growth, it is believed that this solid medium may be of considerable usefulness because of the simplicity of preparing it and because of its superior growth-promoting characteristics. More extensive trials than those so far conducted will be required before this casein hydrolyzate blood agar may be evaluated properly in the laboratory diagnosis of pertussis infection. However, the results obtained seem to indicate that the medium may be able to replace Bordet Gengou agar in all of its normal uses.

SUMMARY

A modification of Hornibrook's liquid casein hydrolyzate medium for the cultivation of *Hemophilus pertussis* has been described, and improved growth conditions have been defined. It was found that culture densities between 20 and 50 billion cells per ml could be obtained regularly within 48 hours, and that organisms grown in this medium were similar culturally to those grown on Bordet Gengou medium. These cultures were suitable for the preparation of vaccines of satisfactory antigenicity as measured by mouse protection tests.

The medium that was developed contains only ingredients that are readily available in a dry, stable form. It can be prepared also as a solid medium by the inclusion of agar. Ten to 20 per cent blood cells had to be added to the solid medium to obtain good growth, but when the blood cells were used, more colonies of *H. pertussis* could be grown with this medium from a dilute bacterial suspension than with either of the two Bordet Gengou agar preparations that were tested. Since these colonies appeared earlier and grew larger than those on the Bordet Gengou plates, it has been suggested that the casein hydrolyzate blood agar might be suitable for use in the laboratory diagnosis of pertussis infection.

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