*J. Hyg., Camb.* (1983), **91**, 167–178
*Printed in Great Britain*



# Interaction of *L. pneumophila* and a free living amoeba (*Acanthamoeba palestinensis*)

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(*Received* 31 *May* 1983; *accepted* 20 *June* 1983)

SUMMARY

Co-cultivation of *Legionella pneumophila* serogroup I and *Acanthamoeba palestinensis* in Neff's medium at 35 °C resulted in the intracellular multiplication of the bacteria as demonstrated by electron microscopy and immunofluorescence. In the closed experimental system used, the number of legionellae rose from $10^7$ colony forming units (c.f.u.)/ml initially to a maximum of $10^{10}$ c.f.u./ml on day 5. Legionellae were seen in expelled phagosomes, in some amoebae filling the cytoplasm and in others in which the process of encystment appeared to have commenced.

At 20 °C the acanthamoebae phagocytosed and digested the legionellae. The bacteria disappeared from the co-cultivation flask by day 2 but reappeared in low numbers ($10^2$ c.f.u./ml) by day 6 suggesting that even at this temperature some intra-amoebal multiplication occurred.

## INTRODUCTION

The members of genus *Legionella*, the aetiological agent of Legionnaires' disease were originally isolated in guinea pigs and in embryonated hen eggs by techniques designed for isolation of Rickettsia (McDade *et al.* 1977). Although legionellae can now be grown on culture media, they are extremely fastidious in their growth requirements *in vitro*. Media supporting growth of legionella require supplementing with ferric salts (Feeley *et al.* 1978) and with several amino acids, including L-cysteine (Wever & Feeley, 1979). Yeast extract, pyruvate and alpha-ketoglutaric acid stimulate growth. The optimum pH of the medium during growth is 6·6–6·9 (Rutter & Maber, 1979). Oleic acid found in agar inhibits growth and this can be counteracted by adding starch or charcoal (Pine *et al.* 1979). It has been suggested that legionellae themselves may be a source of inhibitory fatty acids (Pine *et al.* 1979). The optimum growth temperature is 35 °C with a range between 25 and 42 °C (Feeley *et al.* 1978; Pine *et al.* 1979).

*Legionella* sp. have a world-wide distribution and have been isolated from a variety of aquatic habitats. These include cooling towers and evaporative condensers of air conditioning systems, water and mud rivers and lakes, other natural collections, (Morris *et al.* 1979; Fliermans *et al.* 1981), and potable water systems (Wadowsky *et al.* 1982). The pH of these range between 5·5–8·3 and their

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temperature between 6–63 °C, both of which contrast with the established permissive *in vitro* pH and temperature for growth. Other bacteria found in these waters may be inhibitory to legionellae. A parasitic bacterium *Bdellovibrio bacteriovorus* that replicates inside some Gram-negative bacilli, is present in habitats similar to those from which legionellae have been isolated (Starr & Seidler, 1971), and has been shown to parasitize them (Tomov *et al.* 1982). *L. pneumophila* is also inhibited by staphylococci, streptococci (Flescher *et al.* 1980) *Aspergillus* sp. (Chandler, 1981) and *Bacillus* sp. (Carrington, 1979).

*L. pneumophila* survives in filtered sterilized distilled or tap waters (Skaliy & McEachern, 1979) and autoclaved river waters (Wang *et al.* 1979) for many months, in progressively decreasing numbers. In natural waters, however, the organism multiplies. Although a symbiotic association has been suggested between *L. pneumophila* and certain blue-green algae (Cyanobacteria) (Tison *et al.* 1980), *L. pneumophila* multiplies in places where photosynthesis cannot take place (Kurtz *et al.* 1982).

All these factors suggest that legionellae may not be a free living bacterium. Despite its ability to grow on specialized media *L. pneumophila* appears usually as an intracellular organism in human lung (Glavin, Winn & Craighead, 1979), in yolk sac (Katz & Nash, 1978), in macrophages (Kishimoto *et al.* 1979) in monocytes, (Horwitz & Silverstein, 1980) and in cell cultures (Daisy *et al.* 1981). Rowbotham (1980) suggested that free living amoebae (FLA) of the genera *Nagleria* and *Acanthamoebae* can be hosts to legionellae. These amoebae are ubiquitous in water. They grow over a wide range of temperatures (Griffin, 1972) and their intracellular pH is in the range 6·3–7·4 (Heiple & Taylor, 1980; Drozanski, 1963). It has also been suggested that the outcome of ingestion of *L. pneumophila* by FLA depends upon temperature – at 35 °C the ingested legionellae grow and destroy their host (Rowbotham, 1980), but at 22 °C legionellae are digested by the FLA (Nagington & Smith, 1980).

In this study we have examined the replication, at different temperatures, of an environmental isolate of *L. pneumophila* serogroup I in the presence of free living *Acanthamoeba palestinensis*.

## METHODS

### *Isolation of* L. pneumophila

A 5 l sample of water from a local cooling tower was passed through a 0·3 μm pore size 142 mm membrane. The residue on the membrane was resuspended in 20 ml of the filtrate by vigorous shaking. 0·1 ml was plated onto ACES buffered charcoal yeast extract agar (CYE) containing glycine (3 μg/l) and vancomycin (3 μg/ml), polymyxin (80 u/ml) and cycloheximide (80 μg/ml). The plates were incubated in a moist chamber at 35 °C for 3 days and colonies with morphology typical of legionellae were subcultured and confirmed as *L. pneumophila* by bacteriological methods (Weaver & Feeley, 1979), and by gas liquid chromatography. Serogrouping was done by indirect immunofluorescent microscopy using antisera against *L. pneumophila* serogroups 1–6 (kindly supplied by Dr A. G. Taylor, Division of Microbiological Reagents and Quality Control, Colindale, London). The isolate was stored by freezing in skim milk at −70 °C.

*Isolation of amoebae*

Of the suspension used for the isolation of *L. pneumophila* 0·1 ml was plated on a series of petri dishes containing Stoianovitch's malt yeast extract agar (MYAS) (Page, 1976), previously layered with a suspension of heat-killed *Escherichia coli*. Plates were incubated at 40, 35, 28 and 22 °C, for up to 2 weeks in moist chambers. Growth of amoebae occurred at 35 and 28 °C after 6 days. The amoebae were cloned and a pure isolate of *Acanthamoeba* sp. was identified. This was typed as *Acanthamoeba* (*Mayorella*) *palestinensis* (Dr Warhurst, London School of Tropical Medicine and Hygiene). The acanthamoeba was cultivated axenically and maintained by passage in 4% Neff's medium (Cursons, Brown & Keys, 1980) at 35 °C.

*Kinetics of intracellular growth*

A 48 h growth of *L. pneumophila* on CYE was scraped off and suspended in distilled water. The suspension was then added to give a final concentration of approximately $5 \times 10^6$ colony forming units (c.f.u.) per ml to two each of the following:

(*a*) Neff's medium.

(*b*) Conditioned medium. This was Neff's medium in which *A. palestinensis* had been grown for 3 days at 35 °C and which had then been filtered through a 0·2 $\mu$m membrane.

(*c*) *A. palestinensis* in log phase growth at a concentration of $2 \times 10^5$ amoebae/ml in Neff's medium.

(*d*) Neff's medium containing sonicated *A. palestinensis*. A suspension of amoebae identical to that used in (*c*) was sonicated at an amplitude of 12 $\mu$m for 20 s. Complete disintegration was confirmed by microscopic examination of the sonicate and culturing 0·1 ml on MYAS medium layered with *E. coli* and incubated at 35 °C for 2 weeks.

The mixtures were incubated with gentle agitation, one set at 35 °C and one at 20 °C for up to 2 weeks.

Aliquots were removed at 0, 8, 24 h and daily thereafter and viable *L. pneumophila* were counted on CYE agar by the method of Miles & Misra (1938). A modified Fuchs–Rosenthal counting chamber was used to count amoebal trophozoites and cysts.

*Electron microscopy*

Samples of the amoeba–legionella mixture (c) were removed at 0, 4, 8, 16, 24, 48 and 96 h and centrifuged at 500 r.p.m. for 5 min. The deposit was fixed in 2·5% glutaraldehyde for 1 h at room temperature, washed in phosphate buffered saline, pH 7·2, for 1 h at room temperature and post fixed in 1% osmium tetroxide at 4 °C for 1 h. After passing through a series of graded alcohols to propylene oxide, the pellets were embedded in Spurr epoxy resin. The blocks were polymerized at 60 °C. Sections were cut and stained with uranyl acetate and lead citrate and examined using a Phillips EM301 electron microscope.

*Indirect fluorescence antibody (IFA) staining of sections*

Resins sections, 1 $\mu$m thick, were placed on multispot slides and dried overnight at 60 °C. Sections were de-resinated using saturated alcoholic sodium hydroxide (sodium ethoxide) for 40 min. The sections were then taken down through a series of dilutions of ethyl alcohol to water.

Excess moisture was shaken off the slides and IFA staining carried out using a monoclonal antibody to *L. pneumophila* serogroup 1 (kindly supplied by Mr Ian Watkins, Department of Pathology, University of Oxford), which was applied to the slides undiluted and incubated for 3 h at 35 °C. After washing, the sections were treated with a 1 in 40 dilution of fluorescein-conjugated rabbit anti-mouse immunoglobulin (Dako) for 30 min at 37 °C. After further washing, the sections were mounted in 90% glycerol and examined with a Leitz EB microscope using incident light illumination.

## RESULTS

L. pneumophila *growth kinetics*

Sequential counts of *L. pneumophila* in the four media are shown in Figs 1 and 2.

At 35 °C (Fig. 1) *L. pneumophila* was not isolated from Neff's medium for longer than 24 h. In the medium conditioned by previous growth of *A. palestinensis* and the medium containing fragments of *A. palestinensis*, *L. pneumophila* was isolated in reducing numbers until 3 and 4 days respectively. In contrast, in the medium containing viable *A. palestinensis* the number of *L. pneumophila* increased after a slight initial fall, to a maximum of $5 \times 10^{10}$ c.f.u./ml on day 5. After this, viable bacterial counts decreased slowly and at 12 days a count of $2{\cdot}3 \times 10^{6}$ bacteria per ml was obtained.

At 20 °C (Fig. 2) the numbers of *L. pneumophila* decreased in all four media from an initial count of $4 \times 10^{6}$ to $1 \times 10^{7}$ c.f.u./ml. Survival was prolonged to 4 and 6 days in the conditioned medium and that containing sonicated *A. palestinensis* respectively. On day 6, however, *L. pneumophila* was again present in low numbers ($3 \times 10^{2}$) in the medium containing *A. palestinensis* and was isolated from this source regularly thereafter until the end of the experiment. In the other three media *L. pneumophila* did not reappear.

*Microscopic observations*

Counts and daily observation of the flasks containing *L. pneumophila* and *A. palestinensis* at 35 °C were carried out. Although the total number of amoebae (trophozoites and cysts) did not change significantly during the experimental period, from the initial count of $2 \times 10^{5}$/ml, the proportion of cysts increased from 12% on day 4 to 91% on day 8 and progressively increasing amounts of debris appeared in the medium. By comparison an axenic growth of *A. palestinensis* at 35 °C in Neff's medium with an identical initial inoculum again gave unchanging total counts with 19% cysts on day 4 and 86% cysts on day 8, but there was no accumulation of debris.

In a similar mixture of *A. palestinensis* and *L. pneumophila* incubated at 20 °C the number of *A. palestinensis* did not alter between the fourth ($1 \times 10^{6}$/ml) and

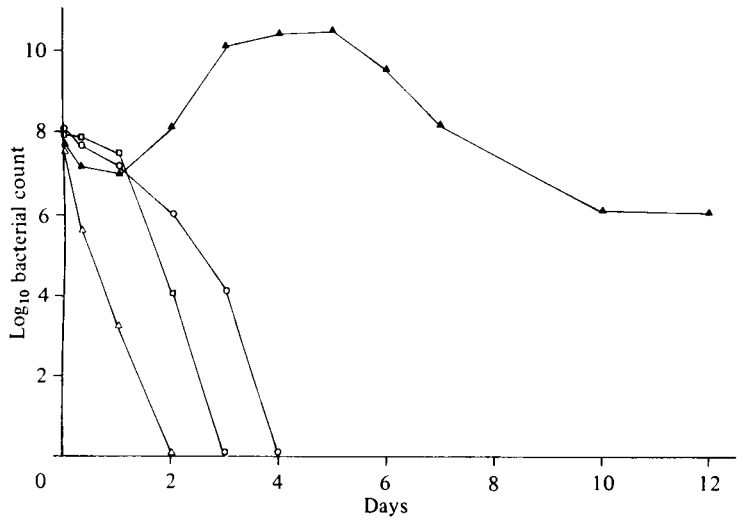


Fig. 1. *L. pneumophila* counts after incubation at 35 °C in: ▲, Neff's medium containing $2 \times 10^5$ *A. palestinensis*; △, Neff's medium only; □, conditioned Neff's medium in which *A. palestinensis* had been grown; ○, Neff's medium with sonicated *A. palestinensis*.

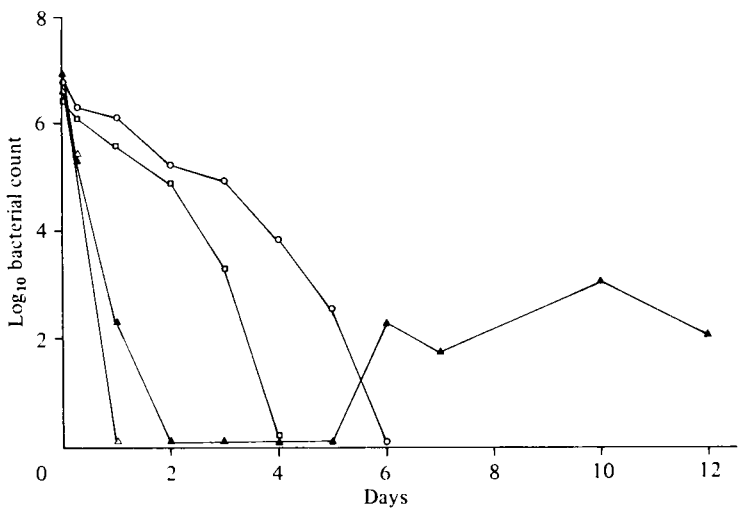


Fig. 2. *L. pneumophila* counts after incubation at 20 °C in: ▲, Neff's medium containing $2 \times 10^5$ *A. palestinensis*; △, Neff's medium only; □, conditioned Neff's medium in which *A. palestinensis* had been grown; ○, Neff's medium with sonicated *A. palestinensis*.

the eighth day ($1 \times 10^6$/ml) and the proportion of cysts increased from 26 to 93% on these respective days. Again the axenic growth of *A. palestinensis* at 20 °C had unchanging total counts with 14 and 73% cysts on days 4 and 8 respectively.

*Electron microscopy*

Electron microscopy of sections of *A. palestinensis–L. pneumophila* mixture incubated at 35 °C demonstrated intracellular bacteria with an ultrastructure consistent with that of *L. pneumophila* (Chandler *et al.* 1979). In early stages of

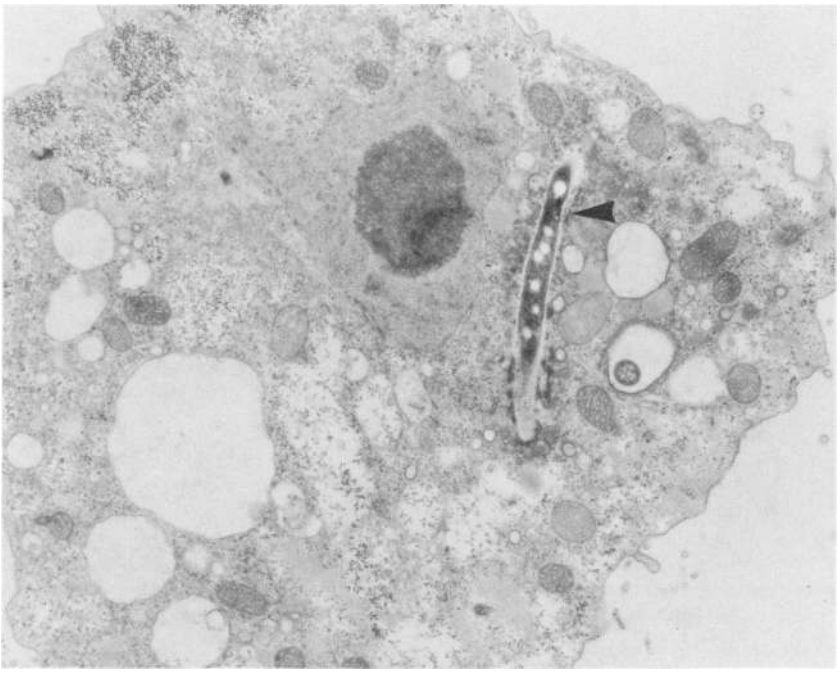

Fig. 3. Electron micrograph showing a single bacterium in a phagosome (◄). Four hour culture of *A. palestinensis* and *L. pneumophila* at 35 °C (×10400).

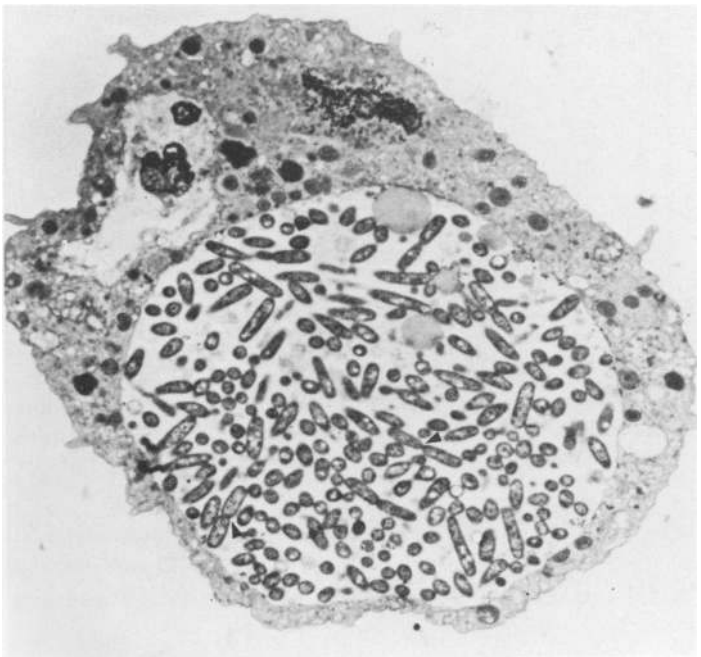

Fig. 4. Electron micrograph showing a phagosome containing many bacteria, some dividing (◄). Sixteen-hour culture of *A. palestinensis* and *L. pneumophila* at 35 °C (×4800).

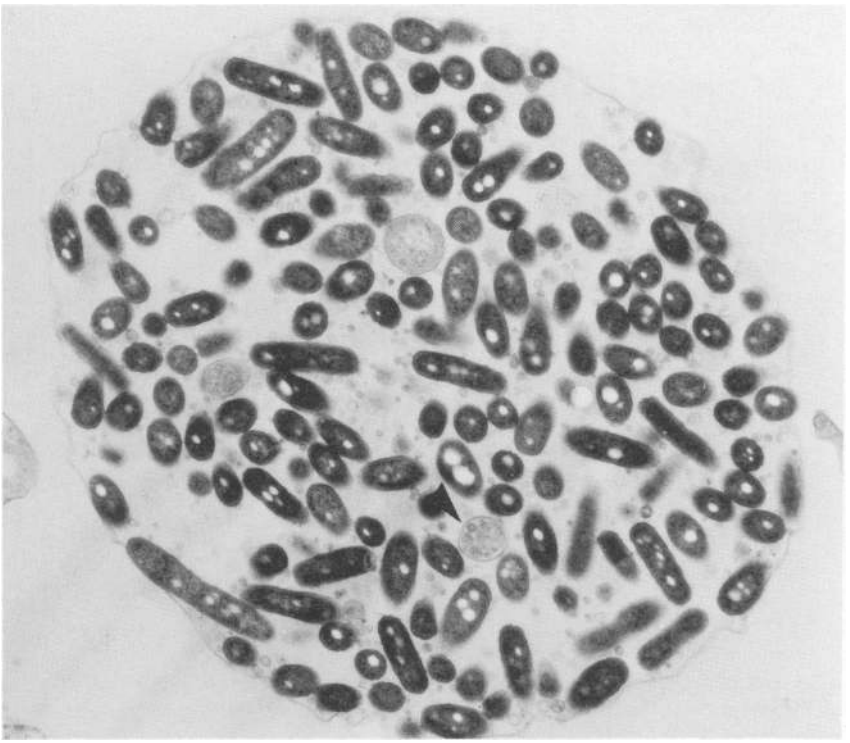

Fig. 5. Electron micrograph showing a rounded up amoeba full of legionellae which are free in cytoplasm. Various organelles and an autolysosome (◄) are seen. Twentyfour-hour culture of *A. palestinensis* and *L. pneumophila* at 35 °C (×13200).

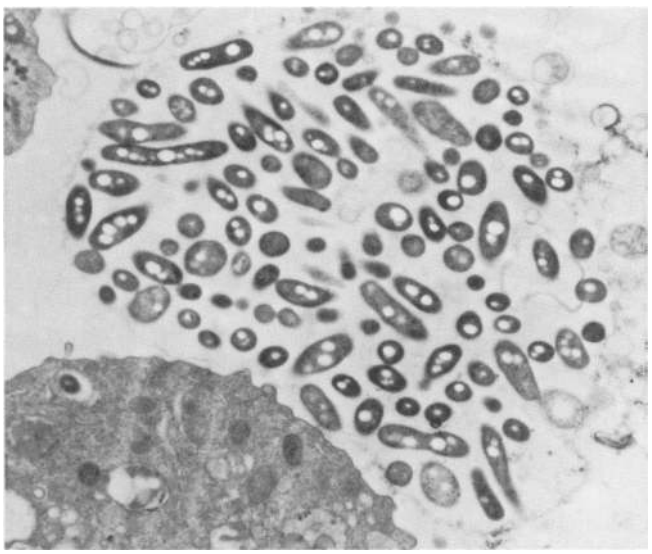

Fig. 6. Electron micrograph showing an extruded phagosome full of legionellae. Ninety six-hour culture of *A. palestinensis* and *L. pneumophila* at 35 °C (×7800).

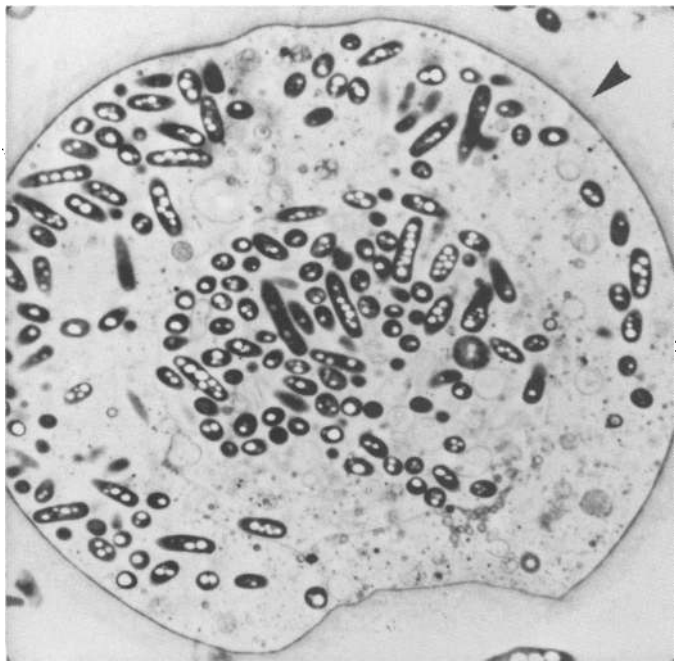

Fig. 7. Electron micrograph showing a rounded up amoeba with accumulation of extracellular material indicating exocyst formation (◄). Ninety six-hour culture of *A. palestinensis* and *L. pneumophila* at 35 °C (×600).

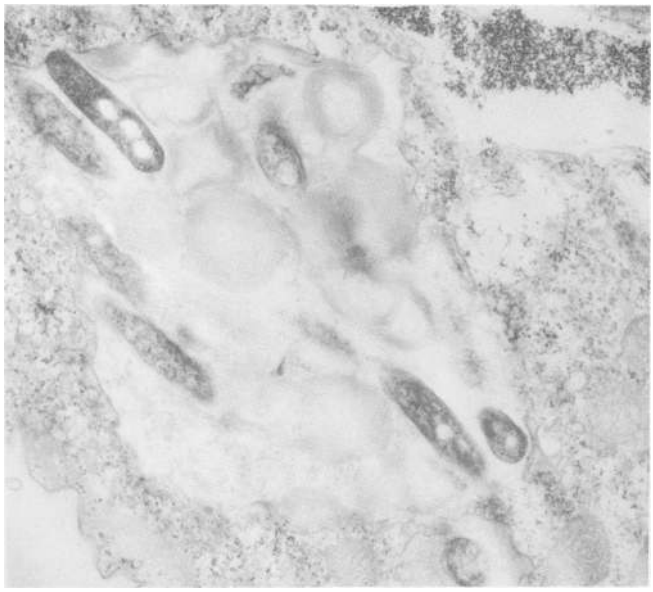

Fig. 8. Electron micrograph showing a phagosome (lamellosome) containing degenerated legionellae and concentric whorls. Twenty four-hour culture of *A. palestinensis* and *L. pneumophila* at 20 °C (×16800).

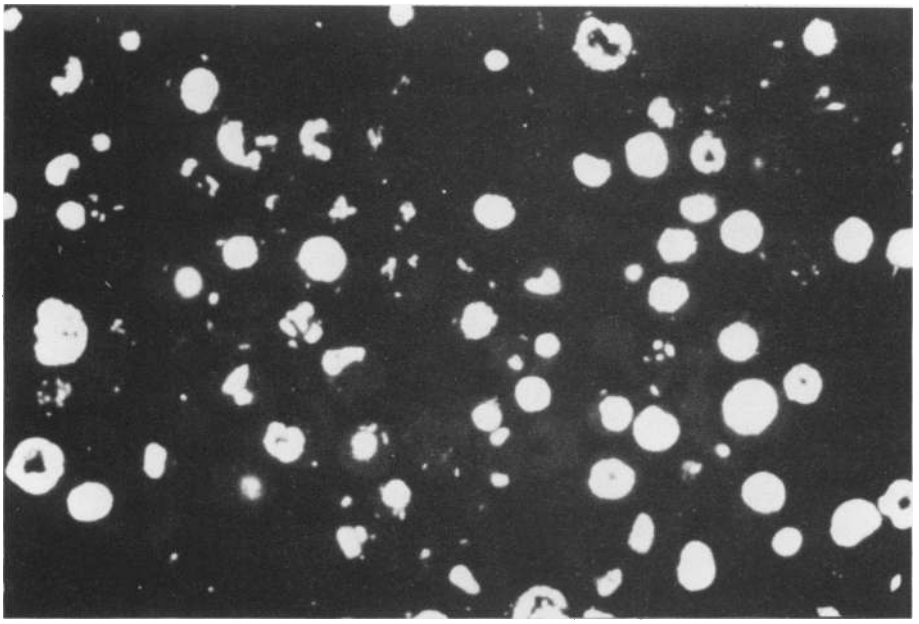

Fig. 9. Fluorescent micrograph of a 24 h culture at (35 °C of *A. palestinensis* and *L. pneumophila* stained with a mouse monoclonal antibody to *L. pneumophila* serogroup 1 and fluorescein-labelled anti-mouse serum. Intracellular bacteria are seen singly and in large aggregates ( × 300).

infection (4 h), single or small numbers of legionella were seen in phagosomes (Fig. 3). At 16 h many amoebae contained large phagosomes full of legionellae, some of which could be seen dividing (Fig. 4). Usually only one phagosome was seen in an infected amoeba which retained its normal shape. Some infected amoebae at 24 h were rounded up and bacteria were seen free in the cytoplasm together with autolysosomes (Fig. 5). Phagosomes full of legionellae could also be seen free outside the amoebae (Fig. 6). At 96 h occasional rounded up amoebae containing legionellae, both inside the phagosome and free in the cytoplasm, showed commencement of cyst wall formation (Fig. 7).

At 20 °C only single or small numbers of legionellae were present within individual phagosomes. Samples taken at 16 and 24 h showed degenerated legionellae and numerous concentric whorls within some phagosomes (Fig. 8).

*Indirect immunofluorescence antibody staining*

The intracellular organisms observed in the electron microscope fluoresced with the monoclonal antibody to *L. pneumophila* serogroup 1 (Fig. 9), but not with the monoclonal antibody to *L. pneumophila* serogroup 3.

When the rabbit antiserum to *L. pneumophila* serogroup 1 was used to stain the semi-thin sections both the ancanthamoeba and *L. pneumophila* fluoresced brightly so that bacteria could not be distinguished inside amoebae.

## DISCUSSION

The results show that *L. pneumophila* multiplied in the presence of a viable acanthamoeba when the two were co-cultivated at 35 °C in Neff's medium. After peaking at 5 days, the decrease in the numbers of legionella was probably due to the reduction in the numbers of trophozoites available for infection in the closed experimental system used, as well as to the limited survival time of the free bacteria in the medium. Indeed, the short survival time of *L. pneumophila* in Neff's medium alone, or with cellular or extracellular products of the acanthamoeba was a notable feature of the experiments.

Intracellular replication was clearly demonstrated by electron microscopy and fluorescent antibody studies and suggests that *L. pneumophila*, after multiplying, is released from the amoebae by expulsion in phagosomes (Fig. 6) and by rupture of the thin cell membrane of heavily infected amoebae (Fig. 5). The bacteria may also remain enclosed in a thicker walled structure (Fig. 7).

At 20 °C *L. pneumophila* disappeared from all four experimental media within 6 days and within 2 days in the presence of acanthamoeba. This suggests that the latter were actively removing the bacteria from the medium by phagocytosis and digestion (Fig. 8), or that the bacteria were dying in the adverse environment of the medium. That after 6 days *L. pneumophila* was again present in low numbers in the medium suggests that at least some of the phagocytosed bacteria were not digested but able to multiply. Tyndall & Domingue (1982) also described the disappearance within 24 h, of *L. pneumophila* when co-cultivated with a strain of *Nagleria* or an acanthamoeba and observed the reappearance of the bacterium some weeks later but they did not mention at what temperature their experiments were carried out.

These findings therefore support both the report of Rowbotham (1980), that intracellular multiplication occurs at 37 °C and that of Nagington & Smith (1980) that legionellae are ingested and digested by amoebae at room temperature. We have, however, extended the latter observation by finding that limited bacterial multiplication also occurs at 20 °C.

It is unclear what significance these laboratory findings might have in an environmental setting. Both legionellae and acanthamoebae are widespread in nature and the organisms that we examined were both isolated from the same water sample. It is therefore quite possible that legionellae infect amoebae in nature and that the rate of multiplication of the legionellae in them is temperature dependent. Sites colonized by amoebae would then act as a focus of multiplication for legionellae.

The finding of collections of *L. pneumophila* within thick-walled structures (Fig. 7), which resemble an early stage of amoebic cyst wall formation (Griffiths, 1970), suggests that such a packet of legionellae may be able to survive more adverse conditions than could free bacteria. It is well known, for example, that amoebic cysts survive the levels of chlorination used to treat domestic water supplies (de Jonckheere & van de Voorde, 1976). If this resistance applied equally to the thick walled amoebae containing legionellae which we have seen, it would explain how the organism is seeded into domestic piped water supplies from which legionella are not uncommonly isolated.

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