APPLIED MICROBIOLOGY
Vol. 12, No. 6, p. 504-507 November, 1964

Blood-Free Medium for the Rapid Growth of *Pasteurella tularensis*

HUGH B. TRESSELT[1] AND MARTHA K. WARD

U.S. Army Medical Unit, Fort Detrick, Frederick, Maryland

Received for publication 29 June 1964

ABSTRACT

TRESSELT, HUGH B. (Fort Detrick, Frederick, Md.), AND MARTHA K. WARD. Blood-free medium for the rapid growth of *Pasteurella tularensis*. Appl. Microbiol. **12**:504–507. 1964.—A medium composed of (in g/100 ml) Tryptose broth with thiamine (Difco), 2.6; cysteine-HCl, 0.12; glucose, 1; $FeSO_4,7H_2O$, 0.005; KCl, 0.02; histidine, 0.1; tris(hydroxymethyl)aminomethane (tris) buffer, 0.3; and agar, 1; will support rapid growth of the fully virulent SCHU-S4 strain of *Pasteurella tularensis*. Although the test organism grew rapidly on medium from which KCl and tris buffer were omitted, these two components increased the stability of the medium upon storage at 4 C. It was necessary to (i) control carefully the relative concentration of the ferrous iron and cysteine-HCl, (ii) incubate the prepared medium overnight prior to use, and (iii) incubate the inoculated plates in an atmosphere of high relative humidity. Rapid growth of the organism was obtained also from very small inocula in the liquid form of the medium. Biochemical studies designed to elucidate the mechanisms involved in the enhancement of growth of *P. tularensis* in this relatively simple blood-free medium were initiated.

A number of media have been described for the growth of *Pasteurella tularensis*. Several contain no blood or blood products (Nagle, Anderson, and Gary, 1960; Snyder et al., 1946; Won, 1958), although the formulas for most included whole blood, one or more of its components, or some product derived from it (Downs et al., 1947; Gibby et al., 1948; Giesken, Guss, and Eigelsbach, 1957; Hood, 1961). In most instances, multiplication of the organism is slow, and a large inoculum is required for initiation of growth.

Earlier work in this laboratory resulted in the development of a blood-containing (Gaspar) medium that markedly enhanced the growth of several strains of *P. tularensis* (Gaspar, Tresselt, and Ward, 1961). Colonies of 1 mm or more in diameter were produced in 29 to 30 hr from an inoculum of 50 to 100 cells.

Our work was undertaken to determine the contribution of blood to the enhancement of growth observed on Gaspar medium. Elimination of blood would greatly simplify biochemical studies designed to elucidate the basic mechanisms involved. In addition, a completely transparent, simple medium for growth of *P. tularensis* would offer many obvious advantages for diagnostic and experimental work requiring the isolation and counting of *P. tularensis* colonies.

Preliminary studies. It was found that the addition of an extract of red blood cells to the basal Gaspar medium gave enhanced growth of *P. tularensis* almost equal to that on medium containing whole blood. The active extract was obtained by boiling lysed erythrocytes for 10 min in the presence of 0.5 g/100 ml of cysteine-HCl, followed by filtration to remove the coagulum. Colonies on the extract-containing medium were only slightly less than 1 mm in diameter after 29 to 30 hr of incubation, whereas those on Gaspar medium were usually 1 mm or more.

Components of the filtrate, identified by the analytical methods used, were iron, cystine, ergothionine, and potassium, in addition to the cysteine which had been added during preparation. An unidentified ninhydrin-positive spot was also observed on paper chromatograms. When equivalent concentrations of iron as $FeSO_4 \cdot 7H_2O$, cystine, ergothionine, and potassium as KCl were substituted for blood in the basal medium of Gaspar, enhanced growth of the organism comparable to that on the blood extract medium was obtained.

Further work indicated that (i) cystine and ergothionine could be eliminated from the medium; (ii) cystine could not be substituted for cysteine without delaying the growth of the organism; (iii) the sodium thioglycolate used by Gaspar was not essential in a blood-free medium; (iv) ferric compounds could not be substituted for the divalent form of iron without delaying growth; and (v) the slightly smaller size of colonies after 29 to 30 hr of incubation of the blood-free medium might be due to inadequate buffering.

The following studies were undertaken to define optimal concentrations of the remaining constituents, and, if possible, to develop a simple blood-free medium which would give growth of *P. tularensis* equal to or better than that observed on Gaspar medium.

MATERIALS AND METHODS

Basal medium. The concentrations (in g/100 ml) of Tryptose broth with thiamine (Difco) (2.6), glucose (1.0), and agar (1.0 to 1.5) originally used were found to be optimal. A concentration of 0.12 g/100 ml of cysteine-HCl was selected for inclusion in the basal medium after titration of this compound in the presence of varying concentrations of ferrous sulfate.

Ferrous sulfate ($FeSO_4 \cdot 7H_2O$). Concentrations of 1.0, 5.0, and 10 mg/100 ml of ferrous sulfate were tested in the above-described basal medium and in modifications of it

[1] Present address: Room 2D233, Pentagon, Washington, D.C.

containing 0.03, 0.06, 0.24, and 0.5 g/100 ml of cysteine-HCl.

Potassium chloride. KCl was tested in all media at concentrations of 0.5, 1.0, 2.0, 4.0, and 8.0 mg/100 ml.

Histidine and tris(hydroxymethyl)aminomethane (tris) buffer. In a medium consisting of basal medium plus 5 mg/100 ml of ferrous sulfate, histidine was tested in final concentrations of 100, 200, 300, 400, 500, and 1,000 mg/100 ml. Concentrations of 100, 200, 300, and 400 mg/100 ml of tris buffer were also tested in the medium alone, and in the presence of 100, 200, 300, and 400 mg/100 ml of histidine.

Inoculum and conditions of incubation. The stock culture of *P. tularensis* strain SCHU-S4 was maintained on glucose-cysteine-blood-agar (GCBA). Inocula were prepared by emulsifying 24- to 48-hr growth in Tryptose (Difco) saline (0.1 g/100 ml of Tryptose, 0.5 g/100 ml of NaCl, pH 7.2 to 7.4). Dilutions were made as described previously (Gaspar et al., 1961). Plates of media were inoculated with approximately 100 organisms contained in 0.2 ml of diluent.

For optimal early growth, an atmosphere of high relative humidity was essential. This was achieved by incubating inoculated plates at 37 ± 0.5 C in closed stainless-steel boxes [8 by 13 by 9 in. (20.3 by 23.0 by 22.8 cm)] containing a cellulose sponge [3 by 4 in. (7.62 by 10.2 cm)] saturated with water and placed in a 600-ml beaker containing 1 in. (2.54 cm) of residual water. This particular method of maintaining high relative humidity proved to be superior to that previously described, in that less variation in colony size was observed on single plates and on the same medium in replicate experiments.

RESULTS

Ferrous sulfate and cysteine-HCl. The optimal concentration of ferrous sulfate was directly dependent upon the concentration of cysteine-HCl. In the basal medium containing 0.12 g/100 ml of cysteine-HCl, a concentration of 0.005 g/100 ml of $FeSO_4 \cdot 7H_2O$ gave best growth of the organism. In cross titrations in which the concentrations of both cysteine-HCl and $FeSO_4 \cdot 7H_2O$ were varied, it was found that other combinations gave equally good results: 0.24 g/100 ml and 10 mg/100 ml or 0.06 g/100 ml and 1 mg/100 ml of cysteine and ferrous sulfate, respectively. At concentrations of cysteine less than 0.06 g/100 ml, growth of the organism was delayed; at levels greater than 0.24 g/100 ml, cystine precipitated in the medium. For convenience, a concentration of 0.12 g/100 ml of cysteine-HCl was chosen for inclusion in the basal medium, and 5.0 mg/100 ml of $FeSO_4 \cdot 7H_2O$ were included for titration of other components.

Potassium chloride. KCl at the concentration tested had no stimulatory effect on growth of the organism in freshly prepared medium; however, the addition of 2 mg/100 ml of this compound appeared to increase the stability of stored medium.

Histidine and tris buffer. The addition of 0.3 % histidine to the basal medium containing 5.0 mg/100 ml of $FeSO_4 \cdot 7H_2O$ provided for enhanced growth completely comparable to that on Gaspar medium. Higher concentrations were without further effect. It was determined that tris buffer could be substituted for part of the histidine. In the presence of 0.3 g/100 ml of tris, the concentration of histidine could be reduced to 0.1 g/100 ml. Subsequent work demonstrated that medium containing tris buffer was more stable on storage than that with histidine alone.

Preparation of the final medium. The medium finally selected (hereafter designated as T medium) was prepared by dissolving 26 g of Tryptose broth with thiamine (Difco), 10 g of glucose (Difco), 1.2 g of cysteine-HCl (Fisher, reagent grade), 0.05 g of ferrous sulfate ($FeSO_4 \cdot 7H_2O$, reagent grade), 1 g of L-histidine monohydrochloride (reagent grade), 3 g of tris, and 0.2 g of KCl (reagent grade) in 1,000 ml of distilled water. The pH was adjusted to 7.0 with concentrated HCl. For solid medium, 1.0 to 1.5 g/100 ml of agar (Difco) were added prior to autoclaving at 121 C for 20 min.

Several important points related to preparation and use of the medium should be noted. (i) It is essential to prepare the medium by weighing out all ingredients into a dry flask before addition of any water. Failure to do so may result in the formation of a precipitate (presumably oxidized cysteine) in the completed medium. (ii) To obtain the enhanced growth described here, plates of prepared medium must be incubated overnight at 37 C before use and, as with Gaspar medium, inoculated plates must be incubated in an atmosphere of high relative humidity.

The results of studies comparing the growth of *P. tularensis* strain SCHU-S4 on several blood-free media are summarized in Table 1. These data show that addition of 0.005 g/100 ml of $FeSO_4$ to the basal medium containing 0.12 g/100 ml of cysteine-HCl (no. 1) gives enhanced growth of the organism practically equal to that on Gaspar

TABLE 1. *Comparison of growth of Pasteurella tularensis strain SCHU-S4 on blood-free media based on colony size*

Medium	Compounds* added to basal medium†				Colony size (mm) after incubation for		
	$FeSO_4$	KCl	Histidine	Tris	29 hr	44 to 48 hr	72 hr
1	0.005				>0.5–1.0	2.0–2.5	3.0–4.0
2	0.005		0.3		1.0	2.0–3.0	3.0–4.0
3	0.005		0.1	0.3	1.0	2.5–3.0	4.0–5.0
T	0.005	0.02	0.1	0.3	1.0	2.5–3.0	4.0–5.0
Gaspar					1.0	2.0–3.0	3.0–4.0

* $FeSO_4$: ferrous sulfate ($FeSO_4 \cdot 7H_2O$) (Fisher, reagent grade); KCl: potassium chloride; histidine: L-histidine monohydrochloride; and tris: tris(hydroxymethyl)aminomethane (Fisher).

† Basal medium (in g/100 ml): Tryptose broth with thiamine (Difco), 2.6; glucose (Difco), 1.0; cysteine-HCl, 0.12; and agar (Difco), 1.0.

medium. Although the difference in colony size is small and at times impossible to measure accurately, a slight difference was consistently apparent when the experimental and control plates were compared side by side. The histidine medium (no. 2) provides the necessary additional stimulatory effect to overcome the slight difference between medium no. 1 and the control (Table 1). No difference in growth could be observed on media in which tris buffer was substituted for part of the histidine or to which KCl had been added (medium no. 3 and T medium). However, as pointed out above, the complete T medium was more stable on storage than were the other variations shown. Growth on plates of T medium after storage for 2 weeks at 4 C was equal to that obtained on freshly prepared plates, whereas considerable variation in colony size was observed on plates of media no. 1, 2, and 3 which had been stored for the same period. Colony counts are not included in Table 1 because no significant difference was observed on the five media used.

Growth of the organism in fluid T medium, without agar, was also investigated, with special attention being given to the ability to support good growth from small inocula. Duplicate flasks containing 50 ml of medium were inoculated with a suspension of cells adjusted to give a final concentration of approximately 1,000 cells per ml of culture. From these initial flasks, four serial tenfold dilutions were made, also in final volumes of 50 ml. One set of the original flasks and 10^{-1} through 10^{-4} dilutions were incubated statically and another on a shaker. Samples were withdrawn from each flask at various time intervals for plate counts. Results of a typical experiment selected from a number of replicates are given in Table 2. Growth as measured by viable counts indicated that this medium in liquid form was capable of supporting rapid growth of *P. tularensis* from an inoculum as small as one organism in 12.5 ml of medium.

DISCUSSION

Although blood or blood products are essential components of many media employed for the growth of *P. tularensis*, this work demonstrated that ferrous sulfate and histidine may be substituted for blood in an appropriate basal medium. Because growth of the organism on medium containing blood was only very slightly better than that in which iron alone was added to essentially the same base, it would appear that the major contribution of blood to the media used is iron. It seems reasonable to assume that the effect of histidine may be attributed largely to its buffering action.

TABLE 2. *Growth of Pasteurella tularensis strain SCHU-S4 from small inocula in liquid T medium**

Shake culture (hr)			Static culture (hr)		
0	20	40	0	20	40
820.0	4.6×10^8	1.0×10^{10}	820.0	6.9×10^7	6.1×10^8
82.0	3.0×10^7	7.3×10^9	82.0	3.8×10^6	5.2×10^8
8.2	3.0×10^6	5.7×10^9	8.2	2.9×10^5	3.9×10^8
0.82	1.8×10^5	4.9×10^9	0.82	1.8×10^4	3.2×10^8
0.08	3.1×10^4	4.5×10^7	0.08	2.7×10^3	2.5×10^8

* Results are expressed as viable cells per milliliter after incubation at 37 C of 50 ml of broth medium in 125-ml flasks.

No satisfactory explanation is apparent for the increased storage stability of the medium containing KCl and tris buffer.

The prime importance of the relative concentrations of certain constituents of media for the enhanced growth of *P. tularensis*, as pointed out by Gaspar et al. (1961), is again emphasized. Furthermore, it cannot be assumed that iron and histidine could be substituted for blood with similar results in another basal medium. The specific contributions of the ingredients of the basal medium to the rapid growth of *P. tularensis* have not been defined. Interesting questions have been raised by the consistent observation that, for optimal growth, it was necessary to incubate plates of T medium overnight prior to use, and to incubate inoculated plates in an atmosphere of high relative humidity. Biochemical studies designed to investigate some of these problems are in progress and will be reported elsewhere.

The medium described was used for the isolation of *P. tularensis* strain SCHU-S4 from simulated pharyngeal washes and in limited clinical studies. The addition of polymyxin B sulfate (300 units per ml), nystatin (100 units per ml), and penicillin G (100 units per ml) to T medium completely suppressed all organisms in the pharyngeal washes tested except *P. tularensis*. Total recovery of the organism was achieved in medium containing antibiotics; however, growth to comparable colony size was delayed for 3 to 6 hr.

T medium was used also for the propagation of a number of other bacteria, including *P. pestis*, *Bacillus anthracis*, *Brucella sp.*, *Salmonella sp.*, *Shigella sp.*, and *Diplococcus pneumoniae*. This medium supported the growth of all organisms tested as well as, or slightly better than, media usually recommended for their culture.

Recent results indicated that certain lots of Tryptose broth with thiamine are not as effective for use in this medium as those originally used. The addition of the recommended antibiotics, however, completely eliminates the lot differences observed. Investigations designed to explain this phenomenon are in progress.

LITERATURE CITED

DOWNS, C. M., L. L. CORIELL, S. S. CHAPMAN, AND A. KLAUBER. 1947. The cultivation of Bacterium tularense in embryonated eggs. J. Bacteriol. **53**:89–100.

GASPAR, A. J., H. B. TRESSELT, AND M. K. WARD. 1961. New solid medium for enhanced growth of *Pasteurella tularensis*. J. Bacteriol. **82**:564–569.

GIBBY, I. W., P. S. NICHOLES, J. T. TAMURA, AND L. FOSHAY.

1948. The effects of an extract of blood cells upon the cultivation of Bacterium tularense in liquid media. J. Bacteriol. **55**:855–863.

GIESKEN, R. F., M. L. GUSS, AND H. T. EIGELSBACH. 1957. A complete, dehydrated agar medium for the isolation and enumeration of Bacterium tularense. Bacteriol. Proc., p. 59.

HOOD, A. M. 1961. A growth medium without blood cells for *Pasteurella tularensis*. J. Gen. Microbiol. **26**:45–48.

NAGLE, S. C., JR., R. W. ANDERSON, AND N. D. GARY. 1960. Chemically defined medium for the growth of *Pasteurella tularensis*. J. Bacteriol. **79**:566–571.

SNYDER, T. L., R. A. PENFIELD, F. B. ENGLEY, JR., AND J. C. CREASY. 1946. Cultivation of *Bacterium tularensis* in peptone media. Proc. Soc. Exptl. Biol. Med. **63**:26–30.

WON, W. D. 1958. New medium for the cultivation of *Pasteurella tularensis*. J. Bacteriol. **75**:237–239.