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Influence of Blue-Green Algae (Cyanobacteria) on Survival of *Legionella pneumophila* in Aerosols

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The fluid in which blue-green algae (cyanobacteria) (*Fischerella* sp.) had been grown (algal extract) was investigated for its effect on aerosols of *Legionella pneumophila*. The bacteria were significantly more stable when suspended in algal extract than in the tryptose-saline solution employed in previously reported experiments. The stabilizing property of the extract disappeared after dialysis, suggesting that a relatively small molecule was involved. The relationship of this observation to the epidemiology of Legionnaires disease is discussed.

Recently, I reported that *Legionella pneumophila* suspended in tryptose-saline solution was unstable in aerosols at 30% relative humidity, but survived for relatively long periods of time at 50 and 80% relative humidity (1). In the same report I also suggested that the influence of natural suspending fluids other than tryptose-saline solution must be investigated.

In several publications, investigators at the Centers for Disease Control and the Savannah River Laboratories of E. I. du Pont de Nemours & Co. have reported isolation of *L. pneumophila* from aquatic habitats (4, 7), and suggested that the algae present in the waters might enhance the growth of *Legionella* (7). Therefore, I initiated an investigation of the effect of algae upon the survival of aerosolized *L. pneumophila* and report herein on the stabilizing effect of *Fischerella* sp.

MATERIALS AND METHODS

***Legionella* suspensions and assay.** The Philadelphia-1 strain of *L. pneumophila* was grown in the yolk sacs of embryonated eggs and then subcultured on charcoal-yeast extract agar as previously described (2). Surface growth was harvested from charcoal-yeast extract plates and diluted in tryptose-saline solution (pH 7.0) to give a final concentration of approximately 10^{10} viable organisms per ml. A 1-ml portion of this suspension was then diluted in 9.0 ml of the suspending medium to be tested. Viable cells were assayed by spreading 0.1 ml of selected dilutions of the organism suspensions on the surface of charcoal-yeast extract plates. After 72 to 96 h of incubation at 37°C, colonies were counted and concentrations were calculated.

Algal extract preparations. A liquid suspension of *Fischerella* sp. was obtained from Carl Fliermans (Savannah River Laboratory, Aiken, S.C.). Castenholz mineral medium (200 ml) (3) was inoculated with 20 ml of *Fischerella* suspension and incubated under fluorescent light on a reciprocal shaker at 45°C for 7 days. A 20-ml portion of the resulting algal suspension was inoculated into 200 ml of fresh medium and incubated for an additional 7 days. The algal suspension was centrifuged and passed through a 0.45-μm filter membrane to remove viable algae, bacteria, and spores. The filtrate was then stored at 4°C until needed. Throughout this report, this filtrate will be referred to as algal extract.

Aerosol dissemination and sampling. The various test suspensions of *L. pneumophila* were disseminated with an FK-8 gun (5) into a 6,200-liter static aerosol chamber that had previously been adjusted to the desired temperature and relative humidity conditions. The chamber employed was similar in configuration to that described by Jemski and Phillips (5). At selected times, the aerosol was sampled with all-glass impingers (8) at 12.5 liters/min. Two samplers, each containing 20 ml of tryptose-saline solution, were operated simultaneously for 1 min, and their contents were pooled and assayed as described previously (5). The results of prior experimentation showed that the median diameter of the aerosol particles in the chamber was 3.8 μm 4 min after dissemination (geometric standard deviation, 2.02).

Effect of algal extract. The survival of *L. pneumophila* suspended in algal extract was compared with that of the organism suspended in tryptose-saline solution or in a combination of 6% raffinose and 0.1% 2,2'-dipyridyl. The latter combination has previously been shown to be an effective stabilizer of several gram-negative bacteria, notably, *Francisella tularensis* (6), and preliminary experiments showed that aerosols of *Legionella* were also stabilized by it. Therefore, I employed tryptose-saline (worst case) and raffinose-dipyridyl (best case) as standards of comparison for the effect of algal extract. Four replicate experiments were carried out at 30% relative humidity only. Samples of each aerosol were obtained at midpoints of 4, 18, and 32 min after dissemination. Data were analyzed by analysis of variance.

As part of this experiment, the respiratory virulence of *L. pneumophila* suspended in algal extract was compared to that of the organism suspended in tryptose-saline. Groups of six guinea pigs each were exposed to graded aerosol doses of the organism suspended in the two fluids by methods that have been

previously described (4). Doses ranged from 9×10^3 to 10^6. Responses were evaluated on the basis of mortality, or, in the event that death did not occur, upon change in weight and febrile response.

Effect of dialysis and mineral broth. A sample of algal extract was dialyzed against frequent changes of distilled water at 4°C for 18 h (seamless dialyzing tubing with molecular weight cutoff of 12,000 k; Fisher Scientific Co.). The dialyzed material, the algal extract from which it was prepared, and a fresh sample of Casteholz mineral broth were then employed as the suspending fluids in aerosol stability tests. This experiment was carried out at 30% relative humidity only. To reduce the number of manipulations required, samples were obtained from the aerosol at 4 and 32 min only. Three replicate experiments were performed and analyzed by analysis of variance.

Effect of water. Since all suspending media were aqueous solutions, the effect of water was compared with that of tryptose-saline. Three replicate experiments were conducted in which samples were obtained at 4, 18, and 32 min at 30% relative humidity only.

Survival in nonaerosolized spray fluids. Determination of the ability of *L. pneumophila* to withstand possible adverse effects, of the various fluids in which it had been suspended during these tests was performed by adding organisms to the fluids to a final concentration of 10^9 organisms per ml. A sample for viable count determination was removed immediately and after 4 h of incubation at 37°C. Organism concentration was determined by conventional diluting and plating procedures.

Calculations. Percent recovery, decay rate, and half-life calculations were performed as follows.

$$\text{No. of organisms/liter disseminated} = \frac{\text{No. of organisms/ml in spray suspension}}{6{,}200} \quad (1)$$

$$\%\text{ of recovery} = \frac{\text{No. of organisms/liter at any time } (t_1)}{\text{No. of organisms/liter at the time of dissemination}} \times 100 \quad (2)$$

$$\text{Decay rate} = 100\,k\ (\%/\text{min}) \quad (3)$$

determined from the simple exponential equation $\%R_{t_i} = \%R_{t_o}e^{-kt}$, in which R_{t_i} and R_{t_o} represent percent of recoveries at times t_i and t_o, respectively, and t is total elapsed time.

$$\text{Half-life } (t_{1/2}) = \frac{0.69}{k} \quad (4)$$

RESULTS

Effect of algal extract. The effect of algal extract is shown in Fig. 1. For comparative purposes, the effects of added raffinose-dipyridyl and tryptose-saline solutions were also determined. Most important here was the observation that the algal extract was as effective as raffinose-dipyridyl solution in stabilizing aerosols of *L. pneumophila*. Neither the 4-min recoveries nor the half-lives of the two solutions were significantly different. In contrast, the recoveries

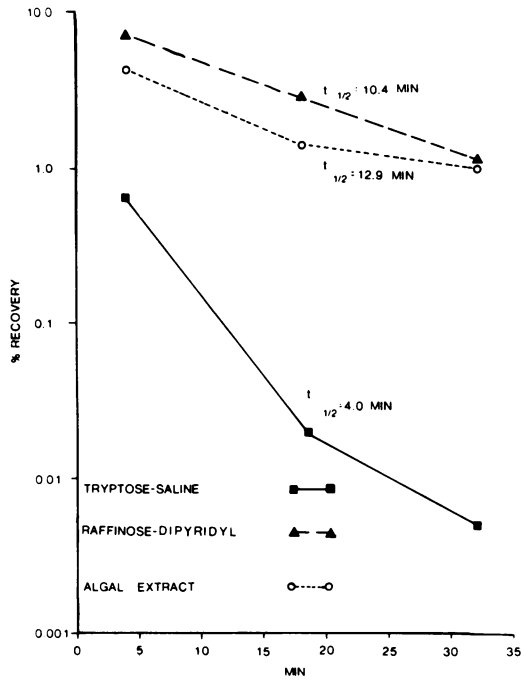

FIG. 1. *Comparison of the stabilizing effect of algal extract with that of tryptose-saline and raffinose-dipyridyl solutions.*

and the half-lives of these two suspensions were significantly greater than those of the tryptose-saline suspensions ($P < 0.001$ for both cases).

Although algal extract provided significant protection for aerosols of *L. pneumophila*, it apparently had no effect upon the respiratory virulence of the organism. Guinea pigs exposed to graded doses of organisms suspended in either tryptose-saline or algal extract showed the same responses in terms of mortality, fever, and weight loss. The calculated 50% lethal dose for both routes was 3×10^5 organisms (95% confidence limits, 1.1×10^5 to 8.5×10^5) per ml.

Effect of dialysis and mineral broth. Preliminary investigation of the nature of the stabilizing substance(s) involved comparison of the effects of dialyzed and nondialyzed algal extracts, as well as the effect of the mineral broth that was routinely used for the culturing of algae. Results are shown in Fig. 2. The stabilizing effect of algal extract was lost by dialysis. The aerosol stability of *Legionella* suspended in the dialyzed product was about the same as previously shown for the tryptose-saline solution. The effect of mineral broth was noteworthy; a highly significant loss of viability ($P < 0.001$ versus algal extract) occurred during the first 4 min, but the organisms that survived the initial stress of aerosolization seemed to be resistant to further decay.

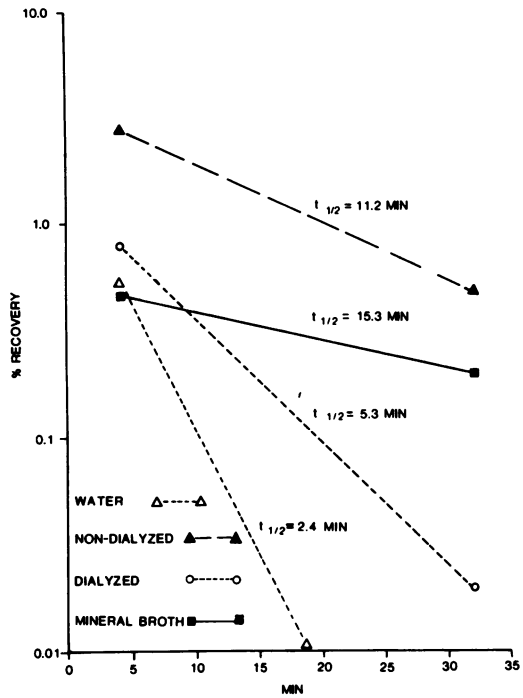

FIG. 2. *Survival of aerosolized L. pneumophila suspended in dialyzed algal extract or in mineral broth.*

Effect of water. The survival of *L. pneumophila* disseminated from water was compared with that of *L. pneumophila* disseminated from tryptose-saline. The organisms were very unstable in water; none were recovered after 18 min. Results with tryptose-saline did not differ significantly from those in the preceding experiment. The water data, therefore, are also presented in Fig. 2.

Survival in nonaerosolized spray fluid. *L. pneumophila* was suspended in tryptose-saline solution, mineral broth, algal extract, and raffinose-dipyridyl and sampled immediately and again after 4 h of incubation at 37°C. Viable concentration of organisms remained unchanged in all fluids over the 4-h span.

DISCUSSION

These data indicate conclusively that *L. pneumophila* may survive much longer in aerosols than was indicated by the results of my previously published experiments (1). Since algae are ubiquitous, it is entirely possible that they play an important role in the transmission of Legionnaires disease. In view of published reports that the growth of *L. pneumophila* under adverse conditions is greatly facilitated when *Fischerella* sp. are present (7), it is likely that the presence of algae in lakes, streams or air-conditioning systems both enhances the growth of and protects *L. pneumophila* when it is aerosolized under natural conditions. Thus, the observations reported here may provide a partial answer to questions concerning the natural transmission of Legionnaires disease.

The nature of the compound(s) that stabilizes *L. pneumophila* is presently unknown. Loss of stability after dialysis suggests that a compound of relatively low molecular weight is involved. The stabilizing effect of mineral media after a large initial loss (at 4 min) suggests that inorganic ions may play a stabilizing role. On the other hand, the stabilizing effect of 2,2′-dipyridyl suggests that the organism may be protected if certain cations are sequestered. All of these factors may interact in the natural condition, but it seems important to control algal growth to prevent dissemination of *Legionella*.

At the present time, I have investigated only *Fischerella* sp. for stabilizing properties. Obviously, other algae should be investigated, as should water obtained from natural sources in which algae are found.

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