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Survival of virulent *Legionella pneumophila* in aerosols

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SUMMARY

Aqueous suspensions of virulent *Legionella pneumophila* grown on solid medium retained virulence and aerosol survival characteristics for several months. Significant numbers of viable organisms were recovered from aerosols held at various relative humidities (r.h.) for up to 2 h. The organisms survived best at 65% r.h. and were least stable at 55% r.h.

Exponential phase broth-grown organisms survived poorly in aerosols in comparison with stationary phase broth cultures or organisms grown on solid medium, suggesting that the metabolic status of *Legionella pneumophila* organisms may be an important factor affecting their ability to survive in aerosols and cause respiratory disease.

INTRODUCTION

Legionella pneumophila is the aetiological agent for the acute human disease legionellois, which appears to have two clinical forms; a pneumonic form which may be fatal, Legionnaires' disease (LD), and a form which appears to be non-fatal, Pontiac fever (Cordes & Fraser, 1980). Since the earliest recognized outbreaks, epidemiological evidence has strongly associated the spread of both forms of the disease with certain types of domestic and evaporative water systems. Thus, many of the outbreaks of LD may have resulted from exposure to droplet clouds generated from air-conditioning cooling towers, evaporative condensers and domestic plumbing systems containing water contaminated with *L. pneumophila* organisms (Cordes & Fraser, 1980; Fraser, 1980; Band, La Venture & Davis, 1981; Tobin, Swann & Bartlett, 1981; Arnow *et al.* 1982), or possibly contaminated dust (Mallison, 1980). There appear to be no reports of spread of legionellosis other than by the airborne route from man-made water installations.

Airborne spread of Pontiac fever was demonstrated experimentally by exposing guinea pigs to air at the site of the first outbreak (Glick *et al.* 1978). Although forms of legionellosis have been experimentally induced in guinea pigs by intraperitoneal injection (McDade *et al.* 1977) or by exposure to aerosols (Berendt *et al.* 1980) they were of little relevance to the human pneumonic disease. Strong supportive evidence for human LD being spread by the airborne route was obtained in 1981 by Baskerville *et al.* who were successful in inducing experimental respiratory

infection similar to human LD in guinea pigs and rhesus monkeys. For their study, animals were infected with aerosols of a strain of *L. pneumophila* which, though isolated from a contaminated domestic water supply, was found to be virulent for guinea pigs.

The first laboratory studies on the survival of airborne legionella were described by Berendt (1980) who examined the recovery of viable organisms from clouds held at different relative humidities; bacteria sprayed from suspensions containing algal extracts generally survived better than those sprayed from tryptose-saline (Berendt, 1981).

In the present work, the survival of virulent *L. pneumophila* strain 74/81 organisms aerosolized from water and held at different r.h. values was investigated. The relevance of the findings for experimental respiratory infection of animals and to the epidemiology of naturally acquired infection is discussed.

MATERIALS AND METHODS

Organisms

The strain of *L. pneumophila* used was the serogroup 1 74/81 strain isolated from a naturally contaminated water supply (Baskerville *et al.* 1981). The organisms were grown on Edelstein's (1981) modification of charcoal-yeast-extract (CYE) agar (Feeley *et al.* 1979) for 4 days at 37 °C in air or, for some experiments, in a liquid medium similar to that described by Ristroph, Hedlund & Allen (1980). The liquid medium, final pH 6·9, contained yeast extract (Oxoid; 10 g/l), ACES (N; 2-acetamido-2-amino ethane-sulphonic acid; 10 g/l), ferric pyrophosphate (2·8 g/l), cysteine hydrochloride (0·4 g/l), α-ketoglutarate (1·0 g/l) and sodium hydroxide (2·8 g/l) and was sterilized by filtration through 0·45 μm pore size membrane filters (Millipore (UK) Ltd, Harrow, Middlesex, HA1 2BR).

Starter liquid cultures were prepared by inoculating 200 ml of medium in a 1l flask with organisms grown on CYE-agar for 3 days; the flask was incubated, with shaking, for 48 h at 37 °C and the purity of the culture checked by Gram's stain, fluorescent antibody stain, ability to grow on CYE-agar and inability to grow on blood agar. For experimental cultures, 200 ml portions of medium were inoculated with 5 ml of a starter culture to give an optical density of about 0·1, then shaken at 37 °C.

A washed suspension of *Bacillus subtilis* var. niger spores was prepared as described previously by Evans & Harris-Smith (1971).

Preparation of spray suspensions and generation of aerosols

Suspensions of *L. pneumophila* organisms grown in liquid medium, or washed from CYE agar with distilled water, were centrifuged (10000 ***g***, 15 min), washed twice in and resuspended in distilled water (about 10^{10} total organisms/ml); washed *B. subtilis* spores were added to such suspensions at about the same concentration. Aerosols were generated with a 3-jet Collison nebulizer into a Henderson apparatus, modified to operate over a range of relative humidity values (Druett, 1969), and stored in a 55 l rotating drum (Goldberg *et al.* 1958). Samples were recovered from aerosols for 1 min, using raised impingers with a flow rate of 10·5 l/min (May & Harper, 1957). Impingers contained 10 ml of one of the

following sterile fluids; distilled water, Page's saline (Page, 1967), PBMA (Cox, 1966) or GPMA (gelatine, 0·2 % w/v in PBMA).

Estimation of the viability of aerosolized bacteria

The viability of aerosolized *L. pneumophila* was determined by the spore tracer technique (Harper, Hood & Morton, 1958). For this, suspensions and impinger samples containing legionella and bacillus organisms were diluted serially in sterile distilled water and quadruplicate portions (0·25 cm^3) inoculated onto tryptose agar, which does not support the growth of *L. pneumophila*, and CYE-agar, containing cefamandole lithium (Eli, Lilly & Co. Ltd; 2 mg/l) and incubated at 37 °C in air. The minimum inhibitory concentrations of cefamandole lithium for *B. subtilis* var. niger. and *L. pneumophila* are 2 mg/ml and 16 mg/l respectively; at 2 mg/l, cefamandole lithium does not affect the growth of *L. pneumophila* 74/81 (P. J. Dennis, unpublished). Colonies of *B. subtilis* on tryptone agar were counted after incubation for 18–24 h and of *L. pneumophila* on CYE-agar after 4 days.

RESULTS

Bacterial suspensions

Aerosols of *L. pneumophila* strain 74/81 grown on a solid medium and suspended in water are infectious for laboratory animals (Baskerville *et al.* 1981) and a similar suspension of this strain, stored at 4 °C, was used for much of the present study. The viability of the suspension was initially about 30 %; no significant changes in the viability or decay rate of aerosols generated from this suspension were detected over a period of several months.

For some experiments, exponential or stationary phase organisms grown in liquid medium were used. A typical growth curve for *L. pneumophila* strain 74/81 is shown in Fig. 1. The stationary phase of such cultures usually lasted for only a few hours, thereafter total and viable numbers decreased fairly rapidly.

The viability of all aqueous bacterial suspensions was unaffected by agitation for up to 10 min in the Collison spray.

Collecting fluid

To evaluate the suitability of various collecting fluids, aerosols of *L. pneumophila* held at 65 % relative humidity (r.h.) for 15 min were sampled into impingers containing either distilled water, Page's saline, PBMA or GPMA. The efficiencies of distilled water, Page's saline and GPMA in recovering viable airborne *L. pneumophila* were similar (Table 1). The viability of bacteria recovered into PBMA were, however, consistently low and when incubated on CYE agar medium these organisms gave rise to a wide range of colony sizes. Consequently, for all other work samples were collected into distilled water.

Survival of airborne L. pneumophila 74/81

The stabilities of CYE-agar-grown *L. pneumophila* strain 74/81 held for up to 2 h at three different r.h. values are shown in Fig. 2. The bacteria were most stable at the intermediate r.h., 65 %, were appreciably less stable at the highest humidity tested (90 % r.h.) and showed least stability in dry air (30 % r.h.). Differences in the stability of aerosols held at 30, 65 and 90 % r.h. were apparent after only 15 min

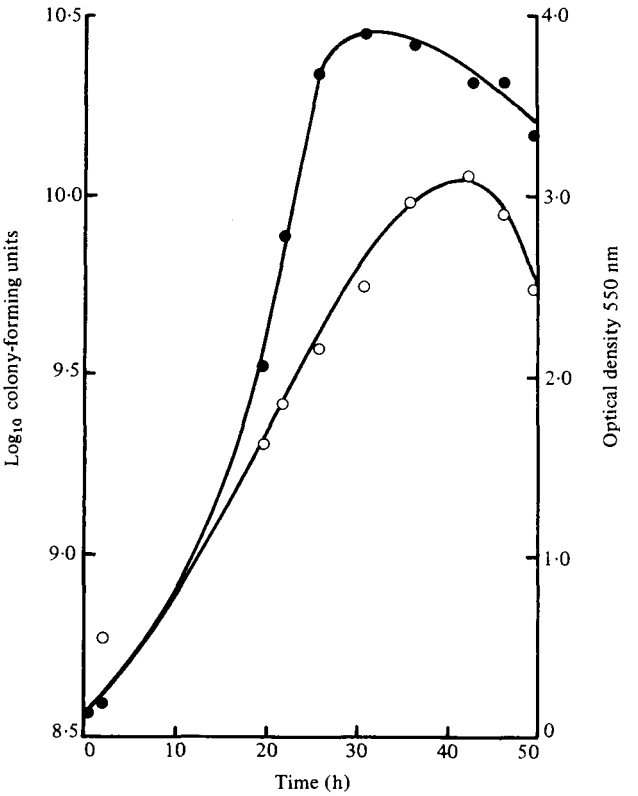

Fig. 1. Growth of *L. pneumophila* in liquid medium. Portions of medium, 200 ml, in 1 l flasks were inoculated and incubated as described in 'Materials and Methods'. ○, Colony-forming units/ml; ●, optical density 550 nm.

Table 1. *Viable numbers and viability of aerosolized* L. pneumophila 74/81 *sprayed from water and recovered after* 1 *s or* 1 *h into various collecting fluids*

	Aerosol age		
Collecting fluid	1 s Viable *L. pneumophila* recovered/ml fluid	1 h Viable *L. pneumophila* recovered/ml fluid	1 h Viability (%)
Distilled water	$1{\cdot}51 \times 10^6$	$9{\cdot}4 \times 10^4$	21
Page's saline	$1{\cdot}2 \times 10^6$	$1{\cdot}1 \times 10^5$	24
PBMA	$4{\cdot}1 \times 10^5$*	$5{\cdot}7 \times 10^4$	11
GPMA	$2{\cdot}1 \times 10^6$	$7{\cdot}7 \times 10^4$	20

* Variable colony size.

(Fig. 2) and in other experiments the effect of storage at a wider range of r.h. values was examined with aerosols aged for 15 min. The results obtained show that after storage for 15 min, airborne *L. pneumophila* is markedly less stable at 55% r.h. than in either drier or more humid atmospheres (Fig. 3).

Aerosols generated from suspensions of broth grown stationary phase cells survived less well at 65% r.h. than those from cultures washed from solid medium (Fig. 4), the rate of loss of viability being similar to that of aerosols of solid medium

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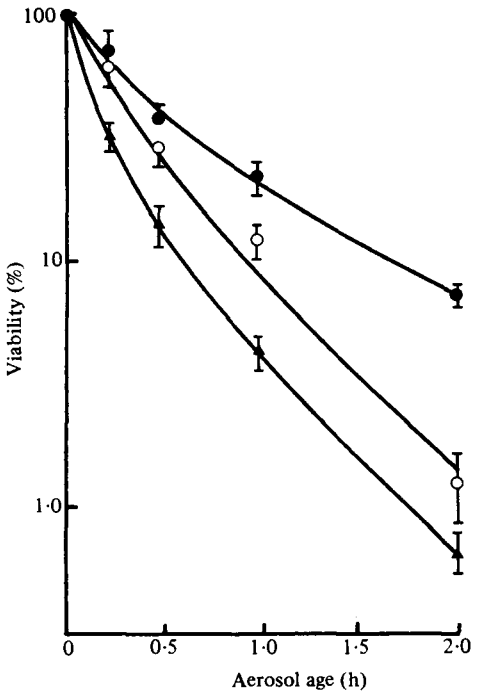

Fig. 2.

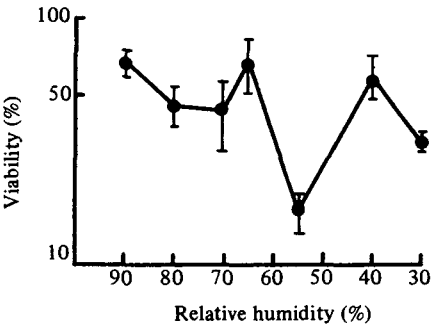

Fig. 3.

Fig. 2. Survival of aerosolized *L. pneumophila* 74/81 grown on solid CYE medium, sprayed from water and held at different relative humidities. ▲, 30% r.h.; ●, 65% r.h.; ○, 90% r.h. Bars indicate the values of the standard error, $n > 6$.

Fig. 3. Survival of aerosolized *L. pneumophila* 74/81 grown on solid CYE medium, sprayed from water and held at different relative humidities for 15 min. Bars indicate the values of the standard error, $n > 4$.

grown bacteria held at 30% r.h. (Fig. 2). The rate of decay of aerosols of exponential phase broth grown organisms was extremely high; the viability fell to 1–2% within 1–2 s and about 0·1% within 5 min; thereafter viable organisms were not recovered.

DISCUSSION

Since the identification of *L. pneumophila* as a causative organism of human respiratory illness (McDade *et al.* 1977) numerous epidemics and sporadic outbreaks of legionellosis have been recognized (Thacker *et al.* 1978; Broome *et al.* 1979; Politi *et al.* 1979; Cordes *et al.* 1980; Dondero *et al.* 1980; Tobin *et al.* 1980; Fisher-Hoch *et al.* 1981). Although *L. pneumophila* has been isolated from many natural water sources (Morris *et al.* 1979; Fliermans *et al.* 1981) it is the occurrence of the organism in domestic and recirculating cooling water systems that seems to be of unique importance in the epidemiology of the human disease. In most of the recognized outbreaks of legionellosis there is strong epidemiological evidence that the disease is acquired by inhalation of bacterial aerosols generated from man-made water systems. There is little or no evidence to support person to person transmission of legionellosis (Mallison, 1980), indeed, sentinel guinea pigs housed with others

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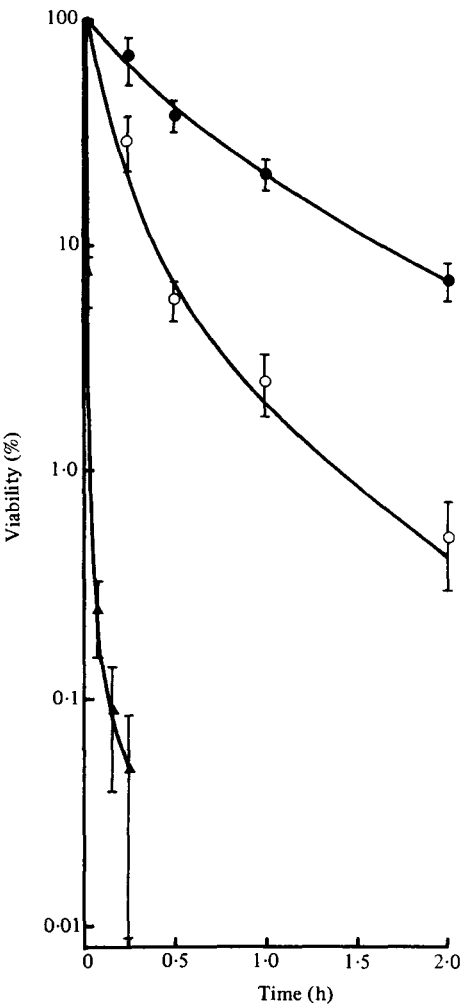

Fig. 4. Aerosol survival of *L. pneumophila* 74/81 sprayed from water and held at 65 % relative humidity (●, grown on solid medium for 4 days; ○, liquid culture, stationary phase; ▲, exponential phase). Bars indicate value of standard error, $n > 4$.

having experimental respiratory legionellosis do not succomb to infection (R. B. Fitzgeorge, unpublished; Katz *et al.* 1982). Confirmation of airborne transmission as the most likely route of infection was provided when Baskerville and co-workers described experimental respiratory infection of guinea pigs and rhesus monkeys (Baskerville *et al.* 1981) and marmosets (Baskerville *et al.* 1982) with aerosols of a virulent strain (74/81) of *L. pneumophila* which gave rise to pneumonic lesions resembling those seen in human legionellosis.

It is likely that the ability of aerosolized legionella to cause disease will depend on the atmospheric conditions prevailing, since it is well known that the survival and infectivity of airborne bacteria are influenced by many environmental factors (see review by Strange & Cox, 1976). Of these factors, the relative humidity of the atmosphere is perhaps most important, although its intrinsic effect is often

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complicated by other factors, such as oxygen tension, temperature and even the method of sampling the microbial cloud or the collecting medium used.

The stability of aerosols of *L. pneumophila* was first investigated by Berendt (1980). The general pattern of survival reported by him was similar to that described here, except that aerosols of the strain used by Berendt appear rather less stable than those of strain 74/81; this may be reflected in the apparently higher virulence of the 74/81 strain. A direct comparison of Berendt's data and ours is difficult, because he used bacteria suspended in tryptose saline rather than distilled water and, furthermore, did not use a stable tracer to compensate for physical losses. The inclusion of sodium chloride in the spray fluid might diminish the aerosol stability of the organism, since Feeley *et al.* (1979) and Dennis, Taylor & Barrow (1981) have shown this substance to be toxic to legionellae except at low concentrations, and sodium chloride can be lethal to airborne bacteria (Anderson & Cox, 1967).

Broadly speaking, airborne *L. pneumophila* strain 74/81 survives relatively well at medium r.h. values (65%) but less well at high r.h. (90%). As was also found with the strain used by Berendt (1980), the 74/81 organisms appear not to survive well in dry atmospheres (30% r.h.). Oxygen is known to be toxic to many bacterial aerosols, but often the effect may only be apparent at low r.h. (Hess, 1965; Cox, 1966; Benbough, 1967, 1969; Webb, 1967); it is possible that the poorer survival of *L. pneumophila* in drier air may be due in part to oxygen toxicity.

One disadvantage of assessing microbial aerosol stability at only three representative r.h. values is that for many bacteria zones of relative instability exist at certain r.h. values (Anderson, 1966; Benbough, 1967; Cox, 1966, 1969, 1971) and similar zones of instability have been found for freeze-dried bacteria (Bateman *et al.* 1961; Dewald *et al.* 1967). The survey of the viability of *L. pneumophila* 74/81 aerosols held for 15 min showed that this organism survives poorly at 55% r.h. compared with its stability at r.h. values slightly above and below this (65 and 40% respectively); this suggests that such zones of instability also exist for legionella.

The viability of aerosolized bacteria may also be affected by the composition of the spray fluid (Anderson & Cox, 1967) indeed, Berendt (1981) reported that survival and infectivity of *L. pneumophila* aerosols was improved by the inclusion of sterile culture filtrates of cyanobacteria. The protection afforded by algal extract was, however, similar to that which resulted when raffinose and dipyridyl were included in the spray fluid and may, in fact, represent non-specific protection, as given to aerosolized bacteria by several compounds (Anderson & Cox, 1967), rather than a specific ecological phenomenon. The effects of spray fluid additives were not investigated here, but a comparison of collecting fluids showed aerosolized *L. pneumophila* to be tolerant of distilled water and Page's saline, but less so of PBMA and GPMA.

It is known that the ability of bacteria to survive in the airborne state is affected by conditions of growth (Benbough *et al.* 1972) and metabolic activity (Hambleton, Strange & Benbough, 1972), bacteria with low metabolic activity having the best ability to survive aerosolization. The present work demonstrates that the survival of airborne legionella similarly may depend on the metabolic state of the organism since stationary phase broth-grown organisms are markedly more stable in aerosols

than exponential phase broth-grown organisms, which presumably have a higher metabolic activity. This implies that the metabolic activity of 4-day colonies on solid medium is diminished in comparison with stationary phase broth cultures since the former exhibit superior survival in aerosols. It is further possible that differences in the aerosol stability of the 74/81 strain and that used by Berendt may reflect differences in the metabolic status of the bacterial suspensions used by the two groups.

This study and those of Baskerville *et al.* (1981, 1982) show clearly that *L. pneumophila* strain 74/81 can retain viability, virulance and aerosol stability when stored in an aqueous environment. The aerosol stability of organisms with low metabolic activity is such that significant numbers of viable organisms can be recovered from bacterial clouds after up to 2 h. Since contaminated evaporative condensers, for example, might be anticipated to generate continuously aerosols containing viable *L. pneumophila* organisms, it is clearly likely that exposed susceptible humans might inhale and retain sufficient viable organisms to acquire an infection.

An unexplained phenomenon is that many contaminated water sources do not give rise to outbreaks of legionellosis and that some outbreaks cease even whilst the source remains contaminated. There are undoubtedly many factors contributing to such features and one may be the metabolic status of the contaminating legionellae, which could affect the ability of these bacteria to survive in the airborne state and subsequently give rise to respiratory infection.

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