Factors affecting growth of *Legionella pneumophila* in liquid media

J. BARKER, I. D. FARRELL AND J. G. P. HUTCHISON

Regional Public Health Laboratory, East Birmingham Hospital, Birmingham B9 5ST

Summary. The growth in liquid media of *Legionella pneumophila* serogroups 1–6 was monitored turbidimetrically and factors affecting growth rate were studied. The presence of inhibitors, use of detoxifying agents and the method of broth preparation each had significant effects on cultivation. Cysteine was essential for growth; the optimal concentration was 100 µg/ml, but supplemental iron had no demonstrable effect.

Introduction

Many factors affect the growth of *Legionella* spp. *in vitro*. Legionellae are reported not to grow on 5% horse-blood agar unless it is enriched with ferric pyrophosphate and L-cysteine (Greaves, 1980). Edelstein (1981) found that a charcoal-yeast extract agar containing iron and cysteine with added α ketoglutarate supported the growth of most strains of *Legionella pneumophila*. Ristroph *et al.* (1980) showed the importance of charcoal in heat-sterilised medium—a heat-sterilised yeast extract broth (without added charcoal) did not support the growth of legionellae whereas the same medium, sterilised by membrane filtration, supported growth without charcoal treatment. Most broth media do not support growth of legionellae.

In this investigation, yeast-extract broths were prepared by various methods in attempts either to avoid the production of toxic components during preparation or to neutralise their effects. The growth of legionellae was monitored turbidimetrically, with estimations of the viable counts at appropriate times. The requirement for supplemental ferric pyrophosphate and L-cysteine was also studied.

Materials and methods

Bacterial strains

Twenty strains of *L. pneumophila* were used, representing serogroups 1–6, isolated from clinical and environmental samples. The strains were grown on Buffered Charcoal-Yeast Extract (BCYE) agar and were suspended in BCYE broth at a concentration of c. 10^{10} cfu/ml and stored at -70°C . When required, the stored strains

were re-cultured on BCYE agar and the inoculum prepared as described below.

Media

BCYE broth was prepared as described by Edelstein (1981). Yeast extract powder 10 g, activated charcoal (Norit A) 1.5 g, plus the Analar-grade chemicals, ACES buffer (Sigma) 10 g, α ketoglutarate monopotassium salt 1 g and potassium hydroxide 2.8 g, were made up in 1 L of distilled water. After sterilising at 121°C for 15 min, Analar-grade L-cysteine hydrochloride 0.4 g and ferric pyrophosphate 0.25 g were added and the pH of the medium was adjusted to 6.9 ± 0.5 at 40°C . The charcoal was sedimented by centrifugation and the supernate used as BCYE broth for the growth studies.

BCYE agar was made according to the above formulation but without removing the charcoal and with the addition of agar (Difco) 12 g/L.

A series of growth studies was performed with several yeast-extract broths (YEB) prepared with the same constituents as were incorporated in BCYE (above), except that charcoal was excluded and, in two of the modifications, the yeast extract component was pre-treated to remove potential growth inhibitors.

YEB 1 was prepared exactly as for BCYE broth (including sterilisation by autoclaving at 121°C for 15 min), except that charcoal was not added.

YEB 2 was made without heat-sterilization of ingredients and without added charcoal. The resulting broth was sterilised by filtration through a $0.45\ \mu\text{m}$ cellulose membrane filter.

YEB 3. The dry yeast-extract powder was shaken in a separating funnel with approximately 10 times its weight of ether; the washings were discarded and the powder dried of all solvent. It was then incorporated with the other BCYE ingredients, except charcoal, into a broth that was autoclaved at 121°C for 15 min.

YEB 4. The yeast extract (10 g) was made up with 100 ml of distilled water, transferred to a 'visking tube bag' and dialysed at 4°C against distilled water for 24 h. It was

then added to the other BCYE constituents, except charcoal, and the resulting broth heat sterilised as above.

YEB 5 was the clear pale straw-coloured fluid exuded from the gel phase of bottled BCYE agar slopes, after they had been stored at 4°C for 5 days. About 1 ml of the fluid ('syneresis fluid') was obtained from each 10 ml slope.

Inoculum

A suspension in distilled water, at *c.* 10^8 cfu/ml, was made from a 3-day *L. pneumophila* culture grown at 37°C on BCYE agar. A 20- μ l inoculum of the suspension was added to 1 ml of each test broth, giving an initial concentration in the broth of *c.* 10^6 cfu/ml.

Growth curves

These were determined turbidimetrically with the Abbott MS-2 Research System, which records changes in optical density (O.D.) in broth cultures every 5 min for up to 35 h and then computes the growth curves. The experiments were set up in cartridges consisting of 11 cuvettes, each holding 1 ml of broth; after inoculation they were incubated at 37°C in the incubator module of the equipment.

Results

Typical growth curves, obtained 30 h after inoculation of test broths (YEB 1–5) with a strain of *L. pneumophila* serogroup 1, are shown in fig 1. All the twenty *L. pneumophila* strains tested, representing serogroups 1–6, showed similar growth patterns. In both BCYE broth and YEB 5 ('syneresis fluid') there was an initial increase in optical density at 15 h and thereafter an exponential increase. After 30 h the viable count was 1.5×10^{10} cfu/ml. Examination of the broth culture at the end of the growth period with phase contrast microscopy showed rods 0.5–1 μ m in width and 2–*c.* 8 μ m in length. A few filamentous forms *c.* 30–40 μ m in length were seen in the 30-h culture, but not in the 15-h culture.

None of the strains of *Legionella* grew within the 30 h incubation period in the heat-sterilised broth without added charcoal (YEB 1). However, when charcoal was subsequently added to this broth, multiplication of legionellae took place after incubation for a further 15 h. Growth of legionellae was first detected turbidimetrically after incubation for 26 h in YEB 4 broth (heat-sterilised, but with

Fig. 1. Growth of *L. pneumophila*, serogroup 1, in six broths: ●—●, YEB 1 (autoclaved); ■—■, YEB 2 (filtered); ○—○, YEB 3 (ether treated); ▲—▲, YEB 4 (dialysed); □—□, YEB 5 (syneresis liquid); △—△, control (BCYE broth). The origin of each curve has been expanded up the ordinate limb of the graph for clarity.

dialysed yeast extract); the viable count after incubation for 30 h was 8.5×10^7 cfu/ml. Similar growth was obtained in YEB 2 broth (sterilised by membrane filtration) and in YEB 3 (heat-sterilised, but with ether-treated yeast extract). Strains grown in BCYE broth usually gave a detectable increase in optical density after an apparent lag phase of *c.* 15 h. At that stage the viable count was *c.* 2.5×10^7 cfu/ml, representing approximately a 10-fold increase over that of the initial inoculum, and a faint turbidity could be seen by visual inspection.

Growth promoters and inhibitors

To portions of YEB 1 (heat-sterilised broth) were added either H₂O-lysed horse red-blood cells (previously washed in saline) 2% v/v, or soluble starch 0.15% w/v. A further portion was shaken with activated charcoal 0.15% w/v and then centrifuged to give a clear broth. These test broths (1 ml) and an untreated control broth, were each inoculated with 10^6 cfu of a strain of *L. pneumophila* serogroup 1 and incubated at 37°C. The charcoal-treated and starch-treated broths produced typical growth curves (as for BCYE broth in fig. 1) with an exponential phase detected turbidmetrically at *c.* 16 h and 20 h, respectively. The lysed-blood broth culture gave no detectable opacity change until 25 h and the control gave none at all within 30 h. This effect was attributable to the horse red-cell lysate rather than any plasma or serum present, because the effect could not subsequently be duplicated with serum or plasma alone.

Inhibitory effect of sodium chloride

NaCl is known to inhibit *L. pneumophila* (Edelstein and Meyer 1984). We tested a series of BCYE broths containing increasing concentrations of NaCl and found that growth was inhibited at 0.03% w/v or greater. Broths prepared in repeatedly re-washed glassware sometimes became inhibitory, possibly due to traces of Na⁺ (or detergent residues) in the glass. Therefore plastic containers were used throughout the main experiments, with satisfactory results.

Effects on growth of iron and cysteine

Three series of doubling dilutions of L-cysteine hydrochloride from 100 to *c.* 0.3 µg/ml were prepared in BCYE broth (deficient in both cysteine and iron). Ferric pyrophosphate was added to give a final concentration in one series of 250 µg/ml, and in

another of 50 µg/ml; the third series contained no added iron. Controls were set up as follows: (i) modified BCYE broth without added iron or cysteine; (ii) standard BCYE broth with ferric pyrophosphate 250 µg/ml; and (iii) BCYE broth without cysteine but containing ferric pyrophosphate 250 µg/ml. The broths (1 ml), inoculated with 10^6 cfu of an exponential-phase broth culture, were shaken in the MS-2 incubator at 37° for 30 h.

Growth curves of an isolate of *L. pneumophila* serogroup 1 are shown in fig. 2. The broths containing iron 250 µg/ml in the presence of cysteine 100 µg/ml produced a detectable change in optical density at 9 h, with viable counts at the end of the experiment at 15 h around 8×10^9 cfu/ml. These were very similar to the counts obtained with the control broth containing iron 250 µg/ml and cysteine 400 µg/ml. There was a marked reduction

Fig. 2. Effect of iron and cysteine concentration on the growth of *L. pneumophila* serogroup 1 in BCYE broth inoculated with an exponential-phase culture containing 10^6 cfu: $\blacktriangle$ — $\blacktriangle$, cysteine 100 µg/ml, iron 250 µg/ml; $\bullet$ — $\bullet$, cysteine 100 µg/ml, no iron, $\triangle$ — $\triangle$, cysteine 25 µg/ml, iron 250 µg/ml; $\circ$ — $\circ$, control (cysteine 400 µg/ml, iron 250 µg/ml). The origin of each curve has been expanded up the ordinate limb of the graph for clarity.

in growth when the cysteine concentration was reduced to 25 µg/ml, as shown in fig. 2 (lower curve). *L. pneumophila* did not grow in the control broths without added cysteine and iron, nor in those without cysteine but with iron 250 µg/ml.

Discussion

L. pneumophila is usually difficult to grow in liquid media, especially those heated during preparation (Ristroph *et al.*, 1980) and the latter report recommended filtration in preference to heat-sterilisation. In the present study, legionellae grew best in BCYE broth that had been heat-sterilised but treated with charcoal to neutralise inhibitory substances produced during preparation. Starch and, to a lesser extent, lysed red-blood cells proved effective alternatives to charcoal.

Although inhibitory factors in broth were less after filter-sterilisation than after autoclaving, they were not completely eliminated, because both bacterial counts and opacity readings were considerably lower than those obtained with the charcoal-treated (BCYE) broth, at all points during the incubation period of the tests.

The disadvantage of using charcoal to neutralise inhibitors in broth was the need to remove it to obtain a clear medium for turbidimetric studies. Although difficult to produce, syneresis fluid (YEB 5) is a clear medium which supports a good growth of *L. pneumophila*, probably because it contains adequate nutrients leached from the gel phase.

Oleic acid and other fatty acids suppress the

growth of legionellae but the effect may be neutralised by the addition of starch to the medium (Pine *et al.*, 1979). However, ether extraction of the yeast extract powder only partially reduced the inhibition, suggesting that not all the inhibitors are ether-soluble or that other substances, e.g., superoxides (Hoffman and Pine, 1983), may be involved. Dialysis treatment of yeast extract also had a limited effect in reducing the inhibitory property of the fluid medium, which suggests that substances of different molecular size and diffusibility may be involved.

The omission of iron supplements from BCYE had no effect on the ability of the broth to support the growth of *L. pneumophila*, suggesting that there is sufficient iron in the unsupplemented medium, although we have found that the concentration is < 1 µg/ml. However, BCYE broth without cysteine did not support growth of legionellae; although minimal growth was observed with a concentration of 25 µg/ml, at least 100 µg/ml appears to be needed for optimal growth.

The reasons for the initial slow bacterial multiplication in these experiments is not clear, but a longer period of adaptation to its environment than is usual amongst the Eubacteriales appears to be necessary before step-up to rapid growth of *L. pneumophila* begins. A means of shortening this long lag phase would greatly facilitate the isolation and study of *L. pneumophila*, but this may be part of the innate biological nature of the organism and not amenable to change.

REFERENCES

- Edelstein P H 1981 Improved semi-selective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. *Journal of Clinical Microbiology* **14**:298-303.
- Edelstein P H, Meyer R D 1984 Legionnaires' Disease. A Review. *Chest* **85**:114-120.
- Greaves P W 1980 New methods for the isolation of *Legionella pneumophila*. *Journal of Clinical Pathology* **33**:581-584.
- Hoffman P S, Pine L 1983 Mechanism of action of charcoal in medium used for culture of *Legionella pneumophila*. *Abstracts of the Annual Meeting of the American Society for Microbiology* p 329.
- Pine L, George J R, Reeves M W, Harrell W K 1979 Development of a chemically defined liquid medium for growth of *Legionella pneumophila*. *Journal of Clinical Microbiology* **9**:615-626.
- Ristroph J D, Hedlund K W, Allen R G 1980 Liquid medium for growth of *Legionella pneumophila*. *Journal of Clinical Microbiology* **11**:19-21.