CULTURE OF HUMAN GENITAL "T-STRAIN" PLEUROPNEUMONIA-LIKE ORGANISMS

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ABSTRACT

FORD, DENYS K. (University of British Columbia, Vancouver, Canada). Culture of human genital "T-strain" pleuropneumonia-like organisms. J. Bacteriol. **84**:1028–1034. 1962.—The conditions under which "T-strain" pleuropneumonia-like organisms, as described by Shepard, are best cultured were investigated. The organisms were found to grow on several types of nutrient agar and broth, of which PPLO medium supplemented with yeast extract and horse serum was the simplest. Subculture was possible through broth cultures, provided the broths were not incubated longer than 16 hr. The organisms on agar required either Fortner's anaerobic atmosphere or 10% CO_2, but broth cultures grew aerobically. "T-strains" grew over a pH range of 6.8 to 7.8, and a temperature range of 30 to 36 C. They were viable after storage for 16 days at 4 C and for 90 days at −20 C, and they resisted lyophilization. They were sensitive to 1.5 μg per ml of tetracycline and streptomycin, but were resistant to ampicillin and penicillin. Quantitative studies showed maximal concentration in broth of 10^6 to 10^7 organisms per ml, and logarithmic multiplication for the first 12 hr of broth culture, with a subsequent rapid decline in number. Colonial morphology was maintained after numerous subcultures.

Shepard (1956) first observed minute colonies of pleuropneumonia-like organisms (PPLO) in agar cultures of urethral scrapings from patients with nongonococcal urethritis. He considered that these "T-strain" PPLO were distinct from the previously recognized large-colony strains, and that "T-strains" were one cause of nongonococcal urethritis (Shepard, 1960). These findings have been confirmed (Ford, Rasmussen, and Minken, 1962), and the present paper describes recent attempts to define more clearly the microbiological properties of these human "T-strain" PPLO. The initial studies of Shepard showed that the organisms were difficult to grow on agar, producing only minute colonies (about 5 to 15 μ in diameter); moreover, they could not be grown in broth or be subcultured. The main objectives of the present investigation were, therefore, the development of methods of subculture and the improvement of the cultural conditions and media so that the organisms could be studied by standard microbiological techniques.

MATERIALS AND METHODS

The starting procedures of the study were those described by Shepard (1956), whose methods were followed closely. Modifications of media and cultural techniques were, therefore, compared with those of Shepard. The method of subculture was developed from fresh isolates on agar cultures of urethral exudate but, subsequently, four propagated "T-strains" originating from different patients were employed for the assessment of modifications to the media and cultural procedures. However, before a medium or procedure was considered an improvement over the Shepard technique, it had to grow "T-strains" from new cases of nongonococcal urethritis better than the preceding method. Except for a few experiments later in the study, the observations were qualitative, and growth was recorded as "improved," "equal," or "worse," according to a subjective evaluation of colony number, size, and staining. Such a subjective impression was necessary because no one parameter appeared adequate, and there was a degree of inverse relationship between colony size and number.

Klieneberger-Nobel's (1959) medium was prepared according to her methods, and she very kindly provided the staphylococcus strain used in her studies. The animal sera were obtained from the Colorado Serum Co., and the human ascitic fluid from patients with neoplastic ascites. The agar-block staining procedure of Dienes, as

detailed by Madoff (1960), was used routinely throughout the study for the observation of "T-strain" colonies, which require a minimum of 100 × magnification for their definition. Two large-colony PPLO strains, isolated in this laboratory from the human genital tract, were employed in the study for comparison with the "T-strains"; whereas "T-strain" agar cultures were incubated for 48 to 72 hr, incubation of large-colony PPLO was continued for 4 or 5 days.

RESULTS

Subculture and growth in broth. The usual procedure, of mycoplasmal subculture on agar by pushing an inverted agar block containing colonies over the surface of new agar, proved unsuccessful. Thus, subculture was first achieved by homogenizing the surface agar of cultures containing "T-strain" colonies on primary isolation from urethral scrapings, a few drops of Shepard broth being added to the homogenate before it was spread on the surface of new agar. It was then found that multiplication of the PPLO would occur in broth incubated for 16 hr, but such broth cultures would deteriorate if incubated longer than 16 hr; a 48-hr incubation usually resulted in loss of viability. Subculture at a 1:10 dilution was, therefore, routinely performed from 16-hr broth cultures, which always remained clear without any trace of opalescence. Because broth cultures would not remain viable when incubated more than 16 hr, the stock broth cultures were routinely stored in a refrigerator over weekends.

Atmospheric conditions of culture. The Shepard technique employs Fortner's anaerobic method of incubation. A comparison was therefore made among the following atmospheric conditions: Fortner's anaerobic method, aerobic incubation, and incubation in 10% CO_2, 5% CO_2, and in a candle-jar. Table 1 shows the comparison of incubations in both 10% CO_2 and atmospheric air with the Fortner technique. "T-strains" did not grow on agar in atmospheric air, but 10% CO_2 gave somewhat better growth than the Fortner atmosphere; moreover, after 10% CO_2 incubation, the colonies stained more uniformly with the Dienes procedure. Candle-jar and 5% CO_2 atmospheres were ineffective in growing the organisms on agar. In broth, the organisms propagated whether the broth was incubated in air or 10% CO_2; oxygenation of broth cultures did not enhance growth.

TABLE 1. *Growth of "T-strain" PPLO incubated at 37 C in 10% CO_2 and atmospheric air as compared with Fortner's method*

Atmospheric condition	Type of culture	No. of cultures	Comparison with Fortner's incubation		
			Improved	Equal	Worse
10% CO_2	Original	74	21	52	1
	Subculture	15	14	1	0
Aerobic	Original	16	0	0	16
	Subculture	12	0	0	12

TABLE 2. *Growth of "T-strain" PPLO in aerobic broth at different pH levels*

pH of broth	No. of subcultures	Comparison with pH 7.8		
		Improved	Equal	Worse
6.8	15	12	3	0
7.1	29	26	3	0
7.4	43	38	5	0
8.0	15	0	0	15

Media pH values. Shepard's agar medium has a pH of 7.8, and growth of "T-strains" on agar was compared at pH 7.0, 7.4, and 7.8, pH adjustments being made with 0.1% NaOH or 0.1% HCl. The cultures, incubated in 10% CO_2, showed no significant differences in growth and, therefore, the original pH of 7.8 was selected for continued usage. Broth cultures, however, propagated better at a lower pH, and, when the pH was determined by the incorporation of phenol red, the broths incubated in 10% CO_2 had a pH of about 7.0. Growth was compared in aerobic broths at pH values varying from 6.8 to 8.0, and optimal growth occurred between pH 7.1 and 7.4 (Table 2); whereas pH 6.8 was compatible with growth, pH 7.8 was unsatisfactory and pH 8.0 was lethal. The rather marked difference between the optimal pH of the broth and agar cultures was unexplained, though the atmosphere of 10% CO_2 in the agar cultures may have reduced the pH of the agar lower than pH 7.8 in the immediate vicinity of the cultures.

Temperatures of incubation and storage. In an attempt to retain viability in broth cultures incubated longer than 16 hr, the temperature of

TABLE 3. *Growth of "T-strain" PPLO after storage of broth cultures at different temperatures*

"T-strain" PPLO	Duration of storage in days												
	Room temp			Refrigerator (4 C)					Deep-freeze (−20 C)					
	1	3	6	1	3	6	9	16	1	3	6	9	16	90
145	+	−	−	+	+	+	+	+	+	+	+	+	+	+
149	+	+	−	+	+	+	+	+	+	+	+	+	+	+
151	+	+	−	+	+	+	+	+	+	+	+	+	+	+
199	+	+	−	+	+	+	+	+	+	+	+	+	+	+

TABLE 4. *Comparisons of yeast extract with Difco Supplement B, and 10% horse serum with 25% ascitic fluid in the supplementation of Shepard's medium*

Variation	Type of culture	No. of cultures	Comparison: (a) with Supplement B; (b) with ascitic fluid		
			Improved	Equal	Worse
(a) 10% Yeast extract	Original	8	6	2	0
	Subculture	49	27	22	0
(b) 10% Horse serum	Original	27	10	17	0
	Subculture	146	52	89	5

incubation was varied: 30, 33, and 36 C. The "T-strains" propagated at each temperature, and, at 30 C, the cultures would remain viable for 48 hr. However, the yield of organisms after 48 hr of incubation at 30 C was not greater than after 16 hr of incubation at 36 C and, therefore, the standard incubation temperature of 36 to 37 C was continued. At room temperature, broth cultures derived from 16 hr of incubation in 10% CO_2 would usually remain viable for 48 hr; at 4 C, for about 2 weeks; and at −20 C, for over 3 months (Table 3). The organisms were also found to be viable after lyophilization.

Nutritional requirements. The Shepard medium was varied in many ways in the hope of improving growth of "T-strains," but only two modifications seemed advantageous. A yeast extract was prepared by boiling 250 g of dried active yeast in 1,000 ml of water for 2 min. The boiled suspension was then centrifuged, and the supernatant was Seitz filtered, after which the extract was added as a 10% supplement to the medium instead of the Supplement B (Difco) employed by Shepard. This extract was somewhat superior to Supplement B in both the growth of primary cultures and subcultures (Table 4). Table 4 also indicates that 10% horse serum was a somewhat better nutritional supplement than 25% human ascitic fluid, 10 of 27 original cultures and 52 of 146 subcultures growing better on the horse serum agar than on the agar supplemented with ascitic fluid.

The supplements incorporated by Shepard were varied by removal from the medium and also by halving and doubling their concentrations, but no observable alteration of growth resulted from these changes (Table 5). In addition, supplementations employed by other workers in the mycoplasmal field were tested but, again, no significant advantage was obtained. The addition of constituents of tissue-culture medium provided no growth stimulus, nor did the replacement of horse serum by 10% human, swine, calf, or rabbit serum; 15% sucrose was incorporated into the medium according to Jonsson (1961) but this, too, was without good effect.

In an attempt to simplify the cultural procedures, Difco PPLO agar was tried as the basal medium, and was, in fact, found to be as effective as the basal medium of Shepard. The Difco PPLO agar was diluted with Difco PPLO broth to give a final agar concentration of 1% when supplemented (Table 6). A 1% Difco Serum Fraction was inadequate to support growth, and both 10% horse serum and 10% yeast extract were required for continued propagation of the organisms. With this supplement, growth of subcultures and primary cultures on Difco agar appeared to be equal to Shepard's medium; moreover, Difco PPLO broth with similar supplements also proved equally as effective as Shepard's broth.

The growth of "T-strains" was also studied on Klieneberger-Nobel medium, on which they grew equally to culture on Shepard or Difco agar. The characteristic morphology of the "T-strain" colonies was not significantly different on any of the three media, and always conformed to that described by Shepard and illustrated in Fig. 1. Moreover, the morphological differentiation of

TABLE 5. *Unsatisfactory modifications of "T-strain" media resulting in no effects on growth*

Nutritional alterations in Shepard's medium		No. of subcultures
Variation of supplements contained in Shepard's medium	Removal of starch	12
	Removal of DNA	16
	Removal, halving, and doubling of glucose content	16
	Removal, halving, and doubling of NaCl content	16
Variation in concentration of horse serum and yeast extract	25% Horse serum compared with 10%	28
	40% and 20% Yeast extract compared with 10%	16
Supplementation with other mammalian sera	10% Human	12
	10% Calf	12
	10% Swine	12
	10% Rabbit	12
Addition of established mycoplasmal supplements	Cholesterol at 0.01 and 0.1 mg/ml	12
	Liver extract at 0.1 and 1%	8
	Difco serum fraction at 1%	12
Addition of constituents of tissue culture media	10% Hanks' solution	12
	10% Medium NCTC 109	12
	10% Lactalbumin hydrolysate	12
	2 mM Glutamine	16
Modification from Jonsson	15% Sucrose	12

large- and T-colony PPLO was also retained on each of the three media. It was noted, however, that Klieneberger-Nobel medium was significantly better for the growth of large-colony human genital PPLO than the other two media.

Quantitative studies of "T-strain" growth in broth. When a fuller understanding of the basic conditions of "T-strain" culture had been obtained and when broth cultures were enabled to grow in a predictable manner, certain quantitative experiments were performed. Broth cultures were serially diluted, and 0.01 ml of drops were placed on agar to cover an area about 2 cm in diameter. These cultures were incubated for 2 days, after which the whole area of the drop was cut out, stained by the Dienes method, and studied under the microscope at 100 × magnification. The PPLO colonies arising from the 0.01 ml of drops were then counted in those dilutions that gave 200 to 5 colonies per drop. The counts from triplicate drops were averaged, and the number of colony-forming units in the original broth calculated from the dilution and the 0.01-ml size of the drop employed. To demonstrate the relationship between colony counts and the size of the inoculum, a series of twofold dilutions from 1:100 to 1:1,600 was prepared from a broth, and 0.01-ml drops were handled as described above. Figure 2 shows a straight-line relationship between the dilutions and the number of colonies obtained.

The above technique with serial tenfold dilutions demonstrated that the maximal concentration of "T-strain" organisms in a 16-hr broth varied around 10^6 to 10^7 particles per ml. This contrasted with counts of 10^8 to 10^9 colony-forming particles in 48-hr broth cultures of the two large-colony PPLO strains under study in the laboratory. A growth curve of "T-strain" PPLO was obtained by 2-hour samplings of an

TABLE 6. *Successful supplementation of Difco PPLO agar*

Media constituents	Presence of growth in three successive subcultures in the same medium		
	1st	2nd	3rd
PPLO agar	−	−	−
PPLO agar + 1% Serum Fraction	−	−	−
PPLO agar + 10% horse serum	+	−	−
PPLO agar + 10% horse serum + 10% yeast extract	+	+	+

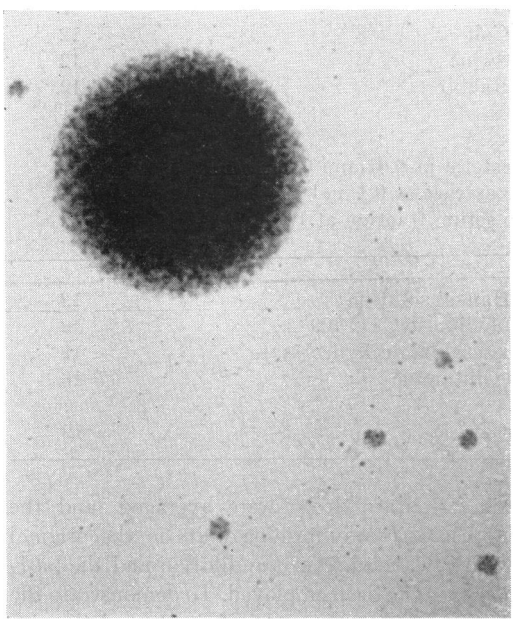

FIG. 1. *Large-colony and "T-strain" PPLO colonies on Klieneberger-Nobel medium, both derived from pure strains subcultured many times and intentionally mixed to obtain this preparation.*

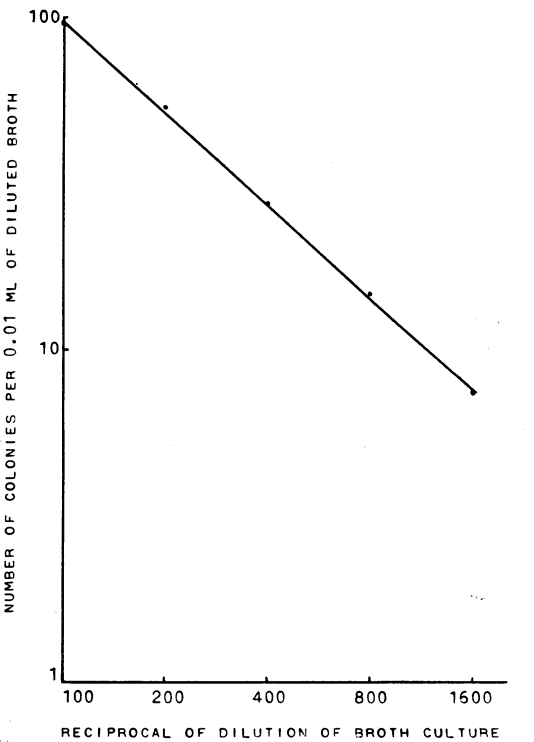

FIG. 2. *Relationship between the number of colony-forming units and the dilution of a "T-strain" broth culture.*

inoculated broth, and this showed (Fig. 3) that the organisms multiplied logarithmically for the first 12 hr; thereafter, growth ceased, and an actual decrease in numbers occurred between 12 and 21 hr. The result, therefore, fully confirmed the earlier qualitative experience with broth cultures, in which a broth culture incubated longer than 16 hr was unpredictable.

Sensitivity to antibiotics. The sensitivity of "T-strain" PPLO to antibiotics is similar to that of human large-colony PPLO of genital origin. Both are resistant to 1,000 units of penicillin per ml, and this antibiotic can, therefore, be employed to obtain pure cultures from contaminated clinical material. Table 7 shows the response of our four propagated "T-strains" to tetracycline, streptomycin, and ampicillin when these agents were incorporated into agar medium in concentrations varying from 100 to 0.18 μg per ml. The organisms were resistant to ampicillin but were sensitive to 1.5 μg per ml of both tetracycline and streptomycin.

DISCUSSION

The present studies allow some definition of the cultural conditions in which "T-strain" PPLO can be propagated with certainty. It is evident that several basic PPLO media will support growth, provided nutritional supplementation includes 25% ascitic fluid or 10% serum (either human or horse) and also a derivative of yeast. At present, it appears that Difco PPLO agar supplemented with 10% horse serum and 10% of a fresh-boiled yeast extract is quite

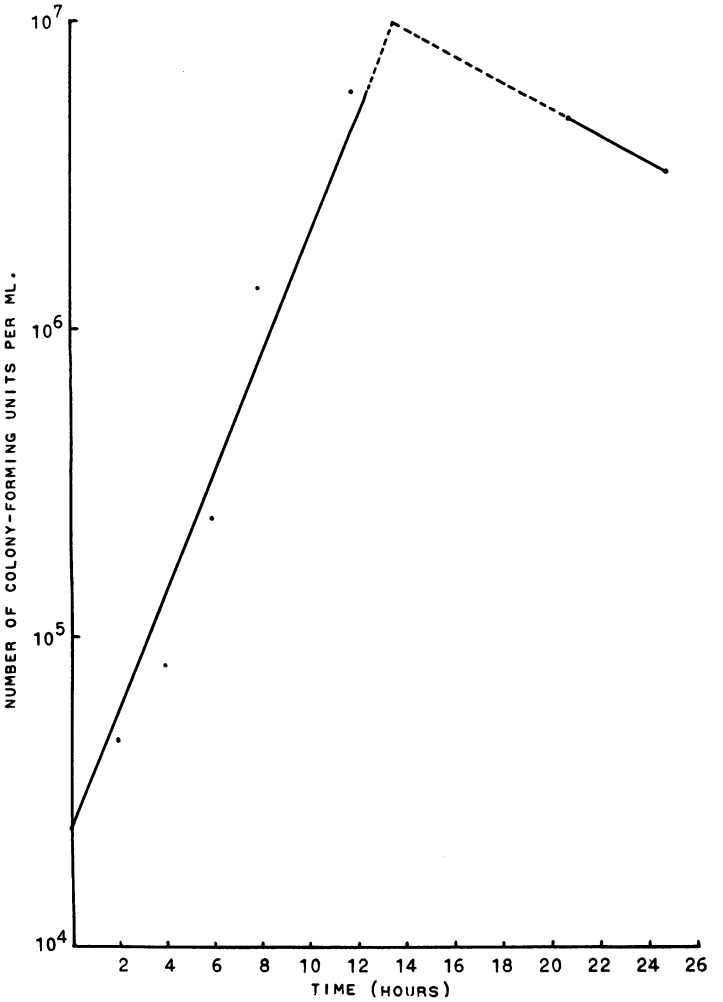

FIG. 3. *Growth-curve of "T-strain" PPLO in broth.*

adequate for "T-strain" growth, and probably the medium of choice due to its simplicity. Horse serum was considered to have two other advantages: a removal of human products from the medium would reduce the chance of incorporating antibody against "T-strains" into the medium; and the removal of human protein from the medium would permit more readily the application of the Coons indirect fluorescent technique, by use of convalescent serum, to the serological study of colonies from agar cultures and "T-strain" antigen from broth cultures. It is also clear that the atmospheric conditions of agar culture are important, with growth occurring only in 10% CO_2 (our preference) or the Fortner anaerobic atmosphere; aerobic conditions, 5% CO_2, and candle-jar atmospheres were inadequate. In contrast, pH and temperature control appear to be less exacting.

TABLE 7. *Growth of "T-strain" PPLO in the presence of tetracycline, streptomycin, and ampicillin*

Antibiotic	Concn of antibiotic in agar medium (μg/ml)									
	100	50	25	12.5	6.2	3.1	1.5	0.75	0.37	0.18
Tetracycline	−	−	−	−	−	−	−	+	+	+
Streptomycin	−	−	−	−	−	−	−	+	+	+
Ampicillin	+	+	+	+	+	+	+	+	+	+

The peculiar growth of "T-strains" in broth

is hard to understand, and the rapid deterioration of broth cultures after 16 hr of incubation is, as yet, both inexplicable and a handicap to the production of adequate antigen for serological investigations. There has been no evidence that a toxic substance is elaborated during multiplication, but, alternatively, a growth-factor deficiency would seem improbable because of the minute mass presented by the maximal concentration of organisms (1 to 10 million per ml). The problem is under continued study.

The maintenance of the typical colonial morphology on different agar and after serial propagation confirms the distinction between "T-strain" and large-colony human genital PPLO. The observation that large-colony PPLO would grow significantly better on Klieneberger-Nobel medium than on Shepard or Difco media was previously noted by Shepard (*personal communication*). This fact probably explains the low incidence of large-colony isolation (10 to 20%) from patients with nongonococcal urethritis who have been studied in Shepard's and this laboratory (Ford et al., 1962), as compared with Klieneberger-Nobel's (1959) laboratory where the incidence was found to be 48%. To obtain a true evaluation of the clinical significance of "T-strain" and large-colony PPLO in the causation of genitourinary disease, it will probably be necessary to study patients with varied genitourinary disorders, in addition to controls, with techniques that are optimal for the isolation of both types of organisms.

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