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THE ASSOCIATION BETWEEN LEGIONELLAE AND AMOEBAE

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DECLARATION

This thesis is the original work of the author:

Reza H. Doust

DEDICATION

This thesis is dedicated to my wife "Mrs. Ashraf M. Mobarez" for her enthusiasm encouragement through my academic career.

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PRESENTATIONS

- Growth of *L. pneumophila* in *Acanthamoeba castellanii*. Presented (poster) at the Postgraduate Symposium of Scottish Microbiology Club (22-23 September 1994).
- Effect of cytochalasin D and methylamine on intracellular growth of *Legionella pneumophila* in *Acanthamoeba castellanii*. Presented (poster) at a meeting of the Royal Society of Tropical Disease and Hygiene- Scottish branch (May 12, 1994).
- The events following entry of Legionellae into Acanthamoebae. Presented (poster) at the International Union of Microbiology Societies (IUMS) Congress. Prague, Czech Republic, July 3-8, 1994.
- Biological association between Legionellae and acanthamoebae, an application of sample enrichment in isolation of Legionnaires' disease bacterium from clinical samples. Abstract; Second conference of Graduate Research on Medical Science. 2-3 April 1994, Leuven, Belgium.

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ABBREVIATIONS

Ab	Antibody
A	Absorbance
Ag	Antigen
Approx.	Approximate
AS	Amoebae saline buffer
ATP	Adenosine triphosphate
Av.	Average
BCYE	Buffered Charcoal Yeast Extract medium
BCYE+	BCYE supplemented with L- cysteine hydrochloride and ferric pyrophosphate
BSA	Bovine serum albumin
CAP	Capsular polysaccharide
CL	Chemiluminescence
Cm	Chloramphenicol
Conc.	Concentration
CS	Culture supernate
DAB	3-3'-diaminobenzidine tetrahydrochloride
dATP	Deoxy-adenosine triphosphate
dCTP	Deoxy-cytosine triphosphate
dGTP	Deoxy-guanosine triphosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dTTP	Deoxy-thymidine triphosphate
d/w	distilled water
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethyleneglycol-bis N,N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron Microscopy
fMLP	Formyl methionine-leucine-phenylalanine
g	Gram
g	gravity
h	Hour

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanosulphonic acid
Hly	Haemolysin
HMPS	Hexose monophosphate shunt
Hrp	Horseradish peroxidase
Ig	Immunoglubulin
IHA	Indirect haemagglutination
kbp	kilobase pairs
kDa	kilo Dalton
L	Legionella
l	Litre
LPS	Lipopolysaccharide
M	Molar
mA	Milliampere (s)
MAb	Monoclonal antibody
mg	Milligram (s)
MIC	minimal inhibitory concentration
min	Minute (s)
ml	Millilitre (s)
mM	Millimolar
mm	Millimetre (s)
Mol. wt	Molecular Weight
mV	Millivolt
NA	Nutrient agar
NB	Nutrient broth
NNA	Non-nutrient agar
OD	Optical density
OM	Outer membrane
OMP	Outer-membrane protein
OZ	Opsonised zymosan
P-K	Proteinase-K
PAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffer saline
PCR	Polymerase chain reaction
pH	hydrogen ion concentration

PLF	Phagosome-lysosome fusion
PMA	Phorbol myristate acetate
PMNL	Polymorphonuclear leukocyte
PMSF	Phenylmethylsuphonyl fluoride
PPG	Proteose peptone glucose
PYG	proteose peptone yeast extract glucose
R	Rough lipopolysaccharide
rcf	Relative centrifugal force (x g)
RNA	Ribonucleic acid
rpm	Revolutions per minute
S	Smooth lipopolysaccharide
SD	Standard deviation
SDS	Sodium dodecyl sulphate
s	second
TEMED	N, N, N', N'-tetramethyl-ethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris hydrochloride
V	Volt (s)
v/v	Volume/volume ratio
w/v	Weight/volume ratio
YE	Yeast extract (broth)
μg	Microgram (s)
μl	Microlitre
μm	Micrometre
°C	Degrees Celsius
cfu	clony forming unit
d	day

SUMMARY

Legionella pneumophila and *Acanthamoeba castellanii* were selected as the model for investigation of amoebae-legionellae interactions.

The number of legionellae recovered from the co-culture, increased with time of incubation, while *E. coli* (serving as particulate food for amoebae in the Laboratory) numbers fell, due to ingestion and digestion by the amoebae during the same period of incubation. Amoebae appeared to restrict the multiplication of legionella at lower temperatures. However, when the temperature was increased (37°C) legionellae multiplied within amoebae and finally lysed the amoebae host cells.

Cytochalasin D and methylamine were used in order to investigate the phagocytosis process in amoebae. Cytochalasin D (2 μM) (an inhibitor of actin filaments), had no significant effect on uptake and intra-amoebal growth of legionella. We concluded that uptake and intra-amoebal multiplication of legionella may occur, in part by a microfilament-independent process. The phagocytic activity of amoebae was inhibited in the presence of methylamine (100 mM) in amoeba -legionella co-culture. We concluded that receptor-mediated pinocytosis may be involve in uptake and intra-amoebal growth of legionella.

The intra-amoeba conditions had abstruse effects on the legionella cell envelope composition. Unique lipopolysaccharide patterns were found in intra-amoeba grown legionella. Some of the major components of the legionella cell envelope, such as MOMP (28 kD) were expressed by either *in vitro* or intra-amoeba grown legionella.

A striking feature of intra-amoeba grown legionella in this study was the expression of a 15 kD novel outer membrane protein. The appearance of new surface components in *in vivo* grown cells, such as the 15 kD protein identified in this study, may fulfill functions such as inhibition of phagosome-lysosome fusion in the amoeba host cell.

We attempted to show whether or not the fusion of lysosomes with phagosomes is inhibited following phagocytosis of legionellae by amoebae. The information obtained by fluorescent microscopy, indicated that legionellae were phagocytosed by amoebae in a manner similar to *E. coli* and latex beads, but legionella-containing phagosomes persisted and failed to fuse with amoebae lysosomes. Further details of phagosome-lysosome fusion at ultra-structural level would be useful to confirm the present conclusion.

Lucigenin-dependent chemiluminescence was used to measure photon emission in amoebae following phagocytic activity. We attempted to compare the magnitude of the oxidative burst following ingestion of *E. coli*, latex beads and *L. pneumophila* by measuring the lucigenin-dependent CL responses. CL in *Acanthamoeba castellanii*, an alternative professional phagocytic cell, generated a low level of lucigenin-dependent CL following phagocytosis of these stimulants. However, this CL response was barely above the background level, and two orders of magnitude lower than the responses found in mammalian phagocytic cells.

The association of legionellae and amoebae was investigated in water samples obtained from different hospitals in the UK.

A novel enrichment method using amoebae suitable for legionella detection in environmental water supplies was developed. Legionellae and free-living amoebae were isolated from 25% of water samples. Different genera of free-living amoeba and ciliates were observed in 46% of water samples. Totally, 49% of water samples were contaminated by potential pathogens, either legionellae or amoebae. Using the above novel enrichment method, we isolated *L. pneumophila* from 15% of water samples which were reported negative by the conventional culture method. We recommend this enrichment method together with PCR (where possible) for detection of legionella, especially in culture-negative water samples.

1- INTRODUCTION

1.1 LEGIONELLACEAE

1.1.1 Nomenclature

The first study about the Legionella began in 1976 with the investigation of a large outbreak of respiratory disease at an American Legion Convention in Philadelphia, Pennsylvania, resulting in the isolation of the causative bacteria agent, *L. pneumophila* (McDade *et al.*, 1977). The family Legionellacae and genus Legionella were defined in 1979 for a single species *L. pneumophila.* (Brenner *et al.*, 1979). Since then 39 other species of Legionella have been identified (Table 1) (Harrison &Saunders, 1994). The original member of the family, *L. pneumophila,* was described as a fastidious, slow growing aerobic, Gram-negative, rod 0.3-0.9μm in width and 2-20μm or more in length. No endospores or microcysts are formed, it is non-encapsulated, and not acid-fast. The organism is motile by one, two or more straight or curved polar or lateral flagella; non-motile strains are occasionally seen (McDade *et al.*, 1977).

New species and serogroups have been confirmed by DNA relatedness. Relatedness between strains of the same species is 75% or higher for all species except *L. bozemanii* for which relatedness is 56%-77%. For all species the related sequences exhibit 3% or less divergence (unpaired bases within heteroduplex DNA) (Brenner *et al.*, 1979).

The family is circumscribed and readily distinguishable from other families on the basis of a unique set of phenotypic characteristics that include Gram-negativity, non-fermentative metabolism, requirement for L-cysteine and iron salts, and predominantly branched chain cellular fatty acids. Relatively large amounts of branched chain hydroxy-substituted branched chain fatty acids are found in flavobacteria, but

these organisms were shown by DNA/DNA hybridisation to be unrelated to legionella (Brenner *et al.*, 1979).

Serological diversity within the species had already been recognised by the time nomenclature was established. Since then many species and serotypes have been discovered. DNA relatedness is the only method whereby one can speciate Legonellae, confirm the existence of new serogoups in existing species, and identify new species. DNA relatedness, however, is not adaptable to routine laboratory use and can be used for those samples which cannot be processed by biochemical, Direct Fluorescence Antibody test (DFA) and fatty acids analysis (Morris *et al.*, 1980).

Table 1 Members of the family legionellaceae (Harrison *et al.*, 1994)

Legionella spp.	Multiple sero-groups	Isolation from patients
1 *L. pneumophila*	14	Y*
2 *L. adelaidensis*		N*
3 *L. anisa*		Y
4 *L. birminghamensis*		Y
5 *L. bozemanii*	2	Y
6 *L .brunensis*		N
7 *L. cherii*		N
8 *L. cincinnatiensis*		Y
9 *L. dumoffii*		N
10 *L. erythra*	2a	N
11 *L. fairfieldensis*		N
12 *L. feeleii*	2	Y
13 *L. geestiana*		N
14 *L. gormanii*		Y
15 *L. gratiana*		N
16 *L. hackeliae*	2	Y
17 *L. israelensis*		N
18 *L. jamestowninsis*		N
19 *L. jordanis*		Y
20 *L. lansingensis*		Y
21 *L. lodiniensis*		N
22 *L. longbeachae*	2	Y
23 *L. maceachernii*		Y
24 *L. micdadei*		Y
25 *L. moravica*		N
26 *L. nautarum*		N
27 *L. oakridgensis*		N
28 *L. parisiensis*		N
29 *L. quarteriensis*		N
30 *L. quinlivanii*	2	N
31 *L. rubrilucens*		N
32 *L. sainthelensis*	2b	Y
33 *L. santicrucis*		N
34 *L. shakespearei*		N
35 *L. spiritensis*	2	N
36 *L. steigerwaltii*		N
37 *L. tucsonensis*		Y
38 *L. wadsworthii*		Y
39 *L. worsleiensis*		N

Y*= Yes, N*= No

a *L. erythra* serogroup 2 is serologically indistinguishable from *L. rubrilucens* (Saunders *et al.*, 1992).

b *L. sainthelensis* serogroup 2 is serologically indistinguishable from *L. santicrucis* (Benson *et al.*, 1990).

1.1.2 Legionellae characters

The summary of biochemical reactions and phenotyping characteristics of *Legionella spp.* is shown in Table 2. As the only genus in the family, the definition of legionella is identical to that of Legionellaceae. Characteristically the organisms appear as small coccobacili, though longer forms can be found. One, two, or occasionally more flagella occur per cell. The flagellae are curved or straight and have polar or lateral arrangement with short pili around the bacterial surface (Rodger , 1983). The bacteria are rod shaped or filamentous; the filament forms are found after growth on agar media, less commonly in yolk sac material, and rarely in human lung or guinea pig tissue. (Chandler *et al.*, 1979). Electron micrographic examination reveals filamentous nucleoids, ribosomes, and sudan black B-staining vacuoles thought to contain poly-ß-hydroxybutyrate granules (Chandler *et al.*, 1979). Cells are enclosed by a double envelope, composed of two three-layered unit membranes; a peptidoglycan layer has been demonstrated for *L. pneumophila*, and for *L. micdadei*. Cell division occurs by a pinching process and growth is by pinching septate fission (Rodger , 1983).

Legionellae are not acid-fast by the Ziehl-Neelsen procedure for mycobacteria, but *L. micdadei* may appear acid-fast in tissue preparations stained by the modified Ziehl--Neelsen stain using 5% acetic acid (Feeley *et al.*, 1979). The bacterial surface is smooth or may be convoluted, sometimes internal globules are evident. In thin section the organisms have a triple layered envelope typical of Gram-negative bacteria with, an outer electron-dense outer membrane, which may be loose and undulating, and an inner less opaque cytoplasmic membrane and an intermediate discontinuous stratum containing

Table 2 Summary of the biochemical reactions and phenotypic characteristics of legionellae.

Test	Results	Relevant species	Comment
Subculture onto BCYE$^+$ agar	Supports growth	All species*	Blood agar is not a suitable substitute for BCYE$^-$, particularly for environmental specimens, as non-legionellae (e.g. some Moraxella spp) may grow on BCYE but not on blood agar.
Subculture onto BCYE (-Cys) agar	Dose not support growth		
Gram stain	Gram-negative	All species	Legionella will readily counterstain with 0.1% basic fuchsin
Catalase	positive	All species (*L. pneumophila* only weakly)	Some species possess a peroxidase rather than a catalase but they still give a positive result in the test if 3% H_2O_2 is used.
Oxidase	Negative	all species	positive results are sometimes recorded but this is probably due to contamination with BCYE medium.
Hippurate hydrolysis	positive	*L. pneumophila*	Some strains, particularly of subspecies *fraseri*, are said to be negative in this test .
	negative	All other species	
Autofluorescence under long-wavelenth UV (~365 nm)	Red fluorescence	*L. erythra* and *L. rubrilucens*	Red fluorescence may only be seen after prolonged or reduced temperature (~30°C) of incubation. These two species were formerly considered to be serologically distinct.
	Blue-white fluorescence	*L. anisa, L. bozemanii, L. cherrii, L. dumoffi, L. gormanii, L. gratiana, l. parisiensis*	Many of these species are phenotypically and antigenically very similar to others in the group
	No fluorescence	All other species	
Bromocresol-purple spot	Positive	*L. micdadei, L. maceachernii*	These species are serologically distinct
	Negative	All other species	

* Except Legionella Like Amoebae Pathogen (LLAP-3)

BCYE ($^-$Cys)= Buffered Charcoal Yeast Extract agar without adding L-cystein.

peptidoglycan. The bacterial cytoplasm, as with other Gram-negative organisms, contains scattered ribosomes and thread-like nuclear elements (Rodgers, 1983).

Legionella cells seem to derive most of their energy from the oxidation of amino acids and related compounds by means of the tricarboxylic acid cycle, which along with a complex electron transport chain, is completely expressed in these organisms. Serine, threonine, and glutamate are especially good substrates for the *Legionella*. In addition, the following organic acids stimulate oxygen consumption by this bacterium: lactate, pyruvate, acetate, malate, fumarate, and oxaloacetate (Dowling *et al.*, 1992).

L. pneumophila is capable of synthesising its own fatty acids. A unique feature of the organism is that it produces mostly branched-chain fatty acids, the predominant one is isopalmitate, a saturated, branched, 16- carbon fatty acid (Moss *et al.*, 1977). The organism also contains small amounts of hydroxy fatty acids, ß-hydroxyisomyristic acid, ß-hydroxyarachidic acid, most of which are confined to cell envelope structures (Mayberry, 1981). The major phospholipids of *L. pneumophila* have been determined to be, in order of decreasing abundance, phosphatidylcholine, cardiolipin (diphosphatidyl glycerol), phosphatidylethanolamine, phosphatidylglycerol, and phosphatidyl dimethylethanolamine (Finnerty *et al.*, 1979).

Legionellaceae have a high lipid content including phosphatidylcholine and fatty acids of which more than 80% are branched-chain fatty acids. Extraction of these acids from saponified organisms with analysis by gas liquid chromatography (GLC) has provided distinctive profiles among the species (Edwards & feltman, 1983). So far only *L. pneumophila* lipopolysaccharide (LPS) has been

throughly analysed (Moll *et al.*, 1992). The lipid A part of this LPS contains 19, 3-hydroxy fatty acids, eight non-hydroxy fatty acids, two 2,3 dihydroxy fatty acids, two (ω -1) oxo fatty acids, two (1,ω)-dioic fatty acids and one (ω-1)-hydroxy fatty acid (Sonesson *et al.*, 1994). Of the total fatty acids of *L. pneumophila*, 85-90% is accounted for by five major acids, the highest concentration being of a saturated branched chain 16- carbon acid with the methyl branch at the penultimate carbon atom, (i-16:0). In decreasing amount are a mono-unsaturated 16-carbon straight- chain acid (16:1), a branched chain 15-carbon (a -15:0), a saturated 14-carbon branched -chain acid (i-14:0) and a saturated 17-carbon branched- chain acid (a-17:0). Other normal straight-chain saturated acids range from 0 - 5 % (Moss & Dees, 1979). Apparently all species of Legionellae share a remarkably complex and distinct pattern of hydroxy fatty acids, all persumbly of LPS origin (Jantzen *et al.*, 1993).

L. longbeachae and *L. oakridgensis* also have i-16:0 as their main branched-chain fatty acid but other acids such as 16:1 or 16:0 in differing proportions make for distinctive profiles. Among other species including *L. bozemanii, L. dumoffii, L. gormanii, L. micdadei, L. jordanis and L. wadsworthii* the predominant acid is a saturated branched- chain 15-carbon acid (a-15:0) with i-16:0 in a lesser position. *L. micdadeii* is distinctive in containing small amounts of mono-unsaturated branched-chain 17- carbon acid, (a-17:1). The cellular fatty acids of recently described species have been stated to qualitatively resemble those of established species (Brenner *et al.*, 1985). A lack of these cellular fatty acids in various other Gram-negative species confirms the independence of the Legionellaceae (Edwards & Felthman 1983).

Carbohydrates are neither fermented nor oxidized by Legionellae (Weaver & Feeley, 1979). Amino acids can presumably serve as the carbon source (Pine *et al.*, 1979). *L. pneumophila* possesses little or no catalase and therefore might be susceptible to damage by low levels of hydrogen peroxide. Low levels of hydrogen peroxide also inhibit growth of a variety of bacteria. Addition of peroxide and superoxide radical scavengers, including enzymes such as catalase and superoxide dismutase (SOD), stimulate growth in the absence of charcoal. (Hoffman *et al.*, 1983).

Lipopolysaccharides of *L. pneumophila* show an unusual banding pattern compared with other Gram-negative organisms and are serogroup-specific antigens (Nolte *et al.*, 1986). Because isoprenoid quinones in bacterial plasma membranes can be of use as chemotaxonomic markers in classification (Collins & Jones, 1981), the detection in Legionella species by reverse phase thin layer chromatography of ubiquinones of ten or more isoprene units has afforded an additional means of grouping them (Kar *et al.*, 1982).

Legionella do not stain with hematoxylin and eosin in formalin-fixed paraffin sections but can be demonstrated easily with Dieterle or Warthin-starry silver impregnation procedures. This technique is not specific and will stain virtually all microrganisms present in addition to Legionellae (Koneman *et al.*, 1992). Legionella can usually be demonstrated in fresh imprints of lung biopsy material stained with Giemsa, or Gram-Weigert stain and it can sometimes be seen in fresh imprints using routine Gram stain procedures with basic fuchin used as a counterstain (Koneman *et al.*, 1992). The findings of weakly acid-fast bacilli using a modified Kinyoun stain may be a clue to the presence of *L. micdadei* (Winn & Myerowitz, 1981).

1.1.3 Cultivation: Legionellae are nutritionally fastidious organisms, and require iron and L- cysteine for growth. As many other pathogens, Legionellae require iron for intracellular growth (Marra & Shuman, 1992). Soluble ferric pyrophosphate, ferric nitrate or haemoglobin can provide the iron. Iso Vitale X (BBL) can serve as a source of L-cysteine. The following amino acids are essential for growth: cysteine, serine, thymine, arginine, valine, leucine, methionine, isoleucine, phenylalanine or tyrosine and threonine; no vitamin or co-factor requirements have been demonstrated (George *et al.*, 1980). Most strains require serine, some also require proline. Serine and threonine can be used as sole sources of carbon and energy (Rowbotham, 1980).

Laboratory media for the growth of *L. pneumophila* require supplementation with L-cysteine and ferric iron, usually ferric pyrophosphate (Rowbotham, 1980). Mueller-Hinton agar supplemented with 1% haemoglobin and 1% IsoVitaleX (MH-IH) was first used for cultivation of *L. pneumophila* (Feeley *et al.*, 1978). MH-IH was first replaced by Feeley-Gorman (F-G) agar for routine culture of *L. pneumophila* (Feeley *et al.*, 1978). Most species will grow on F-G agar only after adaptation (Morris *et al.*, 1980).

Charcoal Yeast Extract (CYE) agar was subsequently developed and found superior to F-G agar. BCYE was then developed which is presently the most sensitive medium (Pasculle *et al.*, 1980). CYE diphasic medium is used to culture blood samples. In all of these media, soluble ferric pyrophosphate or ferric citrate replaces haemoglobin and L-cysteine HCL replaces Iso-VitaleX as growth factors for Legionellae (Morris *et al.*,1980). Yeast extract broth (YE) has also been used for growing large numbers of *L. pneumophila* (Ristroph *et al.*, 1980).

Carbohydrates and many organic acids are not used by legionellae, and some organic acids, for example, citric and oleic acid are reported to have inhibitory effects (Rowbotham, 1980). Growth on solid media is initiated only within a narrow pH range, around 6.9, the optimum pH is between 6.8 and 7.0 (Feeley *et al.*, 1978). Growth of stock cultures obtained from humans occurs at 25°C - 43°C, the optimum temperature has been reported to be 36±1°C (Thacker *et al.*, 1981). In nature or in association with certain algae, Legionellae can grow at 45°C or higher (Fliermann *et al.*, 1981).

Carbon dioxide does not appear to stimulate the growth of Legionellae, with exception of *L. gormanii*. Because of this exception, and also the buffering effect of CO_2 incubation in an atmosphere of air +2.5% CO_2, or in a candle jar has been recommended for all Legionellae, however CO_2 may slightly inhibit growth in BCYE (Pasculie *et al.*, 1980).

All species grow on CYE agar and BCYE agar. Pinpoint colonies appear on buffered charcoal yeast extract (BCYE) agar in about 3 days after primary culture; the colony diameter reaches 3-4 mm after 5-7 days of incubation at 36±1°C. Colonies are grey, glistening convex, and circular with an entire edge (Feeley *et al.*, 1979). Several species exhibit a blue-white autouflourescence, and most species produce a diffusible, brown pigment on tyrosine containing agar (Baine *et al.*, 1978).

Brown pigment is formed on MH-IH agar or on other suitable media that contain tyrosine (Baine *et al.*, 1978) but cannot be observed on BCYE or CYE agar because these media are black (due to charcoal). Some species exhibit yellow fluorescence on F-G agar or in F-G broth after 3-4 d of incubation. Yellow fluorescence is observed only in the dark by using a 365 nm ultraviolet light (Wood's light) (Weaver &

Feeley, 1979). Some species exhibit blue-white autofluorescence on a variety of media when viewed under Wood's light either in daylight or in darkness (Cordes *et al.*, 1979).

1.1.4 Pathogenicity

Legionellae, as facultative intracellular parasites, have to make contact with engulfing cells such as macrophages in the lung alveoli to be pathogenic (Conlan *et al.*, 1986). This occurs after the inhalation of small aerosol particles which contain viable bacteria in a phase of multiplication. The bacteria must attach to the surfaces of susceptible cells by a process of adhesion from either physical or chemical attraction. They may be assisted in this by their motility due to flagellae and pili and then drawn within the cell cytoplasm by cellular microvilli and philopodia (Rodgers & Oldhman, 1984). They may be destroyed by the body's defensive processes. More often they overcome these, probably by releasing secretory enzymes or toxins, and proceed to multiply intracellularly. Destruction of the host cell follows with release of bacteria, toxic products and further cellular invasion then occurs (Rodgers & Oldhman, 1984). More recent studies show that when *L. pneumophila* were grown intracellularly in protozoa , they expressed an increased capacity to attack tissue culture cells (Cirillo *et al.*, 1994).

The lung is the main organ showing pathological damage, though spread to other parts of the body can occur, evidenced by bacteraemia and by the appearance of soluble serogroup antigen in serum and urine. This supports the hypothesis that bacterial toxins can account for fever and other manifestations during the illness (Bibb *et al.*, 1984). Histologically, pulmonary infiltrates containing neutrophils, macrophages, large amounts of fibrin in alveolar spaces, and septic

vasculitis of small blood vessels have been observed. The histopathology is that of bacterial pneumonia and differs from pneumonia caused by chlamydia, mycoplasma, or viruses (Winn & Myerowitz, 1981). In addition to finding focal bronchopneumonia or lobar patterns of pneumonitis with micro abscesses or abscesses of large size (i.e., ≥cm), pulmonary fibrosis may occur as a long-term sequel with persistence of scar tissue in the lungs, and this contributes to persistent chronic problems with pulmonary function in some patients who survive acute legionnaires' disease (Winn, 1988).

L. pneumophila exoproducts, primarily protease, have been demonstrated to have significant inhibitory effects on normal polymorphonuclear (PMN) function and may play an important contributory role in the pathogenesis of legionella disease (Sahney *et al.*, 1990). Though early attempts to associate the severity of Legionella infections with bacterial endotoxin did not succeed in identifying a specific toxic lipopolysaccharide, it is now becoming apparent that several bacterial components may play a synergistic role in both pathogenesis and immunity (Wong & Feeley, 1983). Moffat *et al.* (1994) found that an isogenic Zn-metaloprotease- deficient mutant of *L. pneumophila* appeared to be attenuated in virulence in a guinea-pig model of infection. Proteolytic activity by the organisms has been reported, and proteases may share in the induction of lung lesions (Muller, 1984).

Surface antigens providing serogroup specificity can have endotoxic properties and experimentally can cause pyrogenicity, weight-loss, skin reactions and sometimes death. They can also function as adjuvants by stimulating delayed hypersensitivity as well as antibody booster response (Muller, 1984). At autopsy, varying patterns and

degrees of consolidation are found. Most species produce a severe confluent lobar pneumonia with or without abscesses (Blackmon 1981).

1.1.5 Legionellae exoproducts and toxins

L. pneumophila produces a haemolysin (Bain & Rasheed, 1979), several proteases (Thompson *et al.*, 1981), other exoenzymes (Thorpe &Miller, 1981), an endotoxin (Wong *et al.*, 1979), acid phosphatase, which blocks super oxide-anion production by PMLN (Saha *et al.*, 1985), and a low molecular weight (< 1000 Daltons) heat-stable peptide cytotoxin (Friedman *et al.*, 1980). The cytotoxin killed embryonated hen's eggs and some types of tissue culture cells. PMNLs treated with the cytotoxin show reduced O_2 consumption and NADPH turnover but remained viable and were still capable of phagocytic activity. The mechanism of inhibition does not appear to operate through inhibition of the HMPS, and a role for this toxin in intracellular survival has yet to be demonstrated (Halablab et al., 1990 b). Six extracellular proteases were isolated from *L. pneumophila* (Conlan *et al.*, 1986). Ten strains of *L. pneumophila* produced detectable levels of extracellular protease, phosphatase, lipase, DNase, RNase, and ß-lactamase activity, weak starch hydrolysis was also demonstrated for all strains (Thorpe & Miller, 1981). Legionellae do not produce detectable siderphore, but a periplasmic iron reductase activity has been reported (Dowling *et al.*, 1992).

Administration of tissue-destructive protease (TDP) into the lungs of guinea pigs produced pulmonary lesions which pathologically were similar to those observed in clinical and experimental Legionnaires' disease. Intranasal administration of 20µg of TDP was sufficient to cause

death in experimental animals (Baskerville *et al.*, 1986). Another protease, referred to as major secretory protein (MSP), is a product of *L. pneumophila* that induces protective cell-mediated immunity in guinea-pigs (Blander & Horwitz, 1993). It is a peptide of 13 kD that is methanol-soluble, heat-stable and sensitive to proteolytic enzymes (Friedman *et al.*, 1980).

Live *L. pneumophila* may contain another cell-bound toxin which, at high multiplicities of infection, is capable of causing extensive cytopathic effects on macrophages. Toxicity to macrophages is an important characteristic that may be used to distinguish between virulent and avirulent strains of *L. pneumophila*. Incubating at 4°C or treatment by cytochalasin D inhibits this toxic activity (Caparon & Johnson, 1988). Extracellular *L. pneumophila* has been reported to mediate toxicity for guinea pig peritoneal macrophages and J774 mouse macrophages (Husman & Johnson, 1994). Protein exotoxins of low molecular weight have been extracted from bacterial culture filtrates or from acidified extracts of sonicated bacterial cells. They are lethal to mice, chick embryos and cultured cells including macrophages, interfering with host cell oxidative metabolism and suppressing phagocytosis (Wong & Feeley, 1983).

Total exoproducts from a wild-type strain of *L. pneumophila* markedly inhibited human polymorphonuclear leukocyte (PMN) superoxide anion generation , at sub-lethal concentration, in response to four stimuli, zymosan activated particles (ZAP), phorbol myristate acetate (PMA), calcium ionophore (A 22138), and formyl-methionyl-leucyl-phenylalanine (*f*MLP) (Shaney *et al.*, 1990).

The oxygen consumption and nicotinamide adenine dinucleotide phosphate (NADPH)-turnover during phagocytosis were unimpaired in

PMNL pre-incubated with *L. pneumophila* P-2 toxin at concentrations that did not affect viability or phagocytosis. In toxin-treated PMNL, resting and methylene blue-stimulated activities of the hexose-monophosphate shunt (HMS) were found to be normal. Also the toxin did not directly affect this pathway's capacity to generate NADPH. This toxin selectively impaired activation of the phagocyte superoxide-generation complex without affecting the functional integrity of components of the complex (Friedman *et al.*, 1982).

The complex of the toxin-treated cells could be fully activated by several soluble stimuli indicated that the toxin did not interact directly with a component of the superoxide generation complex to impair its function and that toxin pre-treatment did not lead to exhaustion of the complex. Rather the toxin probably interfered with mechanisms by which particulate stimuli and the Ca^{++} ionophore promote activation of the complex. *L. pneumophila* toxin impairs both depolarisation and HMS activation after exposure to Ca^{++}, but did not affect either the depolarisation of the plasma membrane or HMS activation induced by several other activators (Lochner *et al.*, 1982).

1.1.6 Outer membrane proteins

L. pneumophila outer membranes contain a major protein (MOMP) of 28 kD, which is exposed at the cell surface along with LPS. Legionella LPS appears to be a novel type, which is major antigen recognised by sera from patients who have recovered from legionnaire's disease (Gabay & Horwitz, 1985). In contrast, very few antibodies are produced against the other major component of the cell surface, the legionella MOMP. MOMP is a protein with properties similar to those of *E. coli* outer membrane proteins. The MOMP channel exhibits

selectivity for cations and is voltage-independent. The structure of the Legionella protein appears different from that of the other Gram-negative bacteria (Hindahl & Barbara, 1984). Indeed, as in the case of *Chlamydia psittaci* and *Chlamydia trachomatis* (Newhall & Jones, 1983), it contains interchain disulphide linkages. The disulphide bridges are present in the porin of both *L. pneumophila* and *Chlamydia* sp., two obligate intracellular pathogens, but not in *E.coli* (Nikaido & Nakae, 1979), suggesting the possibility that disulphide bridges may play some special roles in intracellular parasitism (Hindahl & Barbara, 1984 & Gabay & Horwitz, 1985).

1.1.7 Plasmids

Comparison of organisms from epidemic and endemic sources by multilocus enzyme electrophoresis, typing with monoclonal antibodies and plasmid analysis may allow separation of those strains causing disease from others and aid in the selection of sites for disinfection (Edelstein, 1986). Plasmids have a capacity to transfer between organisms not only of similar but of differing bacterial species, a factor which may affect the pathogenic potential of the recipient. They have been found in both clinical and environmental isolates of legionellae though more often in the latter (Brown *et al.*, 1982). The broad host-range plasmids of incompatibility groups P and W have been shown to be transferred to Legionella (Chen *et al.*,1984). Several transposon delivery systems, including those based on thermosensitive replication origins of these plasmids and chimaeric "suicide" plasmids constructed from their origins of transfer plus narrow host-range replicons were tested. Only one (pSUP1021) of several vehicles was found to yield transposon mutants of Legionella at a detectable frequency (Tully *et al.*,

1992a). Transposon Tn5 mutants of *L. pneumophila* showed considerable loss of virulence in an authentic animal model of the pneumonia (Tully *et al.*, 1992b).

Plasmid and pepdide analysis, by providing additional means of distinguishing strains from different sources, is useful epidemiologically in the study of outbreaks (Brown *et al.*, 1985). Plasmid DNA has not, so far, been correlated with any adverse phenotypic traits among these organisms (Aye *et al.*, 1981). One or more cryptic plasmids are present in many, but not all, strains of most species (Mikesell*et al.*, 1981). Neither pathogenicity, antibiotic resistance, nor any other cell function was correlated with the presence of any plasmid (Mikesell *et al.*, 1981). Motile strains of all Legionellae species possess cross-reacting H antigens. O antigens are largely unique for each species although some cross-reactions have been demonstrated. More than one O antigen group has been presented in *L. pneumophila* and *L. longbeachae* (Bibb *et al.*, 1981).

1.1.8 Intra-cellular living

Since Legionellaceae multiply extracellulary on complex laboratory media, they are classified as facultative intracellular bacteria (Dowling *et al.*, 1992). Pulmonary alveolar macrophages are the first line of defence against organisms invading the lungs. These are followed by polymorphonuclear neutrophil leukocytes (PMNL) (Katz & Hashemi, 1982). Avirulent strains appear to resist phagocytosis and when they are phagocytosed, do not replicate within phagocytic cells. The virulent organisms form a distinctive ribosome-lined replicative phagosome and inhibit phagosome-lysosome fusion; avirulent strains neither form a

distinctive phagosome nor inhibit phagosome-lysosome fusion (Horwitz, 1987).

Phagocytosis of *L. pneumophila* occurs by a novel mechanism, in which engulfment of the organism takes place within a pseudopodial coil. A phagocyte pseudopodium coils around the bacterium as the organism is internalised. Human monocytes, alveolar macrophages and polymorphonuclear leukocytes all phagocytose *L. pneumophila* by this unusual process, termed "coiling phagocytosis" and these leukocytes phagocytose not only live *L. pneumophila* in this way, but also formalin-killed, glutaraldehyde-killed, and heat-killed *L. pneumophila*. Treatment of *L. pneumophila* with high-titre anti-*L. pneumophila* antibody abolishes coiling phagocytosis. In contrast , either before or after such treatment , other organisms are phagocytosed by conventional phagocytosis. There is one possibility that a surface component of *L. pneumophila* mediates the unusual response by the phagocyte (Horwitz, 1984). More recently, Rechnitzer & Blom (1989) have reported that the phenomenon of coiling phagocytosis was independent of bacterial virulence and it occurred whether the bacteria were heat-killed or whether they were pre-opsonised with specific antibody .

Legionella contains a surface component on which resides the serogroup-specific antigen of *L. pneumophila*. Johnson *et al.*, (1979) reported that antibody to this fraction was required for phagocytosis of the organism by the mammalian phagocytes (Johnson *et al.*, 1979). Live *L. pneumophila* induced the formation of a unique type of phagosome studded with host-cell ribosomes. Formation of this vacuole entails a complex sequences of cytoplasmic events that take place during the first 4-8 h after engulfment and involves host-cell smooth vesicles,

mitochondria and ribosomes (Horwitz, 1983). The phagosome does not fuse with the primary or secondary lysosomes of the host cell. This inhibition is partly overcome by coating bacteria with antibody or activating the monocytes (Horwitz, 1983). Formalin-killed organisms are rapidly degraded within the phagosome after fusing with host cell granules(Halablab et al., 1990 b). Live organisms maintain a significantly higher pH in the phagocytic vacuoles than do formalin-killed organisms (Horwitz, 1984).

Ingested intracellular pathogens, including *L. pneumophila,* can resist the antimicrobial systems which include the reactive oxygen metabolites generated by the phagocytosis-induced respiratory burst and granule constituents released into the phagocytes during degranulation. Antimicrobial granule components include the iron-chelating protein lactoferrin, hydrogen peroxide and myeloperoxidase, which have multiple roles in bacterial killing together with various acid hydrolases (Halablab et al., 1990 b).

Gamma interferon-activated macrophages inhibit the intracellular proliferation of *L. pneumophila* by reactive oxygen intermediates and reactive nitrogen intermediate-independent mechanisms and partially by nutritionally-dependent mechanisms (Gebran *et al.,* 1994). One inhibitor of glucose metabolism (2-deoxy-glucose), inhibits intracellular multiplication and promotes intracellular killing of *L. pneumophila.* It is assumed that 2dG inhibits the appearance of legionella-associating vacuoles, but the effect of 2dG of accelerating intracellular killing was observed in macrophages of C57 BL/6 mice (Ogawa *et al.,* 1994). The inhibitor of microfilament-dependent phagocytosis cytochalasin D and methylamine (an inhibitor of adsorptive pinocytosis)-sensitive mechanisms may be essential for intracellular multiplication of *L.*

pneumophila in U937 monocytes (King *et al.*, 1991). A global stress protein of *L. pneumophila* was expressed in the intracellular environment of the phagocytic cell and by various *in vitro* stress stimuli, which was controlled by two separately regulated promoters (Kwaik & Engleberg, 1994).

1.1.9 Legionellosis

The term legionellosis has been used by many to indicate all infections caused by *Legionella* . Legionellosis has commonly been recognised as a form of pneumonia. Strains of all species have been directly or, in one case, indirectly implicated in human pneumonia (Brenner *et al.*, 1979). Pneumonia caused by *L. pneumophila,* is commonly referred to as Legionnaires' disease (LD) (Fraser *et al.*, 1977) and pneumonia due to *L. micdadei* is commonly referred to as Pittsburgh pneumonia (Pasculie *et al.*, 1980). At least one *Legionella* species, *L. pneumophila*, can also cause a mild, nonpneumonic, febrile disease termed Pontiac fever (Glick *et al.*, 1978). Although, it is possible that any species of Legionella may cause human disease under the appropriate conditions, most of them have as yet been isolated only from the environmental reservoir (Dowling *et al.*, 1992). A strain of *L. micdadei* was isolated from a guinea pig inoculated with the blood of a soldier, and several strains of *L. pneumophila* were isolated from the blood of patients with Legionnaires' disease (Edelstein *et al.*, 1979). Approximately 85% of cases of Legionellosis are due to *L. pneumophila* , with about 50% of all disease due to *L. pneumophila* serogroup 1 and 10% to serogroup 6. The remainder of cases is due to other serogroups of *L. pneumophila, L. micdadei,* and a number of other species. It is not clear whether this distribution is due to greater

inherent virulence of the more frequently isolated strains or, more likely to the prevalence of the various strains in the environment. In both sporadic incidents and outbreaks of Legionella infection the predominant clinical feature has mostly been a form of pneumonia, often innocuous initially but on occasion developing into a severe and, at times, fatal illness (Hood *et al.,* 1994). Legionellae infections have been recognised in all continents and outbreaks have been reported from several countries(Dowling *et al.,* 1992).

The earliest symptoms of Legionnaires' disease typically include a 'rundown' feeling, muscle aches, and a slight headache. During the first day, patients commonly experience a rapid onset of dry cough and elevated temperature (102°F-104°F or higher is not uncommon) with chills. Abdominal pain and gastrointestinal symptoms (eg, nausea, vomiting, and diarrhoea) occur in many patients. Chest X-ray typically shows patchy infiltrates at the onset that may progress to five-lobe consolidation (Winn, 1988). Pyrexial, often fatal, illness may result, with fibrinopurulent peritonitis containing many organisms in exudates. Other organs could also be affected, with necrotic suppurative foci present (Chandler *et al.,* 1979).

Infiltrates are bilateral in two thirds of patients with pneumonia, and abscess cavities may be present, particularly in immunocompromised patients. Laboratory findings commonly include, in varying combinations, a moderate leukocytosis with a left shift, proteinuria, hyponatemia, azotemia, elevated serum glutamic oxaloacetic transaminase, and high erythrocyte sedimentation rate (Koneman *et al.,* 1992).

In recent years, the clinical spectrum of legionellosis has been expanded. The illness may involve essentially any organ system of the

body, with or without pneumonia. Examples of selective manifestations of extra-pulmonary involvement follow. Bacteramia has been reported, but data on its frequency are lacking (Edelstein *et al.,* 1987). Nearly half the patients with Legionnaires' disease show central nervous system manifestations such as headache, lethargy, confusion, stupor, and other less frequent manifestations, including ataxia, coma, and seizures (Johnson *et al.,* 1981). Other patients have had focal signs and symptoms suggesting a brain abscess or encephalitis mimicking herpes encephalitis. *Legionella* species have been detected by the direct fluorescent antibody test (DFA) in cerebrospinal fluid of one patient and in brain tissue of another who died of Legionellosis (Potasman *et al.,* 1990).

A painful, nonpruritic, macular rash, limited to the pretibial surfaces of the legs, has been reported; however, dermal manifestations are uncommon (Helms *et al.,* 1981). *L. pneumophila* serogroup 1 has been demonstrated in lymph nodes, spleen, kidney, and bone marrow and has been documented in acute myocarditis (Winn, 1981), prosthetic valve endocarditis , pericarditis , and haemodialysis fistula infection (Koneman *et al.,* 1992). *L. pneumophila* serogroup 3 was isolated from a pre-rectal abscess, mixed with multiple species of anaerobic bacteria (Arnow ., 1982). *L. pneumophila* serogroup 4 was shown by DFA in lesions of acute pyelonephritis in a patient who had both pneumonia and pyelonephritis associated with this organism which originated from a cutaneous leg abscess (Dorman *et al.,* 1980).

At least one legionella species , *L. pneumophila* , can also cause a mild, non-pneumonic, febrile disease termed Pontiac fever, with short duration, elevated temperature, myalgia, and headache, few or no respiratory findings and no pneumonia. (Glick *et al.,* 1978, Harrison &

Taylor, 1988). The factors which determine whether Legionellae will cause legionnaire's disease or Pontiac fever remain unclear.The pathogenesis of Pontiac fever is obscure, and viable Legionellae may not be required for its production. Eickhoff (1979) suggested that Pontiac fever results from exposure to dead legionellae because the ilness is often reported as a point-source outbreak with high attack rates but of low severity. Fields *et al.,* (1990) proposed that Pontiac fever is produced by Legionellae strains that are unable to multiply in human cells. However, this observation is inconsistent with the recovery of live legionella, sometime in high concentration, from environmental sites during each of the reported investigations (Fraser *et al.*, 1977; Friedman *et al.*, 1980). Another hypothesis is that Pontiac fever results from hypersensitivity to amoebae (Rowbatham, 1980). The competence of the host immune system has a role in determining which form of legionellosis ensues after exposure to the bacterium. Immunocompromised hosts are particularly susceptible to legionellosis. The role of host factors is also supported by two reported cases of legionellosis, one pneumonic and the other non-pneumonic, which occurred in two individuals who received a common-source exposure while cleaning a cooling tower (Girod *et al.*, 1982).

1.1.10 Laboratory diagnosis

Legionnaires' disease is not a common form of pneumonia and probably comprises less than 5% of acute pneumonia cases requiring hospital admission. Careful analysis of patient characteristics (age, sex, immune status), clinical features, chest radiography and the effect of antibiotics already administered, can often indicate or exclude a preliminary clinical diagnosis of Legionnaires' disease (Bartlett *et al.*,

1986). Therefore it is not necessary that laboratories accept requests to culture Legionella systematically on all patients with chest infection. In patients with suspected Legionella infection, specimens are taken to demonstrate bacteria or antibody. Clinical material for examination may be sputum, tracheal and bronchial aspirates or washing, pleural exudate, lung tissues, blood for culture, urine and serum taken in life or at post mortem. The patients who are treated with appropriate antibiotics within seven days of onset, will recover in most instances even if they are immunocompromised (Dournon, 1988).

As Legionnaires' disease (LD) is primarily a pneumonia, specimens originating from the lower respiratory tract are the most appropriate. However, severe forms of the disease are often associated with bacteraemia and legionella can sometimes be demonstrated in various sites, especially from specimens taken post-mortem. Suitable specimens for culture can be provided from the lower respiratory tract (Harrison *et al.,* 1990). Rowbotham (1983) described the recovery of viable *L. pneumophila* via the amoeba enrichment method. The above method being used successfully in five of six cases of Legionnaires' disease in which *L. pneumophila* was isolated.

Direct fluorescent antibody (DFA) test is highly specific and sensitive for the serological identification of Legionella at both the species and serogroup level. It is used both to determine whether Legionellae are present in clinical and environmental samples, and as a rapid presumptive diagnostic test for the identification of Legionellae (Cherry *et al.,* 1978; Broome & Fraser, 1979).

The DFA test using polyclonal antiserum which is suitably absorbed or with specific monoclonal antibodies is sensitive for the serological identification of Legionellae at both species and serogroup

level. Only ~15% of DFA-positive environmental samples prove culturally positive for Legionellae, but false negative results have not been obtained (Fliermans *et al.,* 1981). Detailed instructions for DFA are given by Cherry, and the test is reported to be more sensitive than the culture method (Cherry & McKinney, 1979). An alternative to DFA is a commercially available DNA probe (Gen-probe) for the detection of Legionella species.

The indirect fluorescent antibody (IFA) test is the standard serological test used to diagnose legionellosis. Human test sera are reacted with heat-killed, whole legionellae cells. A 4-fold or greater rise in titre to $\geq$ 128 in paired acute and convalescent-phase sera is considered definitive evidence of legionellosis. In a single serum, titres of 256 or higher are considered "presumptive" evidence of infection. The details of IFA are given by Wilkinson *et al.,* (1979), although most laboratories use the method of Taylor (Taylor & Fikes, 1980) for IFA.

A slide agglutination test can also be used for the rapid identification of the Legionellae (Wilkinson & Fikes, 1980). This test is specially useful for laboratories that do not have fluorescent antibody capability. Motile Legionellae apparently possess cross-reacting flagellar antigens (Thomason *et al.,* 1979). This property has not been used as a diagnostic tool because flagella are rarely seen in respiratory tract specimens and because fluorescent antibody conjugates prepared from purified flagella preparations are not yet available (Mitchell, 1991). While cultivation of Legionella is the standard method of detection, it has not always been possible to isolate viable bacteria from suspected water samples. Some pathogenic bacteria may enter a state in which they retain viability but can not readily be cultured, which undermines confidence in our ability to isolate this organisms from clinical material

or the environment. Several groups have reported detection of Legionellae by using DNA probes. Grimont *et al.*, (1985) selected and pooled endonuclease restriction fragments of chromosomal DNA from an isolate of *L. pneumophila*, radiolabelled the fragments, and used the pooled fragments to detect legionella which had been seeded onto nitrocellulose membranes and lysed *in situ*. Engleberg *et al.*, (1986) reported a sensitivity limit of $5x10^4$ organisms using another DNA probe to detect legionellae in murine lung tissue homogenates. The development of the polymerase chain reaction (PCR) (Saiki *et al.*, 1985) and the incorporation of the thermostable *taq* DNA polymerase into this process (Saiki *et al.*, 1988) permitted the automated amplification of target DNA sequences. Starnbach *et al.*, (1989) have described a DNA amplification and probe system which was specific for *L. pneumophila*. They detected as few as 35 cfu of *L. pneumophila*, using their primer set and the detection probe. Using Starnbach's system and probe Jaulhac *et al.*, (1992), detected 22 of 30 (75%) Legionella strains.

1.1.11 Sensitivity to antibiotics

The pulmonary alveolar macrophages are the lower respiratory tract's first line of defences against legionella infection. After ingestion by coiling phagocytosis, legionella often resist intra-leukocytic killing. Multiplying within the host cell, the organisms rupture and kill the phagocyte, releasing legionellae, which then infect other macrophages. Because of this subversion of host defence, the ideal antibiotic for treatment of legionnaires' disease should enter the phagocyte and kill intracellular organisms. The early studies showed that Legionella species are susceptible *in vitro* to a variety of anti-microbial agents. Rifampicin

was the most active drug (lowest MIC) tested against these organisms, but they were susceptible *in vitro* to erythromycin, aminoglycoside, sulphamethoxazol-trimethoprim, chloramphenicol, cephoxitin, doxicyclin, and minacyclin (Thornsberry *et al.*, 1978), with the exception of *L. micdadei,* which produce some level of ß-lactamase activity when assayed with the chromogenic cephalosporin test. The ß-lactamase is assumed to be chromosomal rather than of plasmid origin and is more active on cephalosprins than on penicillins (Thornsberry *et al.*, 1978). Erythromycin and rifampcin were the most effective drugs in preventing mortality during *in vivo* egg and animal (Lewis & Knight, 1977). Erythromycin enters macrophages and monocytes but it is not bactericidal for legionella and treatment failure is well documented (Ramirez *et al.*, 1994). Aminoglycosides were effective in hen's egg studies, but not in animal or human studies; ß-lactam antibiotics were not effective in any *in vitro* studies (Lewis & Knight, 1977; Fraser *et al.*, 1977). The correlation between *in vitro* susceptibility and clinical efficacy of antibiotics for Legionellae is poor (Kirby *et al.*, 1980). Cordes & Fraser (1980) suggested cotrimoxazole or doxicyline as the drugs of second choice. Some studies also have found the combination of rifampin and erythromycin suitable when patients respond poorly to erythromycin alone.

A limited number of antibiotics have been shown to be active in animal and cell models of infection and in patients. These include the new macrolides (Arthromycin), rifampicin and a new fluoroquinolone, ciporfloxacin (Vilde *et al.*, 1986). The combination of ampicillin and the ß-lactamase inhibitor sulbactam has been used as experimental therapy for community acquired pneumonia (Castellano, 1988). Ampicillin/sulbactam has a good *in vitro* activity against pathogens

causing community acquired pneumonia, including *Streptococcus pneumonia*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*. The results of *in vitro* studies achieved by Ramirez *et al.*, (1994) suggest that the combination of ampicillin and the ß-lactamase inhibitor sulbactam is able to enter the cells of human macrophage-like cell line (U-937). In addition, bactericidal activity of ampicillin /sulbactam against intracellular *L. pneumophila* was significantly ($p< 0.05$) superior to erythromycin in this model.

The conventional *in vitro* means of appraisal of antibiotic activity, such as minimum inhibitory concentration or minimum bactericidal concentration, are not predictive of the *in vivo* efficacy of antibiotics given in LD (Legionnaire's disease). First, *Legionellae* are facultative intracellular parasites (Horwitz & Silverstein, 1980), and thus some antibiotics that are active *in vitro* are not active *in vivo* because their cellular permeability is poor, *e.g.* beta-lactam antibiotics (Vilde *et al.*, 1986). Second, the media used to culture Legionella inhibit the activity of some antibiotics (Edelstein & Meyer, 1980). Because of these two obstacles, and also the relative rarity of LD and the lack of prospective studies, the optimal antibiotic treatment of LD has not been established. More recent studies (Smith *et al.*, 1994) have suggested that amoxicillin/ clavulanic acid and ticarcilin/ clavulanic acid may be worthy of clinical trial.

1.2 THE ENVIRONMENTAL SOURCE OF LEGIONELLAE

1.2.1 Natural Habitats

Most species of Legionellae have been isolated from the environment. Natural environment isolates are from streams or lakes, often thermally polluted water, or from moist soil adjacent to a body of water (Fliermans *et al.,* 1981). Urban environmental isolates were made from water collected from air conditioning cooling towers and evaporative condensers (Orrison *et al.*, 1981).

Recently isolates have been made from water collected from hospital showers (Tobin, *et al.,* 1981), and from nebulizers (Gorman *et al.*, 1980). *L. pneumophila* was first isolated from the natural environment (Morris *et al.,* 1979), in water of a small creek and from soil of the bank of the river Jordan (Fillerman *et al.,* 1981). Since that time, Legionella species have been found in natural waters from around the world and are widespread in lakes, ponds, and streams.

The Legionella concentration in warm waters (30°C -45°C) appears to be higher, compared with levels in water at cooler temperatures (Wadowsky *et al.,* 1985). Legionella has been recovered from marine waters and from epiphytes in trees (Ortiz-Rogue & Hazen, 1987). The soil has been implicated epidemiologically for Legionella in one of the earlier outbreaks of Legionaries' disease (Broome & Fraser, 1979). However, there have been few reports of attempts to isolate Legionella species from soil, although *L. pneumophila* and *L. bozemanii* have been isolated from samples of wet soil (Morris *et al.,* 1979). More recently , *L. pneumophila*, *L. longbeachae*, and *L. micdadei* were isolated from soil in Australia (Koneman *et al.,* 1992). According to this report, gardening in damp soil, rather than exposure to water

contaminated with *L. longbeachae,* appeared to be the major environmental risk factor associated with Legionellosis . Legionellae (*L. pneumophila, L. longbeachae*) were found in many samples of composted plant matter obtained from home gardeners and from facilities which undertook bulk composting (Hughes & Steele, 1994).

1.2.2 Man-made (Artificial) aquatic habitats

There is ample evidence to show that Legionellae are ubiquitous in man-made water systems, both in the absence as well as in the presence of clinically demonstrated legionellosis. Legionella species are frequently present in water in cooling towers and have been found in tap water , shower water, hot water tanks, the inside of shower heads, rubber gaskets, and metal surfaces within plumbing systems used for domestic potable water supplies. Potable water has been implicated as the most likely source of the organism in many epidemics and sporadic cases of legionellosis that were not epidemiologically associated with cooling towers or faulty air conditioning/heat exchange systems (Arnow , 1982). Whirlpool spas have also been implicated in cases of legionellosis. When whirlpools are inadequately chlorinated, conditions may favour the growth of legionellae and organisms may be aerosolized and inhaled (Fallon & Rowbotham, 1990).

In 1990, an outbreak of community-acquired Legionnaires' disease, involving 33 persons, that was associated with a grocery store moist using machine was reported. The moist using machine continuously generated a tap water aerosol in respirable droplets over the produce display (Koneman *et al.,* 1992). Although Legionellae have been isolated from shower heads and shower water on many occasions, the epidemiological evidence linking bathing or showering

in such water with legionellosis is rather weak. Supportive evidence reported from an outbreak investigation of nosocomial Legionnaires' disease at a hospital strengthened the hypothesis that aerosolized shower water can serve as the vehicle for spread of *L. pneumophila* to patients (Muder *et al.*, 1986; Breiman, 1990).

Legionellosis has also been specifically linked to the use of tap water to clean medication nebulizers and other respiratory therapy equipment. The use of tap water contaminated with *L. pneumophila* to wash medication nebulizers is documented as a major factor that led to an outbreak of nosocomial Legionnaires' disease in patients with chronic obstructive pulmonary disease (Mastro, 1991). Legionellae appear to survive the usual chlorination procedures of municipal water treatment facilities and thus, not unexpectedly, may be present in potable water supplied to homes, apartment buildings, hotels, hospitals, and other buildings. Potable hot water, especially if it does not exceed 55°C, has contained heavy concentrations of Legionella in some instances. In addition to water temperature, the construction of the plumbing system also seems to play an important role; for example, the presence of certain kinds of resins in gaskets, the presence of dead ends or cul-de-sacs, where there is stasis, obstruction, or stagnation in water flow, and the presence of biofilms or slime layers containing other commensal bacteria, protozoa, and algae on the surface of pipes may favour the presence of Legionellae (Koneman *et al.*, 1992).

Legionellaceae have been found in numerous natural aquatic habitats and man-made water systems. *L. pneumophila* survives in filter sterilized distilled or tap waters and autoclaved river waters for many months in progressively decreasing numbers (Anand *et al.*, 1983). It multiplies in places where photosynthesis cannot take place (Oritz-

Rogue & hazen, 1982), such as plumbing systems in buildings (Arnow, 1982). Also *Legionellas* have been found frequently in cooling water systems (Christiansen *et al.*, 1984); other water systems which release water droplets into the air might be considered as potential sources of infection. There may be a lack of amplification factors, the most important of which is a suitable temperature. Although *L. pneumophila* has been isolated from water at temperatures ranging from 5.7 to 63°C, it probably only multiplies actively between 20 -45°C (Fliermans *et al.*, 1981).

1.2.3 Legionella in animals

All Legionella species are potentially pathogenic for humans. There is no information on whether they cause natural infections in animals, but to date Legionellae have not been isolated from naturally infected animals even though more than 400 control guinea pigs have been cultured. Laboratory infections have been successfully induced in embryonated hen's egg and in guinea pigs, gerbils, hamsters, rats, rabbits, and mice. Attempts to infect quail, pigeons, and chickens have not been successful (Brenner *et al.*, 1984).

1.2.4 Legionellae in environmental and domestic water supplies

Legionellae are prevalent in domestic and environmental waters, and legionellosis is transmitted via aerosols generated from these reservoirs (Fiesher-Hoch *et al.*, 1981). Identification of reservoirs requires the detection of *Legionellae* or their products, and culture is the most specific procedure widely available. Culture isolation of *Legionellae* from environmental water samples is acknowledged as the method of choice, though some *Legionellae* spp. or isolates only grow poorly on

artificial media. The results of culture can easily be quantified as cfu,and isolates can be stored and used for exact identification and epidemiological studies (Maiwald *et al.,* 1994). Barbaree *et al.,* (1988) isolated legionellae by water samples treatment including, sample concentration (filtration or centrifugation), and direct inoculation (before and after treatment with heat or acid) on BCYE medium. Sanden *et al.,* (1992) found that water sample incubation markedly improved the sensitivity of culture method for *Legionellae* . Although it is acknowledged that cultural detection is the method of choice for clinical samples its sensitivity has been reported to be 65 - 80% when culture was compared with other available methods (Edelstein *et al.,* 1987).

The isolation of Legionella on culture media is often hampered by their fastidious growth requirements, long incubation period and over growth by other micro-organisms. The problems with cultural detection include contamination with other microorganisms, which significantly inhibit *Legionellae* (Maiwald *et al.,* 1994), and the presence of viable but non-culturable *Legionellae* in some environmental samples (Hussong *et al.,* 1987).

Control of legionellosis however, requires rapid, sensitive and reliable methods to detect the organism in both environmental samples and in clinical specimens. The Polymerase Chain Reaction (PCR) is a method for the sequence specific amplification of minute amounts of nucleic acids. It offers an attractive alternative for the detection of microorganisms which pose problems for environmental culture methods. The principle of the PCR method has been described previously (Loutit & Tompkins, 1993).

Several primer and probe systems for *Legionellae* spp. have been developed. Starnbach *et al.*, (1989) using a randomly-cloned DNA fragment from *L. pneumophila* as target for PCR, could detect as few as 35 cfu of *L. pneumophila* by this method, whereas Mahbubani *et al.*, (1990) chose two primer systems. The first detects the majority of species in the genus *Legionellae* by amplifying part of the gene coding for 5S - ribosomal RNA of *Legionellae* (McDonald & Colwell, 1987). The second set of primers specifically amplifies sequences of the species *L. pneumophila* by utilizing part of the macrophage infectivity potentiator (*mip*) gene (Engleberg *et al.*, 1986). With *Taq* polymerase, DNA amplification of a specific fragment of the macrophage infectivity potentiator (*mip*) gene was used to detect *Legionellae* spp. in bronchoalveolar lavage (BLA) fluid specimens (Jaluhac *et al.*, 1992). The practical value of PCR as a routine technique for the detection of *Legionellae* in environmental samples, however, is not sufficiently assessed. Maiwald *et al.*,(1994) reported the comparison of PCR and culture methods for detection of *Legionellae* from hospital water samples and found both methods equally effective.

The results obtained from PCR methods indicate that PCR method has both advantages and limitations. It is sensitive enough to detect *Legionellae* in filtered 100 ml samples containing less than 1 cfu per ml. As non-viable or non-culturable cells are also detected by PCR, they presumably contributed to the PCR results. However, culture was less affected by rust which can, in some instances, completely inhibit PCR. Introduction of Chelex resin for sample preparation removed the inhibitory effect of rust (Loutit & Tompkins, 1993).

A clear advantage of PCR is its ability to detect *Legionellae* in water samples contaminated by other fast growing bacteria. These bacteria, which may also colonize hospital warm water systems , not only grow faster than *Legionellae*, but also produce bacteriocins which significantly inhibit the growth of *Legionellae* (Gomes-Lus *et al.*, 1992). The non-culturable *Legionellae* could be detected by PCR as well as non-viable *Legionellae* (Maiwald *et al.*, 1994).

1.2.5 The role of biofilms in legionellae maintenance

Biofilms have an important role in the maintenance and survival of micro-organisms in the general environment. They are a major source of Legionellae in both man-made and natural aquatic systems (Marrao *et al.*, 1993; Characklis & Marshall,1990; Rowbotham, 1993). *L. pneumophila* can be detected in greater numbers in the biofilm than in the planktonic phase, although this varies with time and material sampled (Rogers *et al.*, 1994). The biofilm/water interface attracts ciliates, flagellates and amoeba which graze the surface seeking food. The concentration of bacteria within the biofilm provides excellent opportunities for attack by predators, such as protozoa and parasites such as bacteriophages and *Bdellovibrio* species (characklis & Marshall , 1990). Biofilms not only serve to allow the growth of micro-organisms in water system but also protect constituent organisms from antimicrobial substances (Brown & Gilbert, 1993). Copper inhibits both biofilm growth and the inclusion of *L. pneumophila* at different temperatures. Rogers *et al.*, (1994) found that copper ions appear to reduce the planktonic population, and the accumulation of these ions on glass surfaces led to reduced colonization. He suggested the use of copper as a plumbing material to minimize the risk of legionnaires'

disease. Although legionella is an effective parasite of certain species of protozoa, it in turn is susceptible to predation by *Bdellovibrio* (Richardson, 1990) which is a bacterium associated with the biofilm environment (Starr & Seidler, 1971). The predominant protozoa, particularly amoebae, were present in biofilm associated with legionellae were identified as *Rotari neptunia, Hartmanella vermiformis, Hartmanella contabrigiensis* and *Verillifera bacillipedes* (Rogers *et al.,* 1994) Thus, environmental bacteria such as *Legionella, Listeria* and *Vibrio* species have evolved so that they are capable of surviving and multiplying within biofilm predators such as amoeba, offering protection to them in adverse conditions. Indeed resistance to digestion by predatory protozoa may be an evolutionary prerequisite of bacterial pathogenicity and survival mechanisms for bacteria in aquatic environments (King *et al.,* 1988). The greatest number of *L. pneumophila* detected in biofilms developed on the elastomeric materials. The most extensive biofilms occurred on the surface of the elastomeric materials, and this was attributed to the leaching of nutrient from the material, which increased the total viable nutrient in the system (Rogers *et al.,* 1994).

1.3 LEGIONELLA-PROTOZOA INTERACTION

Protozoa are ubiquitous in diverse natural habitats such as fresh and salt water, moist soils and even dry sands. They have a basic role in terrestrial and aquatic environments as predators of bacteria. Protozoa, and especially free-living amoeba, were recognised as the major predators of bacteria in soil, thereby regulating the size of the bacterial population (Weekers *et al.*, 1993). Predation by protozoa has an evident

effect in controlling bacterial populations in soil and the degradation of bacteria undoubtedly contributes to the maintenance of soil fertility (Weekers *et al.,* 1993).

Some bacteria survive in free-living protozoa as endosymbionts. Intracellular symbiosis is common among protozoa and bacterial endosymbionts have been reported in Amoebae The effects of the symbionts on their hosts are generally not known but some confer the ability to produce toxins capable of killing sensitive strains of protozoa ((Hall & Voelz, 1985; Preer & Preer, 1984).

Not all bacteria are suitable for protozoa as food material, some of the non-pigmented enterobacteriaceae, such as *Escherchia coli* and *Klebsiella aerogenes* appear to be excellent food for *A. castelanii*, *Acanthamoeba polyphaga*, and *Hartmanella vermiformis* (Weekers *et al.*, 1993). Some Gram-negative bacteria are able to survive grazing by protozoa, which could be due to the inability of the host cell to ingest and digest internalised bacteria. Some bacteria are able to resist engulfment by defence mechanisms such as toxins, toxic pigments or outer membrane components (Weekers *et al.*, 1993). Normally, non-pathgenic *Legionella* spp. and *Listeria spp.* as well as many other bacteria, serve as food for the secondary part of the microbial food chain. However *Legionella* spp. have succeeded in turning the tables on their predators and growing within the protozoa under appropriate environmental condition (Thi Minh & Muller, 1990).

The discovery that *L. pneumophila* infects and multiplies within some species of free-living amoeba confirmed the ability of bacteria to use normally hostile intracellular environment to survive. Although the interaction between legionellae and amoeba was observed first *in vitro* with axenically grown amoebae (Rowbotham, 1980).

Phagocytosis is not only the means of defense against foreign cells and particles in amoebic protozoa, but also is the principal mode of ingesting food. Indeed, survival and intracellular multiplication of some bacteria in free-living and pathogenic amoeba may well prime these bacteria for virulence. In the aquatic environment, *L. pneumophila* infects and multiplies within a wide range of amoebal hosts (Rowbotham, 1986). *Amoebae* are probably natural host for Legionella and are convenient as an ecological model for laboratory investigation. Intracellular replication of legionellae and inclusion in resistant cysts of amoebae offer a possible survival mechanism in unfavourable conditions and the opportunity of subsequent proliferation. Legionellosis might be the result of an accidental event caused by man in the cycle of aquatic bacteria usually developing in amoebae (Harf & Monteil, 1988).

Particular members of free-living amoebae (*Acanthamoeba* and *Naegleria)* provide either an intracellular niche or extracellular factors for the growth of legionellae (Tyndal & Domingue, 1982). Their possible part in the ecology and epidemiology of Legionellosis was suggested later by Harf & Monteil (1988). Legionellae can remain viable in non-chlorinated tap water for well over a year, but there is no clear indication of how the organisms (which in the laboratory are fastidious in their requirements for growth) are able to survive, or even multiply, in water or mud (Tison *et al.*, 1980).

Among at least 42 Legionella species identified to date, *Legionellae pneumophila* is the primary cause of Legionnaires' disease, and is known to infect five genera of amoeba (Fields, 1993), whereas other species of legionellae have a more specialised host range (Fields *et al.*, 1990). *L. pneumophila* can infect and multiply in *Tetrahymena,*

Hartmanella, *Acanthamoeba* and *Naegleria* spp.(Table 3), which are ubiquitous in moist soil and aquatic environments (Fields *et al.*, 1984).

Some aspects of the *Amoebae*-Legionella relationship were demonstrated first by Rowbotham (1980). He reported that Legionellae were ingested by free-living *Amoebae* (FLA) of the genus *Acanthamoeba* , *in vitro* the infected amoebae were usually rounded up, smaller, and vacuolate; some were lysed. Legionellae in vacuoles were non-motile. There are obvious parallels between *Legionella* uptake and infection of free-living amoebae and similar processes in human phagocytic cells. After ingestion the *Legionellae* within *Amoebae* are confined to vacuoles and under appropriate conditions, multiply to large numbers until the amoeba cells rupture (Henke & Seidel, 1986).

Amoebae isolated directly from river water sedimented contained *L. pneumophila* (Harf & Monfort, 1988). Dry potting soil compost containing soil amoebae (*Vahlkampfia* and *Hartmanella*), have been associated with a number of cases of pneumonia caused by *L. longbeachae* (Steele, 1990). It is suggested that intra-amoebal growth of legionellae is a primary mechanism for the survival and multiplication in natural habitats (Fields *et al.*, 1989).

Following phagocytosis by *Acanthamoeba*, Legionellae multiply within the vacuole, evading the host lysosomal attack and after about 48h a single vesicle of legionellae fills most of the amoebal cell. The final stage of infection is lysis of the host cell and liberation of motile bacteria into the environment (Rowbotham, 1980). After phagocytosis by *Naegleria fowleri* , *L. pneumophila* cells were enclosed in vacuoles enriched by one or more mitochondria opposed to the vacuolar membrane (Horwitz, 1983). Subsequently, ribosomes line the margin of the bacteria-filled vacuole (Newsome *et al.*, 1985).

Table 3 Protozoa supporting growth of Legionella

Category	Species
Amoebae	*Acanthamoeba castellanii*
	A. polyphaga
	A. palestinensis
	A. royreba
	A. culbertsoni
	Naegleria gruberi
	N. fowleri
	N. lovaniensis
	N. jadini
	Hartmanella vermiformis
	H. cantabrigiensis
	Vahlkampfia jugosa
	Echinamoeba exudans
Ciliated protozoa	*Tetrahymena pyriformis*
	T. vorax

Electron micrographs of intra-amoebal legionellae have shown amoeba vesicles of 10 μm diameter, with 90% of the space filled with bacteria, and containing approx. 10^4 bacterial cells (Rowbotham, 1986). The antimicrobial susceptibility and physiological status of intra-amoebal grown *L. pneumophila* was reported by Barker *et al.*, (1992). The recalcitrance of amoeba-grown legionellae might relate to the increased levels of poly- ß–hydroxybutyric acid inclusions (Vandenesh *et al.*, 1990). It is likely that the bacteria are sequestered within the amoeba and that their liberation is dependent upon death of amoeba (Newsome, 1985). Previous studies have suggested the production of a cytotoxic factor by *L. pneumophila* (Friedman *et al.*, 1980). The intra-amoebal legionellae exert a cytopathic effect on the protozoans. It is possible that under growing conditions, amoebae may be able to neutralize the toxin, or components of amoeba culture medium could inhibit the toxicity of legionellae cells (Newsome *et al.*, 1985). Surface proteins of *L. pneumophila* grown in amoebae differ in several respects from those of agar-grown legionellae (Barker *et al.*, 1993).

The morphological changes of Legionellae and its intracellular multiplication are related to the medium in which the amoebae are maintained. When amoebae are maintained in saline, and incubated with legionella, abnormal vacuoles without intracellular multiplication are observed. The ability of legionella to infect protozoa is probably related to bacterial virulence (Rowbotham, 1986). Vacuoles of protozoa infected by *Legionellae* display the same aliment of mitochondria and ribosome-like structures along the vacuole membrane as is seen in infected monocytes. It does not seen likely that the virulence determinants for infection, and multiplication of Legionellae within

amoebae and mammalian phagocytic cells are necessarily the same (Dowling *et al.*, 1992).

1.3.1 Survival of bacteria within amoebae

Legionellae are not the only human pathogens which survive in protozoal hosts. *Listeria monocytogenes* and *Mycobacterium leprae* can infected *A. castellanii* and cause lysis and finally death of the host amoebae cells (Ly & Muller, 1990; Barker *et al.*, 1994).. Environmental strains of the opportunistic mycobacterium, *M. avium*, were reported to survive within *A. castellanii* (Krishna-Prased & Gupta, 1978). *Vibria cholerae* is another example of a bacterial pathogen which survives in free-living amoebae. *V. cholerae* multiplied after ingestion by *Naegleria* and *Acanthamoeba* (Thom *et al.*, 1992). Panikov *et al.*, (1993) showed that *Listeria monocytogenes* could also grow within *T. pyriformis*, the generation time for intracellular growth being14.4 h compared with 7 h for Legionella. Other environmental bacteria including *Edwardsiella* and *Aeromonas salmonicida* are capable of growth in *T. pyriformis* (King *et al.*, 1988).

Although some bacterial species survive ingestion by protozoa, under certain environmental conditions the same organisms are digested. The interaction between legionella and amoeba seems to be temperature-dependent. At low temperatures (22°C) Acanthamoeba may phagocytose and digest *Legionella pneumophila* as food (Anand *et al.*, 1983), whereas at 35°C the bacteria survive and replicate in the amoebae.

Pathogenicity of some strains of Naegleria and Acanthamoeba is directly related to temperature (Griffin, 1972). Mauchline *et al.*, (1993) have reported that in continuous culture at 37°C, *L. pneumophila*

maintains its virulence in animal pathogenicity tests. When the growth temperature was decreased from 37°C to 24°C, virulence was markedly attenuated.

Also at the lower temperature (20°C) *L. pneumophila* disappeared from the culture during the early period of growth of the co-cultivation (viable but non-culturable), but small number of legionellae were grown later under the same experimental conditions (Tyndall & Domingue 1982).

The virulence of *Legionella spp.* is governed by the products of many genes (Miller *et al.*, 1989). The molecules of potential importance to the intracellular survival of *L. pneumophila* have been reviewed (Dowling *et al.*, 1992; Horwitz 1983). Cirillo *et al.*, (1994) reported that amoeba-grown Legionella displayed an increased capacity to enter tissue culture cells. He suggest that *L. pneumophila* grown intracellularly in protozoans may be more infectious. However, the uptake of bacteria by protozoa is an important stage in the infective process. Previous studies have indicated that *L. pneumophila* infects *Hartmanella vermifomis* by a microfilament-independent mechanism because it is not inhibited by cytochalasin D. Conversely, an inhibitor of adsorptive pinocytosis, methylamine, blocked infection of *H. vermiformis*, which suggests that receptor mediated endocytosis is necessary for infection of the amoeba (King *et al.*, 1991). Further works have indicated that entry, not attachment, of virulent *L. pneumophila* is the limiting step in infection of axenically grown *H. vermiformis* (Fields *et al.*, 1993). Receptor-mediated endocytosis has been described as a means of avoiding phagosome-lysosome fusion for *Chlamydia* in mouse fibroblasts. This method of uptake is suspected of directing the organism into vesicles that do not fuse with the lysosomes (Hodinka & Wyrick,

1986). Ultrastructural examination of infected *Hartmanella* revealed single *L. pneumophila* in endosomes immediately after ingestion. In *Hartmanella vermiformis,* the endosome containing legionellae fuses with the endoplasmic reticulum of the host cell and this becomes the site for bacterial multiplication (Fields *et al.,* 1993).

1.3.2 The role of Protozoa in protection of bacteria in environment

Coliform bacteria internalised by protozoa in natural ecosystems may gain protection against external antagonists (King *et al.,* 1988). *Salmonella typhimurium* and *Shigella sonnei* survived ingestion by laboratory strains of *A. castellanii* and *T. pyriformis* and were protected against the activity of free chlorine, since they were cultured from chlorine-treated protozoans well after the time required for 99% inactivation of planktonic bacteria. So, organisms trapped within amoeba could be responsible for the persistence of coliform bacteria in chlorine-treated water supplies (Hudson *et al.,* 1983). The role of free-living amoeba n harbouring environmental bacteria has been confirmed by detecting the presence of heterotrophs, including *Pseudomonas* and *Alkaligenes spp.*, in *Hartmanella* trophozoites and cysts (Tyndall *et al.,* 1991).

Such associations of bacterial pathogens with protozoa have implications in the hospital environment. Eye-wash stations in hospitals were found to contain *L. pneumophila* , *Pseudomonas spp.* and *Acanthamoeba.* The presence of amoeba may have offered protection to the bacteria, and encouraged them to grow (Paszko-kolva *et al.,* 1991). As well as providing physical protection from adverse conditions, growth in amoeba may alter the physiological status of bacteria. *L. pneumophila* grown within *Acanthamoeba* and then freed from the

amoebal host were significantly more resistant to treatment with biocides when compared to bacteria grown on agar (Barker *et al.*, 1992). These results are compatible with the finding that the physiology of Legionella alters as a result of growth within amoebae and produces a radically altered phenotype (Barker *et al.*, 1993). Kwaik *et al.*, (1994) reported that host cell protein synthesis was required for *L. pneumophila* to infect protozoa (*Hartmanella vermiformis*) but not human macrophages (Kwaik *et al.*, 1994). They also found that 33 amoebal proteins were expressed during the infection process; 12 of these were not detected in resting amoeba. The legionella cell membrane changes were studied by Barker *et al.*, (1993) who found that intracellular *L. pneumophila* was capable of expressing new proteins when phagocytosed by the amoeba.

Acanthamoeba containing Legionella encyst, leading to the formation of a precyst or a mature cyst which traps within it motile or non-motile *Legionellae*. Such internalised bacteria may be given unique protection when the protozoa form cysts (Rowbotham, 1986). *Legionella* species have been detected in sewage and concentrations were not appreciably reduced by either primary or secondary treatment processes (Palmer *et al.*, 1993). This finding may be related to the protection provided by Protozoa which are ubiquitous inhabitants of sewage treatment plants. The resistance of amoebal cysts to extremes of temperature (Biddick *et al.*, 1984) and to the effects of biocides (De Jonckheere & Van de Voorde, 1976) may contribute to the difficulties in eradicating *Legionella* from contaminated water system using conventional disinfectant procedures. *A. polyphaga* cysts infected with *L. pneumophila* protected the bacterium from the action of chlorine, and isothiazolone biocides (Kilvington & Price, 1990). The amoeba cysts not

only offer a mechanism for bacteria to evade hostile environmental conditions, but also to spread and colonize new habitats by being blown through the air (Kingston & Warhurst, 1969).

Coliforms and pathogenic bacteria have between 30 and 120 fold increase in resistance to free chlorine residuals when ingested by protozoa (King *et al.*, 1988). *Acanthamoeba* are resistant to 1.25 mg/L (trophozoites) and 50 mg/L (cysts) of free chlorine. *Acanthamoeba* cysts are also resistant to a wide range of biocide chemicals and therefore amoebae cysts containing *Legionellae* survive cooling tower disinfection procedures (Kilvington *et al.*, 1990).

When conditions such as light and temperature become unfavourable for growth of other supporting organisms (Blue-green algae), Amoebae (trophozoite/cyst) provide a reservoir of surviving *Legionellae*. *Legionellae* were recovered from amoebae exposed to 2-10 mg/L (trophozoites) and 50 mg/L (cysts) of free chlorine (Kilvington *et al.*, 1990), while 1 mg/L free chlorine residual has been reported to be 99% bactericidal to all free-living isolates (King *et al.*, 1988). Therefore, chlorination of cooling towers at 15 mg/L for routine cleaning and 50 mg/L to remove cysts has been suggested (Anand *et al.*, 1983). Amoebae-grown legionellae were resistant to CMIT (5-Chloro-*N*-methylisothiazolone), a biocide recommended for water treatment, while this biocide affected the iron-restricted grown legionellae under the same conditions. So iron restriction does not apply within the amoeba (Barker *et al.*, 1992). Polyhexamethylene biguanide (PHMB, an agent routinely used for swimming pool sanitization) was effective not only against the amoeba-grown legionellae but also against the amoeba (Barker *et al.*, 1992).

Acanthamoeba cysts have been recovered from the atmosphere , and colonization of new environments by Legionella through aerial dissemination with cysts may occur (Kingston & Warhurst, 1969). *Legionellae* contained in *Amoebae*, and especially cysts, could survive `environmental temperature extremes, chlorination, and other adverse conditions. Under certain conditions *in vitro,* ingested Legionellae multiply within the vacuoles of free-living amoebae, so that amoebae may serve to amplify the numbers of legionellae in the environment. Furthermore, infected amoebae and amoebal vesicles containing *Legionellae* would be present in the drift from contaminated aquatic environments and provide the vehicles whereby concentrated infectious particles could be delivered to humans (Dowling *et al.*, 1992).

The isolation of *Listeria monocytogenes* from moist surfaces in food processing plant, highlight the problems in cleaning and disinfection (Frank *et al.*, 1990; Nelson 1990) but a contributory factor to the survival of this bacterium in such environments is likely to be through association with adherent biofilms (Ren & Frank 1993). *Listeria* ingested or entrapped within cysts of amoeba would have an increased chance of surviving disinfection procedures. It is widely acknowledged that biofilm bacteria are generally more resistant to treatment with biocides than planktonic bacteria (Gilbert *et al.*, 1990 ; Costerton *et al.*, 1987).

1.3.3 Legionellae-Like Amoebae Pathogens (LLAP)

Legionellae-Like Amoebae Pathogens (LLAPs) are reported to be a group of bacilli that infect and multiply in the cytoplasm of amoebae but have not been grown on routine legionellae media, such as BCYE. LLAPs appear to be of considerable importance, because they cause

pneumonia and induce a serological response (Rowbotham, 1983). The oldest known extant strain of an LLAP is *Sarcobium lyticum*, isolated from Polish soil (Drozanski, 1991). The first human infection case of LLAP was detected in a woman with persistent pneumonia (Rowbotham, 1983). Since 1981, ten strains of LLAP have been isolated from a variety of sources (Rowbotham, 1993). Polymerase chain reaction studies of amplified DNA coding for the 16S rRNA of LLAP3 have shown that this organism is a member of the genus of Legionella (Fry *et al.*, 1991). Based on the 16S rRNA analysis it has been suggested that LLAP3 may be a new group (Figure 42) of Legionellae (Rowbotham, 1993, Springer, 1992) and closely related to *Sarcobium litycum,* an obligate intracellular parasite of various small free-living soil amoeba, first isolated from Polish soil (Drozanski, 1991).

In conclusion, the LLAPs are common and infect a variety of amoebae. They appear to be a new group of legionellae, at least one of which can infect humans. LLAPs may explain the occurrence of Gene-probe-positive specimens from patients with pneumonic illnesses for whom conventional isolation and serological tests for legionellae are negative (Doebbeling *et al.*, 1988). Production of a mature cysts containing motile bacteria has so far only been accomplished with the Legionella-like *Amoebae* Pathogen (LLAP1) (Rowbotham, 1983).

1.4 FREE-LIVING AMOEBAE

Protozoa are important consumers in many aquatic ecosystems including some of economic importance, such as waste water treatment plants. Free-living amoebae previously considered saprophytes, are the most recently discovered protozoa that produce lethal effects in man or domestic animals (Culbertson *et al.*, 1958). Those pathogenic to man

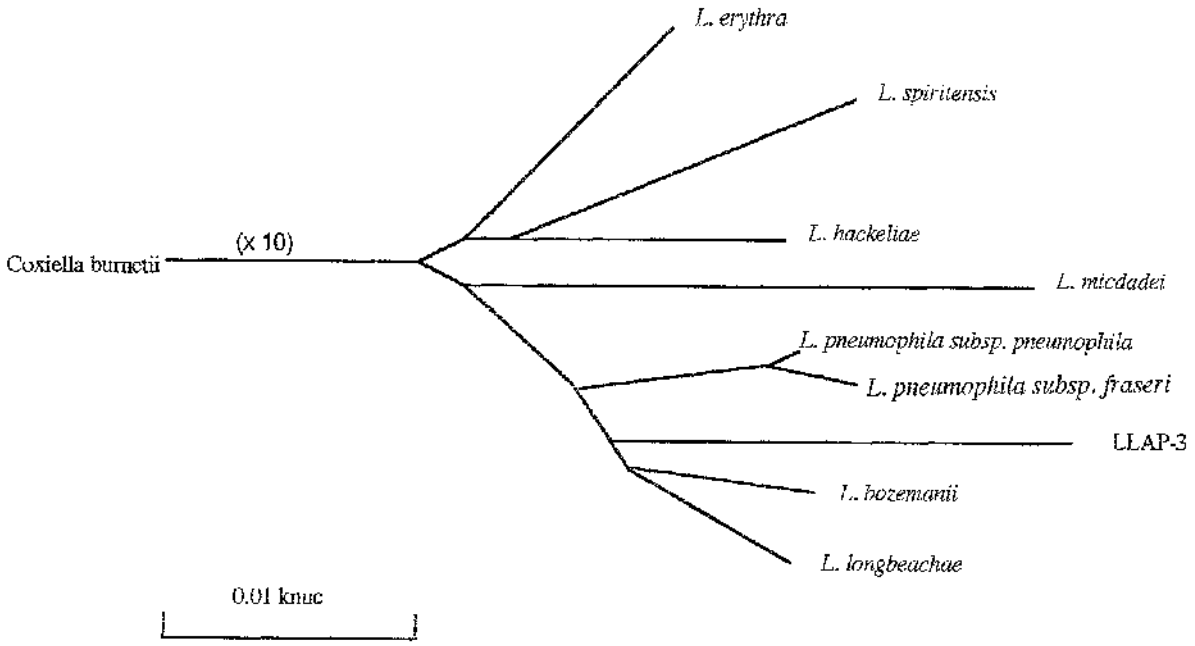


Figure 42 Phylogenic tree showing the position of the legionella -like amoebal pathogen LLA-3 within the *Legionellacea*. The tree is rooted by reference to *Coxiella brnetii*

and laboratory animals belong to two common but very different genera, Naegleria and Acanthamoeba and may be either facultatively endozoic (or parasitic), facultative free-living or opportunistic. They can invade the central nervous system and other organs, causing death or permanent disability (Page, 1988).

Naegleria fowleri, the cause of the rapidly fatal acute primary amoebic meningoencephalitis, has been proved to be responsible for some human fatalities. It occurs in artificially heated waters such as swimming pools and industrial cooling processes, as well as in some naturally heated waters. *N. australiensis* is pathogenic to laboratory animals but was not reported from any human cases until 1988.

The pathogenicity of Acanthamoeba spp. is more widespread and species distinctions are less precise compared with Naegleria (Page 1988). The genus Acanthamoeba was first recognised in 1931 and was mainly of interest to medical ecologists and other workers who occasionally encountered Acanthamoeba as a contaminant of microbial cultures and tissue culture cell lines (Singh, 1975). Members of the genus are the most frequently isolated gymnamoebae, possibly even the most common free-living protozoan. It has often been isolated from salt water and plant matter (Page, 1988). These *Amoebae* are ubiquitous in water, and grow over the wide range of temperature. The intracellular pH of amoebae is between 6.3-7.4. The classification of *Acanthamoeba* is based on morphological characteristics of the trophozoite and cyst stages (Figure 43).

The Acanthamoebae cyst is produced abundantly by most isolates and its wall consists of two layers, the outer wall or ectocyst and the inner wall or endocyst. The first detailed classification of the Acanthamoebae, was achieved in 1967 and seven species were

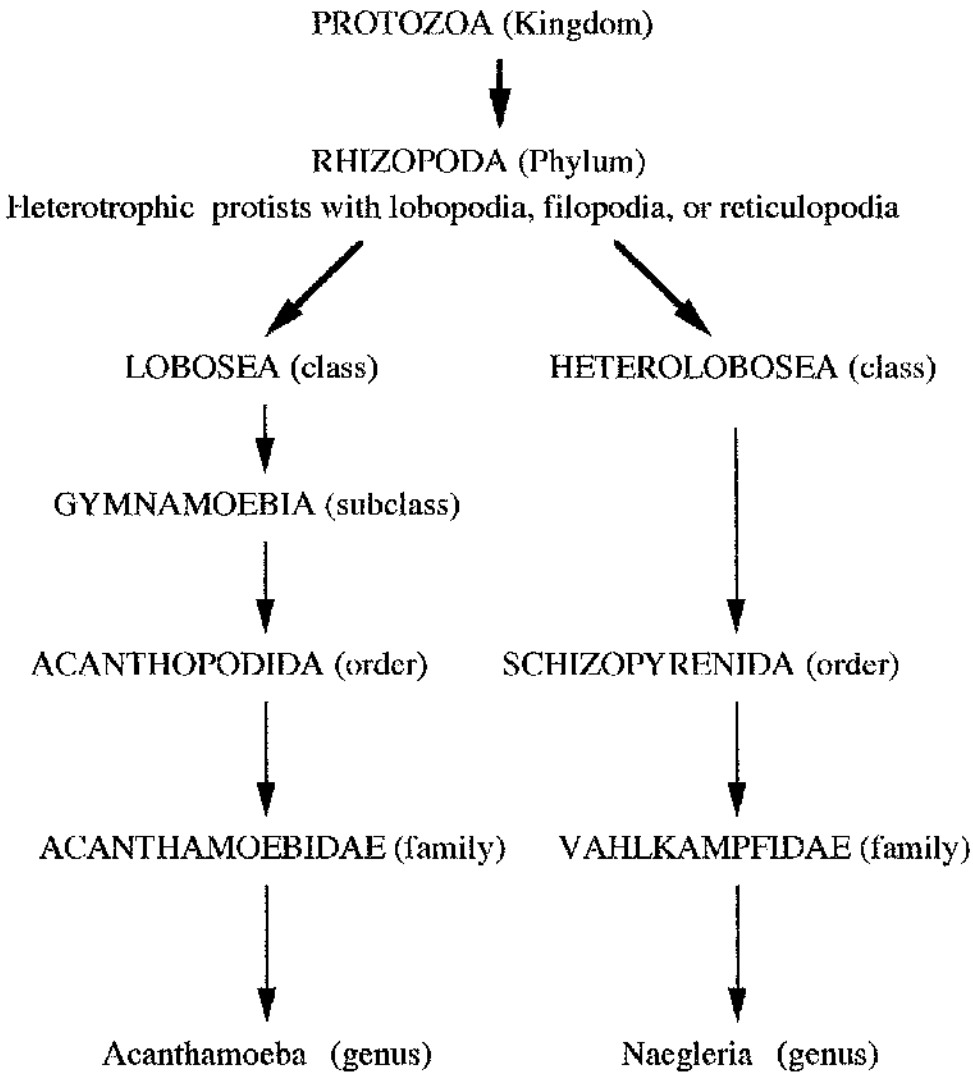


Figure 43 Taxonomic position of *Acanthamoeba* and *Naegleria* (Page, 1988)

recognized, the larger number of strains, were then examined and 18 species were defined based on variation in cyst morphology (Page, 1988, Page, 1967). The species were assigned to three distinct morphological groups (Table 5).

The outer and inner wall layers are most widely separated in group I, where endocyst meets ectocyst at the end of arms or rays. At each point where the two layers meet is a pore or potential opening closed by an operculum. The number of arms and the numbers of pores are therefore the same. The ectocyst is a thin layer in group III, and the more or less rounded endocyst does not produce arms, since the two layers are close together around most of the circumference. Cyst are somewhat intermediate between the two extremes in group II, the endocyst may be somewhat stiletto-like or may be polygonal so that the two wall layers meet at a corner rather than an arm of the endocyst, and the endocyst may even be more or less rounded, though not as consistently as in group III. With the exception of *Acanthamoeba culbertsoni* of group III, all species which have been described as causal agents of human infections belong to group II.

Although the general appearance of the trophozoites and cysts readily defines the genus and allows most species to be separated into one of the three morphological groups, the differentiation of species within the groups is considered subjective and unreliable because of the large variation in cyst morphology that occurs even within clonal populations (De Jonckheere, 1983; Kilvington & White, 1991). Recent studies using isoenzyme or mitochondrial DNA restriction endonuclease analysis have confirmed the present unsatisfactory state of Acanthamoeba taxonomy by demonstrating similarities between strains

assigned to different species (Kilvington *et al.*, 1991, Kilvington &White 1994).

Acanthamoeba is characterized by a trophozoite and cyst stage. Trophozoites are approximately 25-40 μm in length, depending on the species, and numerous needle-like projections from the trophozoite body are termed "acanthopodia" (Page, 1988). A central contractile vacuole is present in the cytoplasm. Trophozoite movement is slow and poly-directional with a hyaline pseudopodium that slowly stretches out and widens. Under adverse conditions cysts are formed from the trophozoites (Page, 1988). These range from approximately 15 to 28 μm , depending on the species, and are double-walled. The irregular joining of the inner wall to the outer gives rise to a polygonal arrangement seen in most species. Pores are evident at the junction of the walls and are sealed by a plug termed the operculum. Both the trophozoite and cyst contain a single nucleus that has a large, dense, centrally-located nucleolus. Trophozoites divide by mitosis, during which the nuclear membrane and nucleolus disappear (Singh, 1975).

Acanthamoeba is one of the most common free-living amoebae found in the environment. The resistance of Acanthamoeba cysts to extremes of temperature (Biddick *et al.*, 1984; Kilvington, 1989), desiccation (Kingston *et al.*, 1969) and disinfection (Kilvington, 1990) accounts for the reported presence of the organism from soil, mud, rivers, ponds, lakes, chlorinated bathing pools, water cooling towers, tap water, and even the atmospher (De Jonckheere, 1991). By their natural predation on bacteria, fungi and other protozoa, these amoebae have a significant effect on microbial ecosystems (Singh, 1975). The predation on cyanobacteria (blue-green algae) by Acanthamoeba has been

Table 5. Three distinct groups of Acanthamoeba species (Pussard *et al.*, 1977)

Group I	Group II	Group III
A. astronixis	*A. castellanii*	*A. Culbertsoni*
A. comandoni	*A. divionensis*	*A. lenticulata*
A.echinulata	*A. grifini*	*A. royeba*
A.tubiashi	*A. hatchetti*	*A. palestinensis*
	A. lugdunensis	
	A. mauritaniensis	
	A. polyphaga	
	A. quina	
	A. rhysodes	
	A. triangularis	

described, and a suggestion has been made as to the potential use of amoeba in the control of toxic cyanobacterial blooms in aquatic sites (Dryden &Wright, 1987).

1.4.1 Pathogenicity of free-living amoeba

Although free-living amoebae, including Acanthamoeba, have been isolated frequently from the faeces of humans and animals, prior to 1958 the only amoeba commonly considered to be pathogenic for man was *Entamoeba histolytica,* the anaerobic parasite which causes amoebic dysentery. The cytopathogenicity of a free-living amoeba, identified as Acanthamoeba, that contaminated monkey kidney tissue culture cell lines, was reported first by Jahnes *et al.,* (1957). The first clear recognition that free-living amoeba could produce disease was made the following year. Working on production of polio virus vacccine, Culberston *et al.,* (1958) inoculated tissue culture cells unknowningly contaminated by Acanthamoeba into the brains or nasal passages of monkeys and mice. This resulted in fatal meningoencephalitis in the animals and a free-living amoeba, subsequently identified as *A. culbertsoni,* was recovered from the brain and lungs of moribund animals.

Acanthamoeba was shown to be a pathogen for man for the first time in 1958. A rising antibody titre to *A. Culbertsoni* in a patient who died from cerebral granulomatous disease was shown by the complement-fixation test (Culbertson *et al.,* 1958). Jager and colleagues used immuno- histopathological staining with antiserum to *Acanthamoeba* spp. to identify trophozoites and cysts of the organism in brain sections of a patient who died with Hodgkin's disease and had received prolonged immunosupressive therapy (Jager & Stamm, 1972).

1.4.1.1 Infection of the central nervous system

Since 1958 there has been an increased interest in small free-living, amphizoic, or limax amoebae because it was discovered that *Naegleria fowleri* and varous species of *Acanthamoeba* are pathogenic and capable of producing fatal meningoencephalitis in man and animals. Infection of the central nervous system (CNS) by Amoebae is termed granulomatous amoebic encephalitis (GAE). Patients have usually a background of immunosuppression from chemotherapy , alcohol or chronic disease (Martinez, 1985; Martinez, 1991).

In 1965, Fowler and Carter reported acute pyogenic meningitis which they attributed to Acanthamoeba (Fowler & Carter 1965). GAE is also reported as a primary cause of death in patients with acquired immunodeficiency syndrome (AIDS) (Visvesvara & Stehr-Green, 1990). *A. culbertsoni* has been considered to be the principal but not only amoebal pathogen of the central nervous system. Approximately 50 cases of GAE have been reported worldwide, all of which have been fatal (Martinez, 1991). Because amoebae are not seen in the cerebrospinal fluid in GAE the diagnosis is usually made post-mortem. The role of infection in GAE is unclear but is thought to be the result of haematogenous spread of the organism from a primary infection elsewhere in the body, possibly the lungs (Warhurst, 1988). Because GAE is usually diagnosed post-mortem, no cases have been reported where specific anti-Acanthamoebal therapy has been initiated. Several antimicrobial agents, namely chlorhexidine and polyhexamethylene biguanide (PHMD) have been shown to have the uniform amoebacidal activity (Hay *et al.*, 1994). Sulphadiazine and rifampicin have been shown to protect animals from experimental infections but there is no

evidence whether these have any effect in cases of human disease (Das, 1991).

1.4.1.2 Acanthamoeba Keratitis

The first case of Acanthamoeba keratitis was diagnosed in 1973, in a Texan farmer with a history of ocular trauma from straw fragments and rinsing the affected eye with tap water (Janes *et al.*, 1975). Occasional reports of the disease followed, and Acanthamoeba keratitis was considered an extremely rare opportunistic infection arising from accidental injury to the eye (Ma *et al.*, 1981). However, a significant increase in the incidence of the disease has been recognized since 1985 , particularly among wearers of soft contact lenses (Moore *et al.*, 1987, Seal, 1991; Kilvington & white, 1994). The inclusive distribution of Acanthamoeba in the environment presents a constant challenge to the wearers of contact lenses. Such sites include domestic tap water, swimming baths, hydrotherapy and spa pools (Seal & Dart, 1990). Contact lens-associated Acanthamoeba keratitis is almost invariably associated with poor hygiene practices and it seems likely that it results from primary contamination of the contact lens storage case (Seal *et al.*, 1992). This may occur from any environmental source but most probably arises from rinsing the lenses in tap water or non- sterile saline, or wearing them while bathing. Inside the storage case amoebal growth and replication occurs with feeding on the associated bacterial Population Trophozoites and cysts of Acanthamoeba can then adhere to the surface of the lenses and so become inoculated onto the cornea (Kilvington & White, 1994). Whether it is the trophozoite or cyst form of Acanthamoeba which is responsible for causing keratitis and the precise mechanism by which it occurs, is unclear. The highest rate of infection

was found with cyst inocula, in an experimental model (Larkin *et al.*, 1990). While it has been assumed that infection results from minor epithelial trauma from the presence of the contact lens, studies have shown that trophozoites of Acanthamoeba can rapidly penetrate intact corneal epithelium by causing a separation of the interacellular junctions (Moore *et al.*, 1991). Non-contact lens-associated Acanthamoeba keratitis accounts for approximately 10% of reported cases and is usually connected with history of ocular trauma and associated environmental contamination. More recent reports suggest that the combination of chlorhexidine and propamidine to have effective amoebacidal activity within the infected cornea (Hay *et al.*, 1994).

Although clinical findings such as intense pain and ring infiltrates in the cornea in a patient who is a contact lens wearer or has a history of ocular trauma would suggest Acanthamoeba keratitis, diagnosis can be achieved only through laboratory investigation. This is achieved by the culture or histological staining of corneal scrapings or biopsy material. Culture isolation is made by inoculating tissue onto non-nutrient agar (NNA) seeded with a suspension of *E. coli* (Page, 1988 & Kilvington *et al.*, 1990).

1.4.2 Cultivation and laboratory diagnosis of pathogenic free-living amoebae

For the isolation and identification of the amoebae that cause CNS disease and keratitis, cerebrospinal fluid (CSF) and small pieces of tissue, e.g., brain, lungs, cornea, taken at biopsy from the affected areas must be obtained. The specimens should be kept at room temperature (24°C-28°C) until processed. Direct microscopic examination of the

CSF as a wet-mount preparation is of utmost importance for the rapid diagnosis of primary amoebic meningoencephalitis (PAM) and granulomatous amoebic encephalitis (GAE) and keratitis caused by these amoebae. Beside the suitable medium, a few other conditions are required for growing of free-living amoeba. Fresh water and soil amoebae can be cultured at room temperature. The temperature and pH of the culture media have a direct influence on the growth of pathogenic free-living amoeba (Visvesvara *et al.*, 1983, Martinez, 1985). The pathogenic Naegleria are thermophilic and grow better at higher temperature, while Acanthamoebae grow better at room temperature. These organisms normally grow in either indirect light or in the dark, but cultures in saline solutions should be kept in the dark to prevent algal growth. The amoeba saline solutions are used as non-nutrient media, with regular addition of food and as transfer liquids for amoebae grown on agar. Culture isolation is made by inoculating specimens onto non-nutrient agar (NNA) seeded with a suspension of *Escherchia coli* (Page, 1988). Chang's serum casein glucose yeast extract medium (SCGYEM) is recommended for growth of *Naegleria* and proteose peptone glucose (PPG) is suitable for growth of *Acanthamoeba* . Mice are generally recommended as model systems for the isolation and propagation of pathogenic free-living amoebae. Other techniques such as histochemical staining, immunohistochemical techniques, indirect immunofluorescence technique (IIF), immunoperoxidase technique, and indirect staphylococcus protein A coagglutination (ISPAC) have been used to detect amoebae (Visvesvara *et al.*, 1983).

AIMS OF RESEARCH

- To investigate the influence of environmental temperature on uptake and intracellular fate of legionellae in Acanthamoeba.

- To study the mechanism of uptake of Legionella by Acanthamoeba by use of inhibitors.

- To compare surface properties of legionellae grown *in vivo* (within amoebae) and *in vitro*.

- To study the events which immediately follow internalisation of legionellae in amoebae, particularly phagosome-lysosome fusion.

- To assess the importance of legionella-amoebae interactions in hospital water supplies and their significance in detection methodologies and eradication protocols.

2- MATERIALS & METHODS

2.1 Organisms

A virulent strain of *L. pneumophila* serogroup 1 (subtype Pontiac), derived from a hospital water supply, was obtained from the Wolfson Center of Bacteriology, Glasgow (Patterson *et al.*, 1994). Legionellae were either stored freeze-dried (Appendix 4) or preserved on glass beads as described previously (Appendix 4). It was cultured on supplemented Buffered Charcoal Yeast Extract ($BCYE^+$) agar (Appendix 2) before each experiment. *E. coli* (C118) was obtained from the Laboratory of Microbiology, University of Glasgow . It was grown on Lauria agar at 35°C and stored as a cell suspension at -70°C.

A. castellanii was originally isolated from the eye of a patient with Acanthamoeba keratitis (Hay *et al.*, 1994). It was obtained from the Wolfson Center for Bacteriology, Glasgow . *Acanthamoeba* was maintained on 1.5% non-nutrient agar covered with heat-killed *E. coli* (Robotham, 1983). It was cloned and grown axenically as previously described (Page, 1988). Cultures stored on such Petri plates at 4°C in sealed plastic bags survived for at least 6 months. Acanthamoebae were also maintained axenically in PYG amoeba broth (Appendix 1) in sterile plastic tissue culture flasks (25 cm^2, canted neck-rectangular type, Corning Labs.) at 30°C for routine laboratory work. In this case, the organisms were subcultured every week.

2.2 The effect of temperature on uptake of *L. pneumophila* by amoebae

Acanthamoeba-Legionella co-cultures were prepared by the modification previously described by Moffat &Tompkins (1992). Briefly, amoebae were grown in 25 cm^2 tissue culture flasks at 30°C to confluence on the flask (4-5 days). Axenic *A. castellanii* was raised at room temperature (23-25°C) in 15-ml screw-cap tubes (Falcon 2025; Becton Dickinson & Co., Lincoln Park, N. J.) in 5 ml of PYG broth (Appendix 1). To harvest the amoebae, flasks were vortexed vigorously to detach amoebae, and then the cell suspension was centrifuged at 500 x *g* for 5 min in an MSE bench centrifuge. The cells were resuspended in amoebae saline (Appendix-1) to a concentration of 2x 10^5 cell /ml; 1 ml was then placed in each well of a 24-well tissue culture plate (Falcon 3047). The amoebae were allowed to adhere to the wells and equilibrate for 1 h at 30°C before the bacteria were added.

L. pneumophila (serogroup 1) was recovered from frozen stocks (-70°C) on supplemented BCYE agar prior to each assay. After incubation, *L. pneumophila* were harvested from the BCYE agar plates in sterile distilled water, washed twice and resuspended to approximately 10^9 cfu per ml in sterile distilled water. Bacterial suspension (10 µl) was added to each well containing amoeba in 1 ml of amoeba saline (AS) to give a ratio of approximately 200 bacteria/amoeba. Incubation was carried out under different conditions over the course of 4 h. Bacterial invasion was permitted to continue for different times (1h, 2h, 3h, and 4h) at 37°C, after which time the buffer was aspirated and wells were washed once with warm amoeba saline and the supernatant fluid replaced with AS containing 80 µg/ml of gentamycin (Sigma). After 2h incubation at 37°C, which was sufficient to kill all extracellular legionellae, the wells were washed twice with fresh

amoeba buffer without gentamycin. Distilled water (1 ml) was added to each well and amoeba cells were detached and aspirated. Acanthamoeba containing phagocytosed bacteria were lysed by drawing the suspension through a 27-gauge needle three times to break up the destabilized amoebae. Aliquots of 0.1 ml of amoeba lysate were plated onto BCYE agar in duplicate. The BCYE plates were then incubated at 37°C in 5% CO_2 for 4 d, and colony counts (cfu) were carried out to determine the number of legionellae taken up by the Acanthamoebae.

The effects of temperature on growth of legionellae were studied during a period of 10 d of amoebae/legionella co-cultivation. *A. castellanii* were grown axenically at 30°C in PYG broth (Appendix 1) in 25 cm^2 sterile tissue culture flasks (Corning) containing shallow (1-2mm) levels of liquid. After 4-5 d of incubation at 30°C, amoebae were washed twice with AS (Appendix 1), and suspended in AS to give cell densities of 10^5 /ml as assessed by direct count in a haemocytometer. *L. pneumophila* (serogroup 1) were scraped from four-day $BCYE^+$ plate cultures into amoeba saline (pH. 6.9), and washed twice with sterile distilled water (2000 x *g* for 15 min at room temperature, Microfuge, MH2, Sarstedt). The suspension of Acanthamoeba (10^5 cells/ml) in AS were inoculated with 10^3 /ml Legionella cells and the bacteria-amoeba co-cultures were incubated for 10 d at different temperatures. Aliquots (0.1 ml) of the mixed suspensions were taken at times (1, 3, 5, 7, and, 10 d) and serial dilutions spread on pre-dried $BCYE^+$ agar plates which were incubated for 3-4 d at 37 °C in the present of 5% CO_2 before Legionella colonies were counted.

2.3 Effect of phagocytosis inhibitors on bacterial uptake and intra-amoeba replication

Cytochalasin D and methylamine have been used to investigate bacterial invasion of different host cells. Cytoskeletal and metabolic inhibitors were used in this experiment. *A. castellanii* were cultivated in PYG medium in 25 cm^2 sterile tissue culture flasks at 30°C, until a confluent monolayer of amoebae was established. Amoebae were harvested by centrifugation (500 xg for 5 min) and resuspended in AS (pH 6.9). Trophozoites were enumerated with a haemocytometer and diluted to a cell density of 10^6/ ml before use. Trypan blue (0.1% w/v) exclusion was assessed to ensure viability of cells. *L. pneumophila* growth was harvested in amoebae saline (pH. 6.9) and adjusted to an OD_{660} of 0.9 with a spectrophotometer (PYE, Unicam, SP6 series). Suspensions were serially diluted in AS to a concentration of approximately 10^4 cfu/ml before inoculation into cultures of amoeba.

A modification of the method previously described by King *et al.*, (1991) was used to assess the effect of inhibitors on intra-amoeba growth of Legionella. Briefly, a stock solution (1 mM) of cytochalasin D (Sigma) was diluted in assay medium (pH 6.9) to a concentration of 2 or 1 µM. A stock solution of methylamine (500 mM) (Sigma) was diluted in assay medium (pH 6.9) to achieve 100, 50 and 10 mM concentrations. Aliquots of assay medium containing inhibitors were then filter-sterilised (0.22 µm pore size) and warmed to 37°C in separate tissue culture flasks before the addition of Acanthamoebae.

Amoebae were diluted to 10^5/ml with assay medium containing cytochalasin D (2 or 1 μM) or methylamine (100, 50, and 10 mM) in tissue culture flasks. The cells were incubated for 1h at 37°C , inoculated with 1 ml of suspensions of 10^4 *L. pneumophila* /ml, and incubated for 7 d. Aliquots of these mixed cell suspensions were removed at 0, 3 and 7 d to determine the number of cfu of *L. pneumophila*. Control cultures of the amoebae in the assay medium without inhibitors were incubated and inoculated with *L. pneumophila* in a similar manner.

The effect of each inhibitor on the growth of *L. pneumophila* in PYG medium and yeast extract broth (YB) (Ristroph *et al.*, 1980), was tested. Bacteria were inoculated into assay medium containing cytochalasin D and methylamine to achieve 10^3 cfu/ml and plated to determine the number of cfu at 0, 3, and 7 d. Legionellae were also grown in YB broth (pH 6.75) and incubated in the presence and absence of cytochlasin D or methylamine on a rotary shaker (100 rpm) at 37°C, and enumerated as above. Experiments with YB medium were performed to determine whether the bacteria could multiply in the presence of these inhibitors under favourable conditions. All tests were performed in duplicate.

Intracellular multiplication of *L. pneumophila* in *A. castellanii* was defined as an increase in numbers of an inoculum of *L. pneumophila* in static co-incubation with amoebae. Controls were used to show that viable amoebae were required for growth of Legionellae in the absence of supplemented growth medium. Control cultures of the amoebae in the assay medium, but without inhibitors, were incubated and inoculated with *L. pneumophila* in a similar manner. The effect of each inhibitor on the survival of *L. pneumophila* in the assay medium was tested. *L. pneumophila* was inoculated into the assay medium containing cytochalasin D and

methylamine and plated out in order to determined the number of cfu. at 1, 3, and 7 d. All tests were performed in duplicate.

2.4 Cell envelope analysis

2.4.1 Preparation of intra-amoebal Legionella

A. castellanii cells were grown in 25cm^2 tissue culture flasks containing PYG amoeba broth (pH 6.9). Ameobae (10^5/ml) were inoculated with *L. pneumophila* to give a bacteria /amoeba ratio of 200. The co-cultures were incubated for 12h at 35°C to allow bacterial invasion before antibiotic treatment. After the invasion time, gentamycin (80 µg/ml final concentration) was added to the co-culture before incubation for 2 h at 37°C. The antibiotic was washed from the amoeba monolayer with 3 washes of AS. The tissue culture flasks containing Acanthamoeba were incubated in fresh amoeba broth (Appendix 1) at different temperatures (30°C and 37°C) for a further 48-72 h. Growth of *L. pneumophila* in amoebae was monitored under light inverted microscopy (Olympus CK2). After incubation, the amoeba medium was replaced by sterile distilled water and intracellular legionellae were released by disruption of amoebae by 3 cycles of freezing and thawing. The resulting suspension was centrifuged 3 times (each cycle of 5 min at 500 x *g*) to sediment amoeba debris. *L. pneumophila* were then separated from the supernatant by further centrifugation at 2000 x *g* for 15 min. The culture purity checks were performed by plating the recovered bacteria onto BCYE$^+$ and blood agar. Bacteria were stored at -70°C until used.

2.4.2 Outer membrane preparations

Envelope preparations of intra-amoeba grown Legionellae were prepared as described previously (Barker *et al.*, 1993). Briefly, 1.5 ml of bacterial suspension were disrupted by 10 cycles of 30 s sonication (with 30 s cooling in each cycle). Na-lauryl sarcosinate (20% w/v final conc) was added to the sonicated cells and the mixture incubated for 30 min at room temperature to dissolve the cytoplasmic membrane (Lambert, 1988). The outer membranes were harvested from the mixture by centrifuging at 11600 x *g* for 2 h at 4°C. Protein concentrations were determined by a modification of the Lowry method (Appendix 5).

Lipopolysaccharide (LPS) was prepared from whole-cell lysate by digestion with proteinase K (Protease Type XI-S from *Tritirachium albus* ; Sigma). The method was essentially as described by Barker *et al.*, (1993). Briefly, Legionellae were grown either on BCYE$^+$ agar or in Acanthamoeba intracellularly, harvested as described above, resuspended in cold PBS, pH 7.0 and washed once (2000x g for 15 min). The washed bacteria were resuspended in PBS and the A_{660} was adjusted to between 1.0 and 1.2. This suspension (approx. 1.5 ml) was centrifuged and 50µl of sample buffer (2% w/v SDS; 4% v/v 2-mercaptoethanol; 10% v/v glycerol; and 0.01% w/v bromophenol blue; in 1 M Tris buffer pH 6.8) was mixed with the cell pellet before heating at 100°C for 10 min. The samples were then cooled and 10 µl of proteinase K (2.5 mg/ml w/v in lysing buffer) was added to the bacterial lysate before incubation at 60°C for 60 min in a water bath prior to LPS analysis on polyacrylamide gels. Outer membranes and lipopolysaccharide of *L. pneumophila* grown on BCYE$^+$ at different temperatures were prepared and used as controls.

2.4.3 Sodium dodecyl sulphate- polyacrylamide gel (SDS PAGE) electrophoresis and detection of outer membrane proteins

The discontinuous method of gel electrophoresis (Laemmli 1970) was used for SDS-PAGE analysis. A 4% w/v acrylamide stacking gel and a 12% w/v acrylamide resolving gel (Appendix 4) were used. Equal volumes of outer membrane protein (OMP) preparations (protein concentration approximately 2 mg/ml) were heated at 100°C for 10 min in sample buffer (see 2.4.2), and 25µg protein were loaded per well. Electrophoresis was performed in a vertical slab gel apparatus (Protean II; BIO Rad) unless stated otherwise, in buffer comprising 25 mM Tris, 192 mM glycine and 0.1% w/v SDS (pH 8.3) at a constant current (per 1.5 mm thick gel) of 20 mA through the stacking gel and 35 mA in the separating gel, until the dye front reached the bottom of the gel.

Proteins in the electrophoresed gels were visualised by staining with 0.1% w/v Coomassie blue R-250 (Sigma) in 10% v/v acetic acid and 45%v/v methanol in distilled water for several hours to overnight. Gels were destained with several changes of destaining solution containing 10% v/v acetic acid and 30% v/v methanol in distilled water. All steps were performed at room temperature with gentle shaking.

2.4.4 Determination of molecular weight of proteins

The molecular weight (M_W) of the SDS-PAGE resolved polypeptides was determined by comparison of mobility with standard marker protein mixtures of known M_W, (SDS-6H SDS-7 Sigma). The standard protein mixture, SDS-6H contained: rabbit myosin (205 kD), *E. coli* ß-galactosidase (116 kD), rabbit muscle phosphorylase b (97.4 kD), bovine albumin (66 kD), egg albumin (45 kD) and bovine erythrocyte carbonic anhydrase (29

kD). The SDS-7 contained: bovine albumin (66 kD), egg albumin (45 kD), rabbit muscle glyceraldehyde-3- phosphate dehydrogenase (36 kD), bovine erythrocytes carbonic anhydrase (29 kD), bovine pancreas trypsinogen (24 kD), soybean trypsin inhibitor (20.1 kD) and bovine milk α-lactalbumin (14.2 kD). After measuring the relative mobility values of the marker proteins, standard curves were generated either by plotting the relative mobility of each standard protein against its MW on a log scale, or by generating computer-assisted curves (Apple Macintosh, Cricket Graph version 1.3). These curves were used to estimate the MW of proteins under characterisation.

2.4.5 Western blotting (immunoblotting)

Cell proteins of *L. pneumophila* grown under different conditions and separated by SDS-PAGE as described above, were transferred onto nitrocellulose membranes (0.45 μm pore size; Bio-Rad Laboratories Ltd., Watford, UK), using the Western blotting (immunoblotting) procedure described by Towbin *et al.* (1979). Transfer was carried out at pH 8.3 in 25 mM Tris buffer containing 192 mM glycine and 20% (v/v) methanol, using a Bio-Rad Trans-Blot Cell at 100 V for 1h at 4°C. To ensure that efficient transfer of proteins had taken place, the gels were stained with Coomassie blue after transfer. The nitrocellulose blots were blocked by washing in TTBS (Tween-Tris-buffered saline; 0.3% [v/v] Tween 20, 0.9% [w/v] NaCl in 10 mM Tris-HCl, pH 7.4) for 1h at room temperature. The blots were then given three 5-min washes in TBS (Tris-buffered saline; 0.9% NaCl in 10 mM Tris-HCl, pH 7.4).

Reaction with antisera was carried out overnight at 4°C by immersing the blot in serum diluted 1:200 in TTBS. The anti-legionella serum was obtained from Central Public Health Laboratory, Colindale, London NW9 UK. It was raised against formalin-killed whole cells grown on BCYE+ agar. After probing, the blots were given three 5-min washes in TBS and immersed in TTBS containing 0.25 μg of staphylococcal protein A-horseradish peroxidase conjugate (Sigma) per ml for 3h at room temperature. The blots were washed again in TBS as described above, and the bound enzyme was detected by the addition of freshly-prepared substrate solution (0.03% w/v 3-3-diaminobenzidine tetrahydrochloride dihydrate [DAB, Aldrich Chemical Co] in aqueous 1.5% w/v $CoCl_2$ [5 ml], with 100 μl hydrogen peroxide added per 100 ml of the substrate solution). The blots were developed as required and the reactions stopped by immersing the developed blots in distilled water for 10 min. The membrane was finally rinsed and air dried for photography.

2.4.6 LPS analysis of intra-amoeba Legionella

Lipopolysaccharides obtained by the proteinase K digestion of whole-cell lysates or outer membrane preparations were electrophoretically analysed on polyacrylamide gels in the discontinuous buffer system of Leammli (1970) using a 4.0% v/v acrylamide stacking gel and 15% v/v acrylamide resolving gel in 4M urea (final concentration). Approximately 5 μg of purified LPS, heated at 100°C for 5 min in sample buffer, or the equivalent in proteinase K-treated whole cells or outer membranes, were loaded per well. The electrophoresis was performed in a vertical slab gel apparatus (Protean II: BIO Rad) in buffer comprising 25 mM Tris, 192 mM glycine and 0.1% w/v SDS (pH 8.3), at a constant current (per 1.5 mm thick

16 x 14 cm gel) of 20 mA through the stacking gel and at 30 mA in the separating gel, until the dye front reached the bottom of the gel.

2.4.7 Detection of lipopolysaccharides in gels

The method of Tsai & Frasch (1982) was used for visualisation of LPS profiles in SDS PAGE gels. Briefly, the gels were fixed overnight in 200 ml (per gel) of 40% v/v ethanol and 5% v/v acetic acid in distilled water . Fixing solution was replaced with 0.7% periodic acid in 40% v/v ethanol and 5% v/v acetic acid for 5 min. After this, three 15 min washes were performed with 500-1000 ml distilled water per wash. Gels were then treated for 10 min with freshly prepared reagent (150 ml/gel) containing 2 ml conc NH_4OH, 28 ml 0.1N NaOH, 5ml 20% w/v $AgNO_3$ in 115 ml water. Gels were then washed again three times as above. After the last rinse, gels were developed with formaldehyde developer (200 ml/gel) containing 10 mg citric acid and 100 µl of 37% v/v formaldehyde and finally stored in distilled water after washing. All steps were performed at room temperature with gentle shaking. The control gels were checked for undigested proteins by staining with Coomassie blue R-250. The LPS standards routinely included on the gels were: smooth LPS from *Escherichia coli* 0111: B4 and rough LPS from *Salmonella typhimurium* Ra mutant TV 119 (both from Sigma).

2.5 Superoxide generation during phagocytic activity of Acanthamoeba (chemiluminescence)

Luminescence is the emission of light by a non-thermal process. In the case of chemiluminescence (CL) analysis, the light is produced by a chemical reaction. CL has been used to study mammalian phagocytic cells' activity. In 1990 lucigenin was recognised as a suitable probe for investigation of superoxide generation in the amoeba model (Davis *et al.*, 1991). Luminol-dependent CL is dependent on oxidase as well as degranulation (myeloperoxidase) while lucigenin monitors oxidase activity independently of the extent of degranulation (Edwards, 1987) .

We used lucigenin in a CL study of the interaction between Acanthamoeba and Legionella at the superoxide generation stage. *A. castellanii* were grown to the mid-exponential phase in 25 cm^2 tissue culture flasks containing 5 ml amoeba broth (Appendix 1). Starved amoebae were used in all CL experiments unless otherwise stated. For starvation, Acanthamoebae were harvested, resuspended in PBS (phosphate-buffred saline: 0.9% NaCl; 10 mM potassium phosphate buffer, pH 7.4) and incubated at 4°C for about 20 h . Under these experimental conditions no encystment of the amoeba cells was observed and trypan blue exclusion showed > 95% cell viability. Previous work has shown that Acanthamoeba has enhanced phagocytic activity after starvation (Davis *et al.*, 1991). We found that greater CL was evident when amoebae were starved. CL measurements were made in an LKB Wallac 1251 Luminometer connected to an IBM-PC computer, in 1.0 ml of suspension containing 25 µM lucigenin (Sigma) and $>10^5$ Acanthamoeba trophozoites per ml. Stimulant (100µl of either *L. pneumophila* , *E. coli* or latex beads at a density of 10^7 particles/ ml of reaction suspension) was added to the reaction cuvettes at

10 min after measurement started. The CL emission was measured in mV at 37°C or 30°C for 30 min. All CL experiments were performed in duplicate.

2.6 Phagolysosome fusion assay

L. pneumophila has been reported to inhibit the fusion of phagosomes with lysosomes in mammalian phagocytes, in common with other intracellular pathogens such as *Toxoplasma gondii* and *Mycobacterium tuberculosis, Chlamydia psittaci* and, *Encephalitozoon cuniculi* (Horwitz, 1983). Several types of phagosome-lysosome (P-L) fusion assays have been described. Electron microscopy has been used to look for the appearance of lysosomal marker acid phosphatase in the phagocytic vacuole. Fluorescent markers have also been used to label lysosomes, and phagosome-lysosome fusion has been followed by fluorescent microscopy (Kielian, 1986). We used acridine orange (AO), a fluorescent vital dye to label Acanthamoeba lysosomes. The modification of the method used by other researchers (Ishibashi & Yamashita, 1982) was used in this study to label the Acanthamoeba lysosomes and follow the events during phagocytosis of Legionellae by Acanthamoebae.

2.6.1 Fluorescent microscopy with acridine orange

The stock solution of acridine orange (AO) (Sigma) was prepared at a concentration of 100 µg/ml in PBS, sterilized by filtration (0.2µm pore size, Millipore Ltd) and stored in the dark at 4°C. *A. castellanii* were grown in tissue culture flasks in amoebae broth (Appendix 1), and trophozoites were harvested and washed twice in AS (Appendix 1). The viability of amoebae was assessed by Trypan blue exclusion. Coverslips (12mm) for microscopy were cleaned in 90% v/v ethanol, rinsed in distilled water and

sterilised by flaming in ethanol. Amoebae were resuspended in AS to approx. 5 x 10^6 cell/ml and 100µl (5 x 10^5 cells) layered onto the coverslips previously sterilized.

Amoebae were allowed to adhere by incubating the coverslips in a moist chamber for 1 h at 30°C. The coverslips were then washed gently with pre-warmed AS to remove non-adherent cells. Lysosomes were labeled by adding AO solution (5 µg/ml final concentration in AS) to the coverslips and incubating for 20-30 min at 37°C in the moist chamber. The stained amoebae on the coverslips were then washed with AS, drained and covered with amoeba broth (Appendix 1) for 30 min to allow internalization of AO located on the cell surface. The medium was aspirated and replaced with 100µl of diluted suspension of particles (live *L. pneumophila* , live *E. coli* or latex beads) in amoeba saline (approx. 10^7 particles/ml). The particles were centrifuged onto the cell monolayer by placing the coverslips into wells of a 24-well tissue culture plate in a centrifuge plate carrier (Cooke Laboratory Products, Alexandria, VA) and centrifuging for 10 min in an MSE Mistral 6L Centrifuge at 1000 xg at 4°C. Under these conditions, particles became closely associated with the amoeba cell membrane, and unbound particles were removed by washing each coverslip with 1 ml of AS. Each well (containing one coverslip) was then given 1 ml of prewarmed AS and at time zero the coverslips were incubated at different temperatures (22°C, 30°C, and 37°C) for 4 h. After incubation, the coverslips were washed with AS and allowed to air dry, followed by UV irradiation for 5 min to kill the bacteria. The coverslips were then inverted onto microscope slides in one drop of anti-fading liquid (Appendix 7) or PBS, blotted and rimmed with nail polish. Slides were viewed immediately in a Zeiss photomicroscope with a mercury lamp adjusted for epi-illumination , and fitted with BG12

and BG38 filters, a fluorescein dichroic mirror for excitation, and a K510 barrier filter. The intracellular particles displaying bright orange fluorescence were counted as positive for P-L fusion; faintly green-fluorescent particles were scored as negative(Kielian, 1986). For each time point, at least 10 different microscope fields and duplicate coverslips were examined, and a total of >100 particles counted. A parallel set of samples, without AO, were prepared and stained by Giemsa before examination by bright-field microscopy (x100). The intracellular particles were counted directly from Giems-stained coverslips to assess the phagocytosis activity of Acanthamoeba. The fusion index and the phagocytic index were defined as the percentage of positive fusions x average number of fused phagosomes per cell and the percentage of positive phagocytosis x the average number of phagocytized particles per cell respectively(Ishibashi & Yamashita, 1982).

2.6.2 Acid phosphatase staining and transmission electron microscopy

The co-culture of *A. castellanii* and particles (*L. pneumophila* , *E. coli* or latex beads) was prepared and incubated for 12 h. at 37°C. Amoebae were then washed 3 times with pre-warmed (AS) to remove extracellular particles. Amoebae were then fixed in 0.1M cacodylate buffered fixative (pH 7.4) at 4°C on a rotator shaker for 30 min. The cells were rinsed 6 times (5 min each rinse) in 0.1M cacodylate buffer (Appendix 6) and incubated in the same buffer over night at 4°C. Amoebae were rinsed twice (15 min each rinse) in 0.05M acetate buffer, pH 5.0 the following day before addition of incubation medium. Incubation medium was prepared according to the method of Lewis & knight (1977), and sodium ß-glycerophosphate was used as a substrate for acid phosphatase. Amoebae were incubated in incubation medium for 30 min at 37°C in a water bath followed by washing over a

period of 40-60 min in 5% sucrose in 0.05M acetate buffer (pH 5.0) and rinsed once in 0.1 M cocadylate buffer. Amoebae were fixed with 1% w/v OsO_4 in distilled water for 1 h at room temperature.

2.7 Association of Legionellae with amoebae in hospital water supplies

2.7.1 Sample preparation

As water systems of large buildings such as hospitals are often contaminated with Legionellae, there exists a potential danger to patients. Several reports have shown a clear association between the presence of Legionellae in water systems (e.g. hot water supplies) and the occurrence of legionellosis. The problem becomes particularly important for immunocompromised patients. In order to gather information about the presence of Legionellae and amoebae in hospital water supplies, water samples were collected from hot and cold supplies from 69 hospitals. Two samples (cold and hot water) were collected in 5 litre plastic containers from the water supplies of each hospital.

Water samples were filtered through Multipore nylon membrane filters, pore size 0.22 - 0.45 μm (Supro 200, 142 mm membranes, product No. 60305). Five litres of each sample was filtered using a peristaltic pump. Turbid samples or those containing particulate debris were filtered through a 1.5 μm pre-filter prior to the main filtration step. After filtration of each sample, the filter stand and pipework systems were pasteurised by passing boiling water through the assembly. Boiling water passed in this manner for approximately 5 minutes eradicated any contaminating vegetative organisms. The assembly was then left to cool or cooled by passage of cold

sterile distilled water through the system. The filtration assembly and flask were cleaned and sterilised daily and rinsed with fresh sterile distilled water. The filter membranes were placed in a 50 ml aliquot of filtrate collected in a suitable container (e.g. a sterile honey jar) and shaken vigorously to remove any retentate from the membrane before storage at 4°C until required.

2.7.2 Samples screening for preliminary diagnosis of legionellae

Two 10 ml aliquots were removed from each 50 ml sample; one was treated with an acid buffer (HCl/KCl, pH 2.2) for 10 min, the other with heat (56°C) for 15 min. Buffered charcoal yeast extract agar (BCYE+) and BCYE with antibiotics (glycine, vancomycin, polymyxin and cyclohexamide) (Harrison & Taylor, 1988) was then inoculated in duplicate with aliquots (0.1 ml) from each heat-treated and acid-treated sample. Plates were incubated for up to 14 days at 37°C in the presence of 5% CO_2 in humid air and growth recorded on the 3rd, 5th, 7th, 10th, and 14th d. Direct culture of the 50 ml aliquots containing the filters was also performed in duplicate as above. Colonies of Legionellae were recognised by their characteristic ground glass appearance and characteristic fluoresecence under UV light. Colonies were verified as being *Legionellae spp.* by failing to grow on subculture to non-supplemented BCYE agar (Oxoid Ltd.).

At least one colony per plate was confirmed and serotyped using immunofluorescent sera supplied by Public Health Laboratory Service, Colindale, London. Briefly, bacterial growth from the plate was emulsified in 1% formal saline and left for about 2 h at room temperature. To the well of a Hendley-Essex or flow PTFE- coated slide was added 5µl of bacterial antigen as prepared above. The slides were dried at 37°C (20 min) and fixed in acetone at room temperature (15 min). Rabbit antisera were diluted and

10 µl of dilution added to each well. The slides were incubated in a wet box at 37°C for 30 min, rinsed in PBS and then washed in PBS for 15 min with two changes of buffer before a final rinse in distilled water. After drying at 37°C for 10 min, the slides were stained with 5µl of donkey anti-rabbit FITC conjugate (working titre of 1/40) followed by incubation in a wet box at 37°C for 30 min. After washing and drying as described above, preparations were mounted in glycerol mounting medium (Appendix 7) and examined at x400 by incident - light fluorescence microscopy.

2.7.3 Isolation of free-living amoeba from hospital water supplies

Aliquots (2 ml) from each 50 ml filtered water sample (see paragraph 2.7.1) was mixed with an equal volume of AS in a 5 ml glass Bijou bottle and kept at 4°C until required. Samples from these Bijous (0.1 ml) were inoculated onto the surface of non-nutrient agar plates seeded with heat-killed *E. coli* to grow free-living amoeba (see Appendix 1). The plates were left to air dry, and incubated at 30°C for up to 15 d, with daily examination by inverted light microscopy after the first week for growth of amoebae. The positive samples were chosen for subculture onto fresh plates for mixed cloning and identification. A clear zone of agar bearing a small numbers of uniform cysts or trophozoites was cut with a sterile scalpel and put upside down onto a fresh plate, and incubated for a further period. Plates showing amoebal growth were sealed with tape and kept at 4 °C in plastic bags until used.

All amoebae were identified by their cyst and trophozoite morphology as seen by inverted light microscopy (Olympus - Model CK2). After such analysis, all water samples could be put into one of four different categories (see Table 13).

Table 13) Grouping of water samples on the basis of the presence of amoeba or Legionella .

Groups	Amoebae*	Legionellae	%
G1	+	+	19
G2	+	-	27
G3	-	+	3
G4	-	-	51

Amoebae*: Means free-living amoebae identified as *Acanthamoeba spp., Vahlkampfidae spp.* or *Hartmanella spp.* by morphological characteristics.

Group 2 (27%), Legionella-negative by routine culturing method and amoebae-positive, were selected for further investigation.

2.7.4 Processing amoeba-positive and legionella-negative Group 2 samples by an enrichment method

Legionellae-negative, amoebae-positive (Group 2) hospital water samples (2 ml each sample) were incubated at 30°C for one week in the presence of acid washed cysts (1% HCl for 2 min) of cloned amoeba species in AS (2 ml), originally isolated from the same water sample, in a 5 ml glass bijou . After incubation, an aliquot of 0.1 ml of each mixture was directly inoculated onto a BCYE$^+$ agar plate. The plates were incubated at 37°C in 5% CO_2 in air for up to 14 d and examined for growth on the 3rd, 5th, 7th, 10th and 14th day. Legionella colonies were initially recognised by their characteristic ground glass appearance and characteristic fluorescence on exposure to UV light. Colonies were verified as *Legionellae* initially by failure to grow on subculture on blood agar (Oxoid). Legionella-like clonies were also stained by Gram's method and confirmed as Legionellae by the Microscreen legionella latex test.

The Microscreen Legionella Latex Test (Mercia Diagnostics Ltd., Mercia House, Bradford Park, Shalford, Guildford, Survey GU4 8EW, UK), was done as follows. Sample preparation, and detection were carried out as specified by the manufacturer. Briefly, one drop of isotonic saline (about 30 µl) was dispensed onto two wells of a microscreen slide. Legionella-like clonies were selected from BCYE$^+$ plates, mixed with the saline and emulsified to form a smooth , heavy suspension, by spreading the liquid over the entire surface of the well. Latex beads coated with either anti-*L. pneumophila* type 1 antibody or anti-*L. pneumophila* types 2-12 antibody, were added (onc drop) to each well. The slide was gently rocked for 30 s and a positive result was indicated by latex agglutination or

clumping. *E. coli* and standard *L. pneumophila* types 1 and 5 were used as negative and positive controls.

2.7.5 Polymerase Chain Reaction (PCR)

The Group 2 water samples (Table 13), were subjected to PCR for *L. pneumophila* before and after the enrichment procedure described above. PCR involves the use of two short oligonucleotide primers which flank the target sequence of DNA and are complementary to it. The presence of a target sequence was detected by its positive amplification during successive rounds of the PCR reaction sequence.

One ml of each (pre-enrichment) samples was taken from the original 50 ml water sample filtrate (see paragraph 2.7.1) and placed in a 1.5 ml Eppendorf tube. DNA extraction was carried out by 3 x freezing-boiling (5 min each). Water samples (post-enrichment) from which legionella were isolated were also placed in a 1.5 ml Eppendorf tube, and DNA was extracted by Proteinase K digestion. The latter method proved more sensitive and was adopted for routine use.

Bacterial DNA was amplified by PCR as described by Starnbach *et al.*, (1989), utilising two 19-mer primers (LEG 1 [5'-GTC ATG AGG AAT CTC GCT G-3'] and LEG 2[5'-CTG GCT TCT TCC AGC TTC A-3']) specific for a *L. pneumophila* chromosomal DNA sequence of unknown function. The legionella oligonucleotides (Leg 1, Leg 2, and Leg 3) were prepared as solutions in ammonium hydroxide and purified by the ethanol percipitation method (Appendix 8). Thirty five cycles of amplification were done, each cycle comprised of denaturation for 1 min at 93°C(start at 92°C), annealing for 1 min at 55°C (start at 56°C) and extension for 1.5 min at

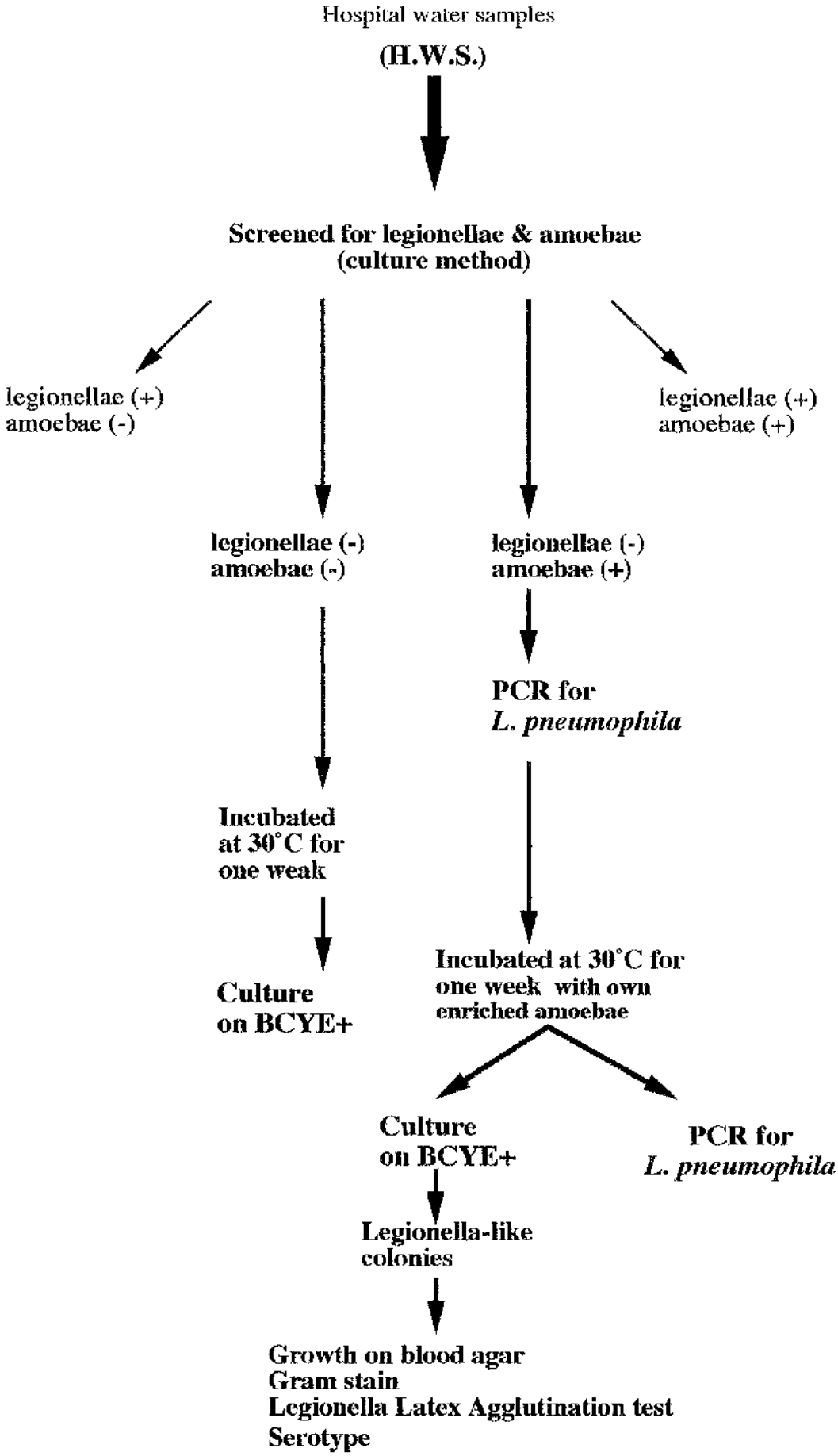
74°C (start at 71°C) followed by a final 7 min incubation at 74°C to maximise extension of amplified products. The PCR reagents were obtained as a Gene Amp PCR reagent kit (Perkin-Elmer Cetus, Norwalk, Connecticus, USA) and were used as recommended by the manufacturer. Controls used were primers incubated with sterile, non-pyrogenic water (Sterile water for irrigation, Baxter Healthcare Ltd, Thelford, Norfolk, UK), Gene Amp positive control were as supplied, and *L. pneumophila* serogroup 1 positive controls were prepared as for the experimental samples. Sterile pyrogen-free water was used as a negative control. A 1 kb DNA ladder (Gibco BRL, Life Technologies Ltd, Paisley, Scotland) was used as a marker.

2.7.6 Dot blotting and probing of amplified DNA

Hybridization with a radiolabeled probe is the most sensitive method to detect specific DNA sequences. Thus, dot blots of the amplified products were prepared and probed with a specific 25-mer radio-labeled oligodeoxynucleotide (LEG-3 [5'-GTC CGT TAT GGG GTA TTG ATC ACC A-3']) end-labeled with γ [$^{-32}$ P] dATP by polynucleotide kinase. Samples for blotting were removed from the reaction tubes after PCR amplification and boiled for 10 min. Then 30 µl was spotted onto a nitrocellulose membrane (Zeta- Probe GT, BIO-RAD, Biorad Laboratories Ltd, Hemel Hempstead. Hertfordshire, UK) and the membrane baked under vacuum at 80°C for 2h followed by incubation in a prehybridization buffer of 6x SSC, 5x Denhardt solution, and 0.1% SDS (Starnbach *et al.*, 1989). Controls were pyrogen-free water and *L. pneumophila* (serogroup 1) as before. The specificity of the reaction for *L. pneumophila* had previously been confirmed using a range of bacterial species, and the sensitivity

estimated as approximately 35 cfu (Starnbach *et al.*, 1989, Hay *et al.*, 1995).

Figure 44 Hospital water sample treatment for study of legionellae-amoebae interaction



3- RESULTS

3.1 The effect of temperature on uptake of Legionellae & multiplication in amoebae

Acanthamoeba spp. grow and multiply optimally between 25 and 30°C while legionellae have an optimum growth temperature of 36-37°C. When *L. pneumophila* was co-cultivated with *A. castellanii* , bacteria were taken up by amoeba host cells. The uptake kinetics at two different temperatures (30°C and 37°C) are shown in Figure 2. At the higher temperature, more legionella cells were recovered from the co-culture suspension than at the lower temperature. Possible explanations of this observation are (1) that numbers of extracellular legionellae increase by growth and division during the incubation period of the experiment and a *pro rata* increase in uptake occurs without a change in rate of uptake (2) that elevated temperature stimulates the rate of uptake of bacteria *per se* and that bacterial multiplication occurs predominantly *in vivo* (3) that a combination of (1) and (2) occurs.

Although the number of Legionellae recovered from the co-culture at 30°C was slightly less than those recovered after incubation at 37°C in two replicated experiments, the observed differences were not statistically significant by the Student's T test. When uptake of legionella by amoeba was compared with uptake of *E.coli* (30 min readings in Fig. 1) the rate for Legionellae was over twice that of *E. coli.* During the subsequent 2 h of incubation, viable intracellular bacterial numbers recovered differed markedly for the two bacterial species. Legionellae multiplied rapidly, in the intracellular environment whereas numbers of *E. coli* fell rapidly in the second hour. This probably reflects susceptibility of this species to killing

mechanisms of Acanthamoeba, and the lack of specific adaptive mechanisms for intracellular survival.

In order to obtain more information on Legionella-Acanthamoeba interaction, the effect of temperature on uptake kinetics of Legionallae by Acanthamoebae was investigated (Figures; 3, 4, and 5). Temperatures between 22-37°C were selected because this range encompasses the natural environmental temperatures of these micro-organisms. When legionellae and amoebae were incubated at 22°C, a 3-fold increase in bacterial numbers was evident after 10 d. Increasing the incubation temperature to 30°C or 37°C caused the number of recovered Legionellae to increase considerably and by day 10, a 5 to 6 fold increase in bacterial numbers had occurred.

Legionellae alone were incubated in the assay medium as a control, to assess the role of amoeba in legionella growth. In this case, bacterial numbers decreased progressively with incubation time at all temperatures tested , but more rapidly at 22°C than at 30°C and 37°C. Indeed, after 10 d incubation at 22°C, no bacteria were recovered in the sample. Controls incubated at 30°C and 37°C indicated that although Legionella remained viable over the incubation period of 10 d, they failed to multiply in the absence of amoeba.

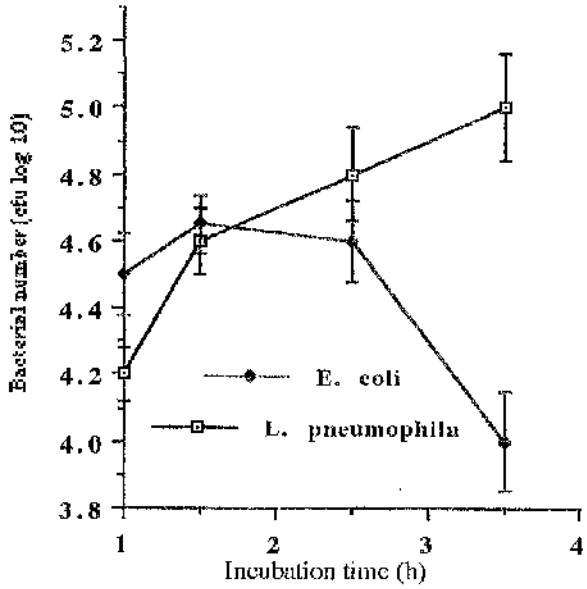
At 22°C and 37°C in co-culture, Legionellae numbers levelled off after 7 d incubation (Fig. 3 & 5), whereas at 30°C bacterial numbers recovered from the co-culture were still increasing at 10 d (Fig. 4). Interestingly, most of the amoeba host cells containing motile

legionellae were rounded up by day 7 and some amoebae had encysted by this time.

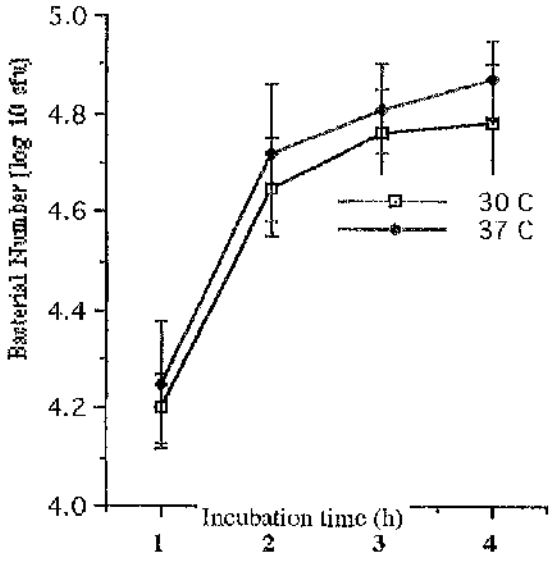
Figure 1. *L. pneumophila* and, *E. coli* numbers recovered from co-cultures with Acanthamoeba.
Mixtures of each bacterium and Acanthamoeba were incubated for given time points at 37°C followed by the addition of Gentamycin to kill bacteria not internalised by the amoebae (see Methods). After washing, aliquots of 0.1 ml of the amoeba suspension were lysed and plated for viable counts. The points on the graph represent the mean values from two experiments.

Figure 2. Uptake of *L. pneumophila* by *A. castellanii* at different temperatures.
L. pneumophila were co-cultivated with *Acanthamoeba* and incubated for up to 4 h at 30°C and 37°C. After Gentamycin treatment to kill extracellular bacteria, amoebae were washed and aliquots of 0.1 ml of amoeba suspension were lysed before bacterial numbers were determined by viable counts. The points on the graph represent the mean values from 2 experiments.

(Figure 1)

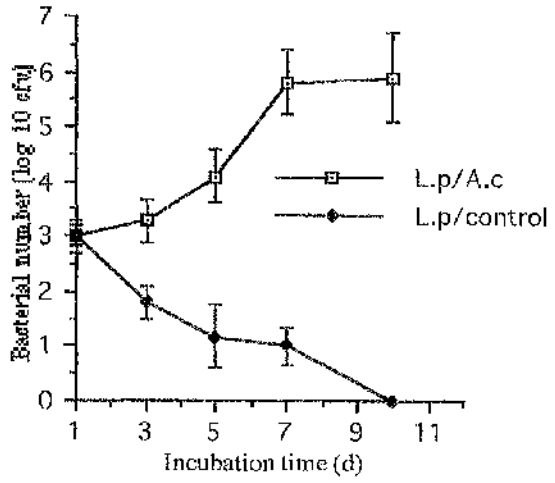


(Figure 2)

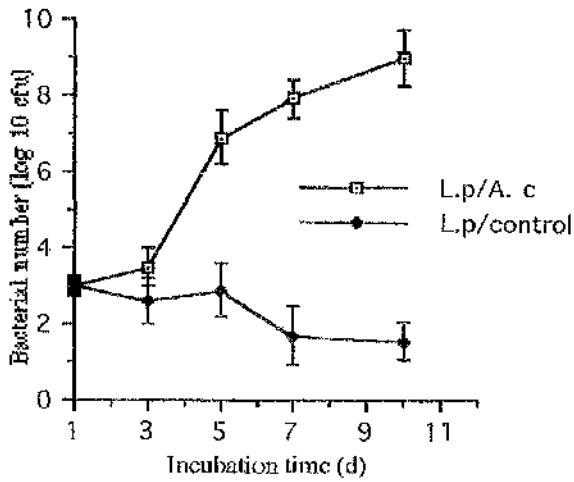


Figures 3, 4, and 5. Viable counts of *L. pneumophila* from co-cultures of *L pneumophila* and *A. castellanii* incubated at 22°C, 30°C, and 37°C. Controls consisted of *L. pneumophila* incubated under identical conditions in the absence of amoebae. The points on the graphs represent the mean values from 2 experiments. L. p/Ac = co-culture of *A. castellanii* and *L. pneumophila*, L. p = *L. pneumophila* alone.

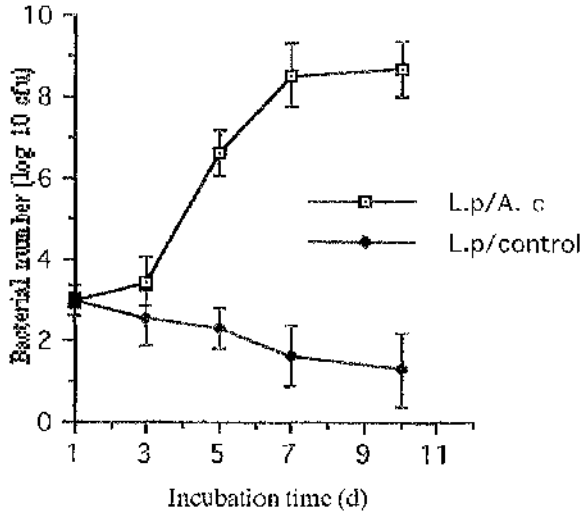
(Fig. 3)



(Fig. 4)



(Figure 5)



3.2 Effect of phagocytosis inhibitors on uptake of *L.pneumophila* and growth within Acanthamoeba

Cytoskelctal and metabolic inhibitors are useful probes for investigation of invasion of different host cells by pathogens (King *et al.* 1991). Free-living amoebae have proved useful in understanding the pathogenesis of legionella, particularly for other mammalian host cell types. The reason for this is, first, that bacterial multiplication within amoebae is not complicated by the intricate interaction of the host cytokine pathways and serum proteins (e.g., complement and antibody). Second, free-living amoebae are relevant because of the close relationship of these organisms with *Legionella spp.* in the environment (King *et al.* 1991).

Cytochalasin D is an inhibitor of microfilament-dependent phagocytosis and methylamine is an adsorptive phagocytosis inhibitor. In order to find out whether or not the phagocytosis of legionella by Acanthamoeba is either microfilament- -dependent or an adsorptive pinocytotic process, we investigated the effects of cytochalasin D and methylamine on uptake and intra-amoebal multiplication of *L. pneumophila*. The effect of inhibitors alone on the viability of Acanthamoebae and of Legionellae was assessed by enumeration of live amoebae or colony forming units (cfu) after different exposure times. The effect of inhibitors on Acanthamoeba viability was assessed by trypan blue exclusion (King *et al.* 1991). Figures 7 and 9 show the effects of cytochalasin D (2 μM) and methylamine (100 mM) respectively on the viability of Acanthamoebae over a 7 d experimental period. Neither cytochalasin D nor methylamine at these concentrations reduced viability or

inhibited amoeba growth in the assay medium, when compared with controls. The growth of Legionella in co-culture was not affected by cytochalasin D in the concentration ranges tested (Fig 6). There was no significant effect on growth of Legionellae when co-cultured with amoebae in the the presence of 1 or 2 μM cytochalasin D. When amoebae were omitted from the co-culture, Legionellae failed to grow as expected. The number of viable amoebae did not appear to be affected by either concentration of inhibitor in Page's medium (1988), when compared with non-treated amoebae in the same medium (Fig. 7).

The uptake of *L. pneumophila* and its subsequent multiplication in *A. castellanii* was affected by methylamine (Fig. 8). Methylamine at 50 mM and 100 mM inhibited the multiplication of Legionellae in amoebae. This inhibition was most marked at 7 d incubation, with no significant difference in bacterial numbers between the treated and untreated cultures in samples taken at day 0 and day 3. The inhibitory effect of methylamine at 100 mM was 10 fold greater than at 50mM. No significant growth-inhibitory effect of methylamine was observed at 10 mM in the assay medium.

The data provided here show that cytochalasin D has no effect on uptake and intracellular multiplication of *L. pneumophila* in *A. castellanii*. These findings are in agreement with similar studies carried out by King and colleagues (1991) on *Hartmanella vermiformis*. Methylamine at concentrations of greater than 50 mM inhibited uptake and subsequent intracellular multiplication of *L. pneumophila* suggesting the involvement of a microfilament-independent process, such as receptor-mediated pinocytosis.

Figure 6. Effect of cytochalasin D on bacterial numbers during co-cultivation of *L. pneumophila* with *A. castellanii.* Lp/Ac = co-culture of Acanthamoebae and Legionellae; cD^-A^+ = bacterial numbers (cfu) when no cytochalasin was added to co-culture. $cD^+ A^-$ = bacterial numbers (cfu) in the presence of cytochalasin D, but without amoebae, cD^-A^- = bacterial numbers (cfu) in absent of both inhibitor and amoebae.

Figure 7. The effect of cytochalasin D on growth of Acanthamoeba ; (1) viable cell count in the absence of cytochalasin D, (2) viable cell count in the presence of cytochalasin D (2 μM). Ac = *A. castellanii.*

(Fig. 6)
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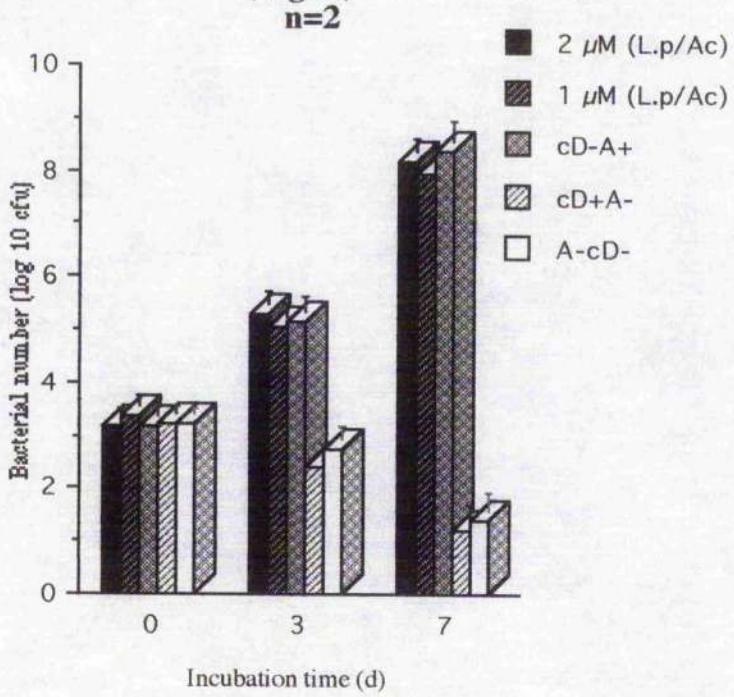

(Figure 7)
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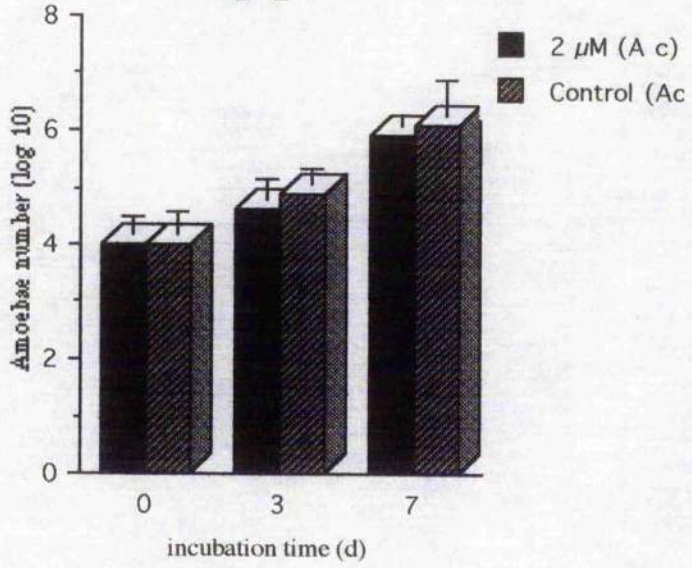

(Fig. 8)
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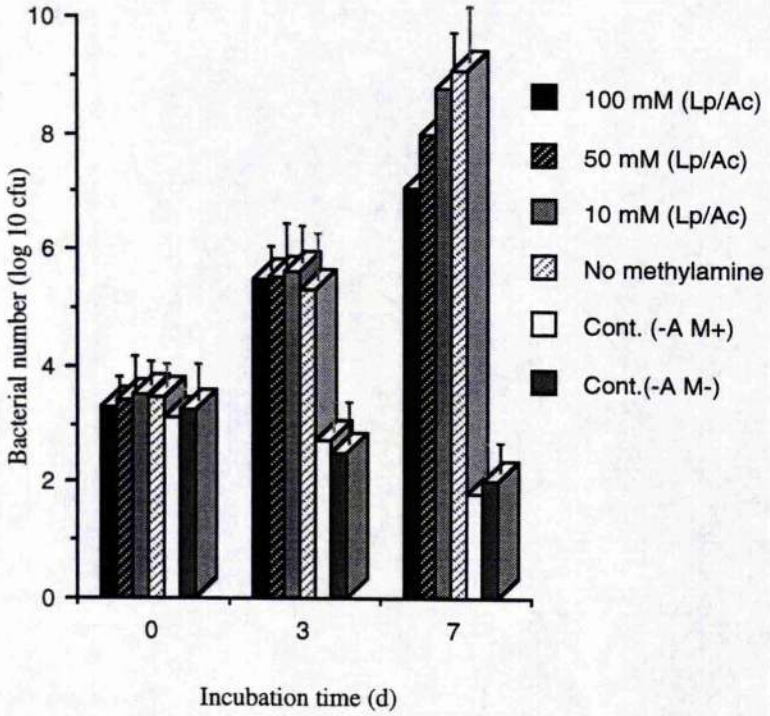


(Figure 9)
n=2

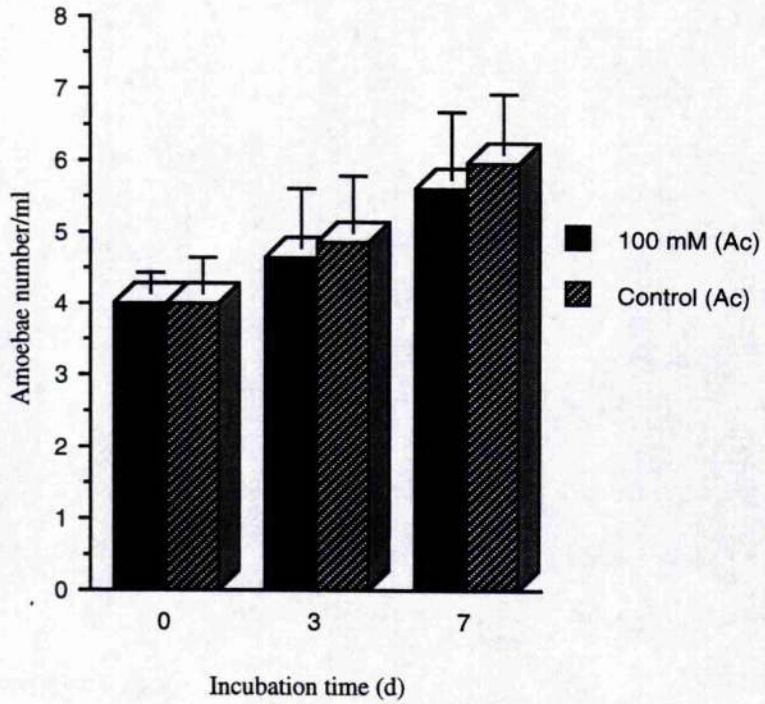


Figure 8. The effect of methylamine on growth of *L. pneumophila* co-cultivated with *A. castellanii*. Bacterial numbers (cfu) in the presence of 100 mM, 50 mM, 10 mM, and 0 mM methylamine in co-culture medium. As a control, bacterial numbers were determined in the presence and absence of inhibitor in the assay medium without amoebae . Lp/Ac = co-culture of amoebae and legionellae. -A, +M = bacterial numbers in the presence of methylamine, but without amoebae, -A, -M = bacterial numbers in absence of both of inhibitor and amoebae.

Figure 9. Growth of Acanthamoeba in the absence and present of methylamine (100 mM). *Ac; A. castellanii*

3.3 Legionellae cell envelope analysis

Lipopolysaccharide profiles

LPS was prepared from Legionellae by proteinase K digestion of whole cell lysates (Barker *et al.*, 1992). SDS-PAGE analysis and silver staining showed a compact ladder pattern of bands which was much tighter than comparable preparations from *S. enteritidis* and *E. coli*, used as standards (Fig. 12). This banding pattern of the legionella LPS was seen only when samples were prepared by the protease K digestion method of Nolte *et al.* (1986) and analyzed in 15% w/v acrylamide gels. Lower concentrations of acrylamide in the separating gel produced an unresolved, densely stained band at the gel front in the position associated with rough LPS of enterobacteria.

Although the LPS profiles of Legionella grown under various conditions were basically similar, there were some minor differences. Intra-amoeba-grown cells had about ten bands in the LPS ladder, whereas cells grown on BCYE$^+$ plates had only 7-8 bands (Fig. 12 & 13). Quantitative comparison of the LPS profiles of cells grown under different conditions was made on the basis of equivalent cell numbers in each preparation. The LPS content of the cell suspensions was not measured before proteinase K digestion.

Protein composition of Legionella

SDS PAGE analysis of proteins from cell envelopes and whole cells of *L. pneumophila* revealed that whole cells contained major proteins of apparent molecular weight 28kD, 45kD and a somewhat less intense band of 65kD. Protein profiles of Sarkosyl-prepared OMs from *in vitro* and intra-amoeba-grown legionellae are shown in Fig.

14. Based on staining intensity, a band of 28 kD appears to be the most abundant outer membrane protein (Gabay and Horwitz,1985). As with the LPS profiles, comparison of protein profiles of the variously grown cells was based on samples which contained approx. equivalent numbers of cells and equivalent protein concentrations. The estimation of the total protein content of the OM preparations of intra-amoeba-grown legionella was impossible because of the small amount of material available. Therefore, SDS-PAGE analysis revealed by Coomassie blue staining showed only the relative amounts of different proteins present in these preparations.

A 29 kDa protein has been identifed as the major porin MOMP (Ehret *et al.*, 1986), and is expressed under all growth conditions tested. The presence of a 15 kDa protein, characteristic of intra-amoeba-grown cells was a notable finding (lanes 6,7), although the relative amount of this protein varied between batches of cells (data not shown). This protein was not usually expressed in legionellae grown *in vitro* (however see Fig. 14, lane 7). Proteins in the range of 45-97 kDa were not major components in intra-amoeba-grown bacteria. SDS PAGE profiles of whole amoebae(lane 3) and amoebae subjected to identical procedures to those used for isolation of OM from legionellae (Lane 2) showed that amoebae possess a protein of approximately 15 kDa. The question then arises as to the origin of the 15 kDa band seen in amoeba-grown legionella. When *in vitro*-grown legionella ($BCYE^+$) were incubated with a sonicated extract of amoebae (data not shown), a 15 kDa protein was not detected in OMs prepared from such bacteria.

Western blotting of envelope proteins.

Immunoblots of Sarkosyl-insoluble legionella OMs (from cells grown under different conditions) and amoebae membranes were probed with rabbit anti-*L. pneumophila* (serogroup 1) polyclonal antibodies followed by Donkey anti-rabbit IgG-HRP conjugate (Fig. 15). Comparison of the immunoblots showed that the anti-legionella antibodies did not recognize any amoebal proteins (Lane 1). However, three proteins of intra-amoeba-grown *L. pneumophila* (including a 15 kDa component) were recognised by the probing antibodies (Lane 4, 5). In outer membrane preparations from *in vitro* grown *L. pneumophila*, a 66 kDa protein was recognised, with a weak band showing at approx. 45 kDa.

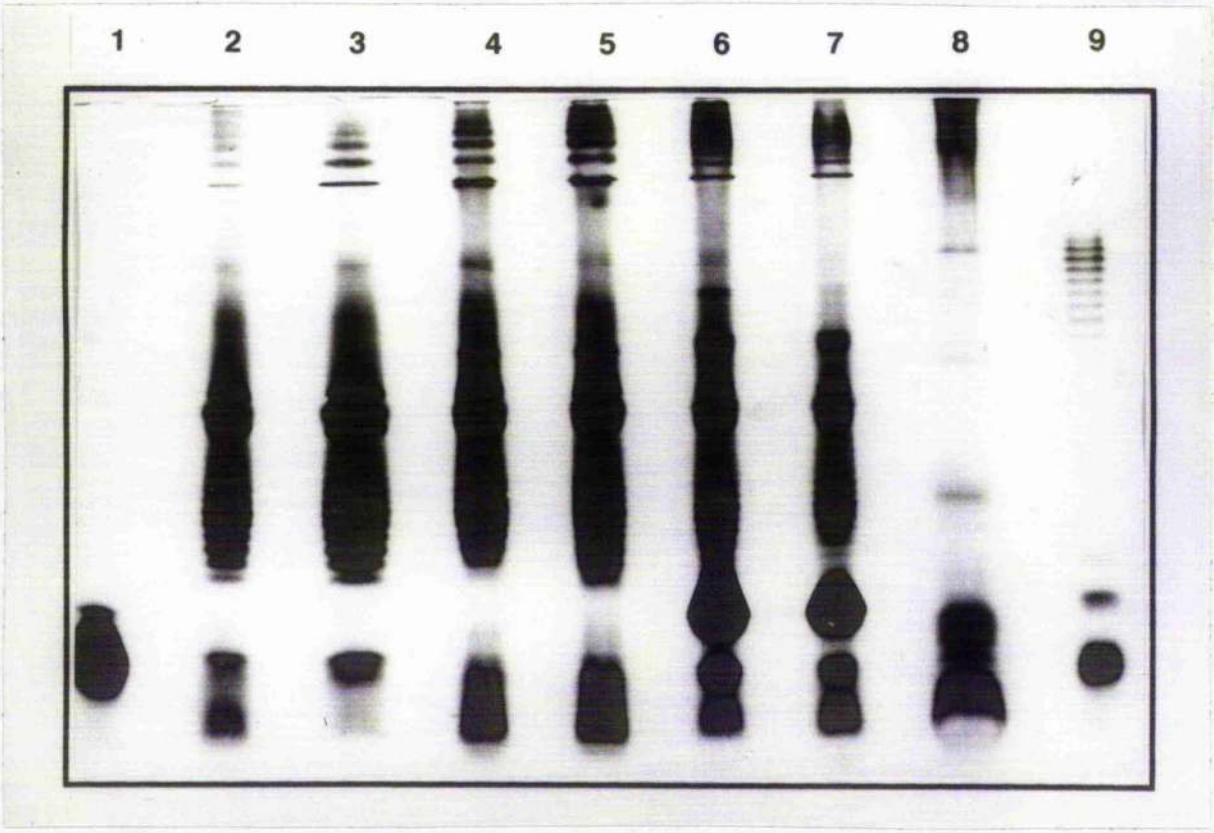


Figure 12 SDS PAGE analysis of lipopolysaccharides (proteinase K digestion) of *L. pneumophila* grown in BCYE+ at different temperatures, lane 1 = Purified rough *Salmonella typhimurium* LPS; lane 2 = *L. pneumophila* (cell envelopes) grown at 37°C ; lane 3 = *L. pneumophila* (outer membranes) grown at 37°C; lane 4 = *L. pneumophila* (whole cell) grown at 37°C; lane 5 = *L. pneumophila* (whole cell) grown at 30°C; lane 6 = *L. pneumophila* (outer membranes) grown at 30°C; lane 7 = *L. pneumophila* (cell envelopes) grown at 37°C; lane 8 = whole amoeba control; lane 9 = purified smooth LPS from *E. coli*.

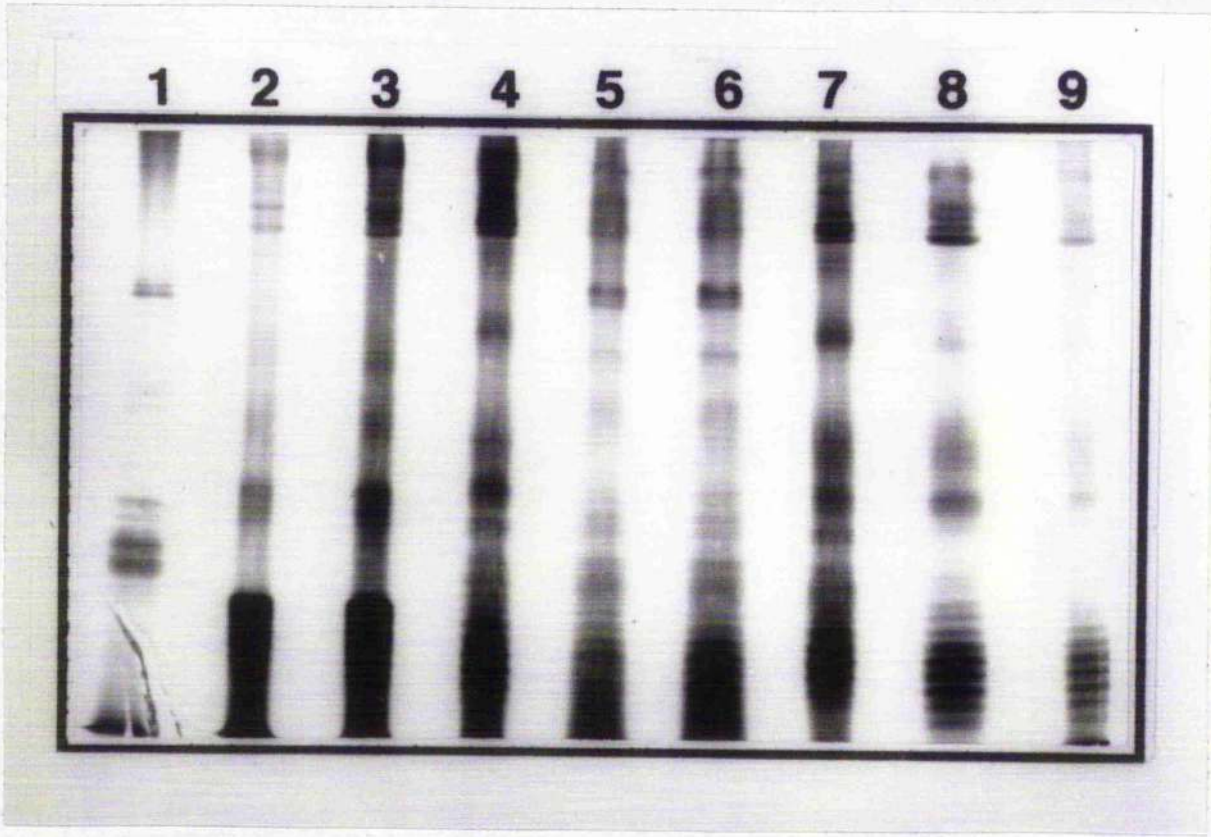


Figure 13 SDS PAGE analysis of lipopolysaccharides (proteinase K digestion) of *L. pneumophila* grown under different conditions,

lane 1 = *L. pneumophila* grown in amoebae at 22°C; lane 2 = *L. pneumophila* (grown on BCYE$^+$) at room temperature, lane 3 = *L. pneumophila* grown on BCYE$^+$ at 30°C; lane 4 = *L. pneumophila* grown on (BCYE+) at 37°C; lane 5 = *L. pneumophila* grown in amoebae at 30°C; lane 6 = *L. pneumophila* grown in amoebae at 37°C; lane 7 = outer membrane of *L. pneumophila* grown on BCYE+ at 37°C; lane 8 = outer membrane of *L. pneumophila* grown (BCYE+) at 30°C; lane 9 = amoeba whole cells

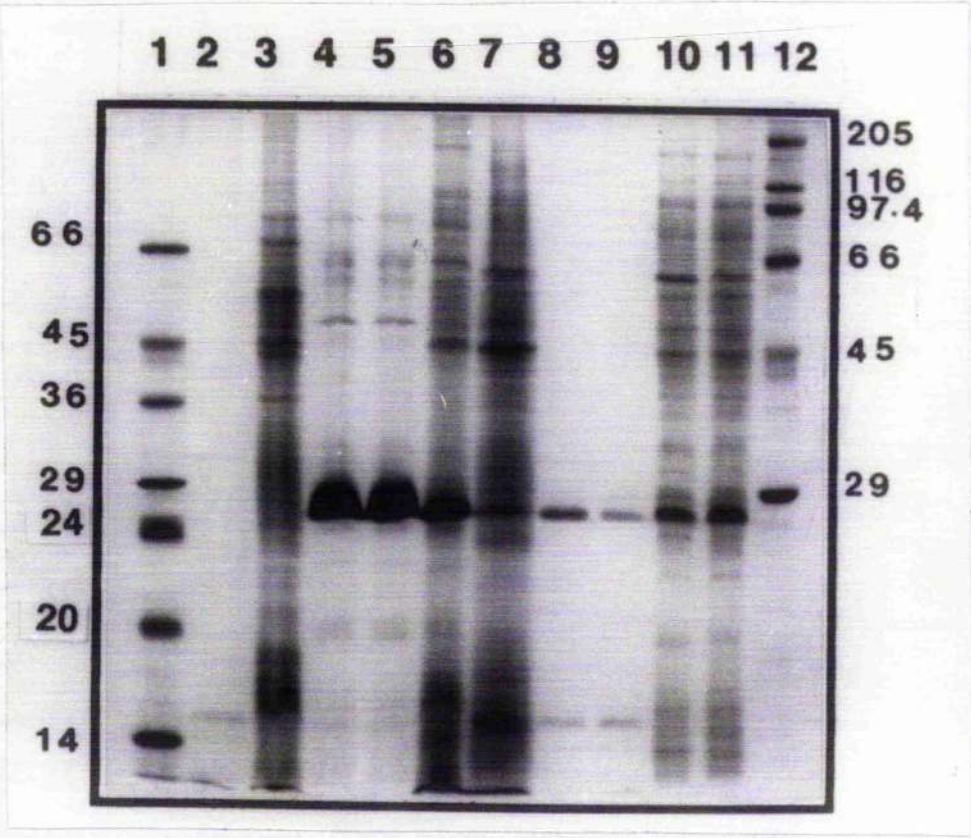


Figure 14 SDS PAGE protein profiles of cell envelopes of *L. pneumophila* grown under different conditions.

Lane 1 and 12 show molecular weigth marker (SDS 7 & 6H respectively). Figures on each sideof the gel represent molecular weight in kDa.; lane 2 - amoeba membrane preparation; lane 3 = amoeba whole cells; lane 4 = *L. pneumophila* (outer membranes) grown on BCYE at 37°C; lane 5 = *L. pneumophila* (outer membranes) grown on BCYE at 30°C; lane 6 = *L. pneumophila* (whole cells) grown on BCYE at 37°C; lane 7 = *L. pneumophila*(whole cells) grown on BCYE at 30°C; lane 8 = *L. pneumophila*(outer membranes) grown in amoebae at 37°C; lane 9 = *L. pneumophila* (outer membranes) grown in amoebae at 30°C; lane 10 = *L. pneumophila* (whole cells) grown in amoebae at 37°C; lane 11 = *L. pneumophila* (whole cells) grown in amoebae at 30°C.

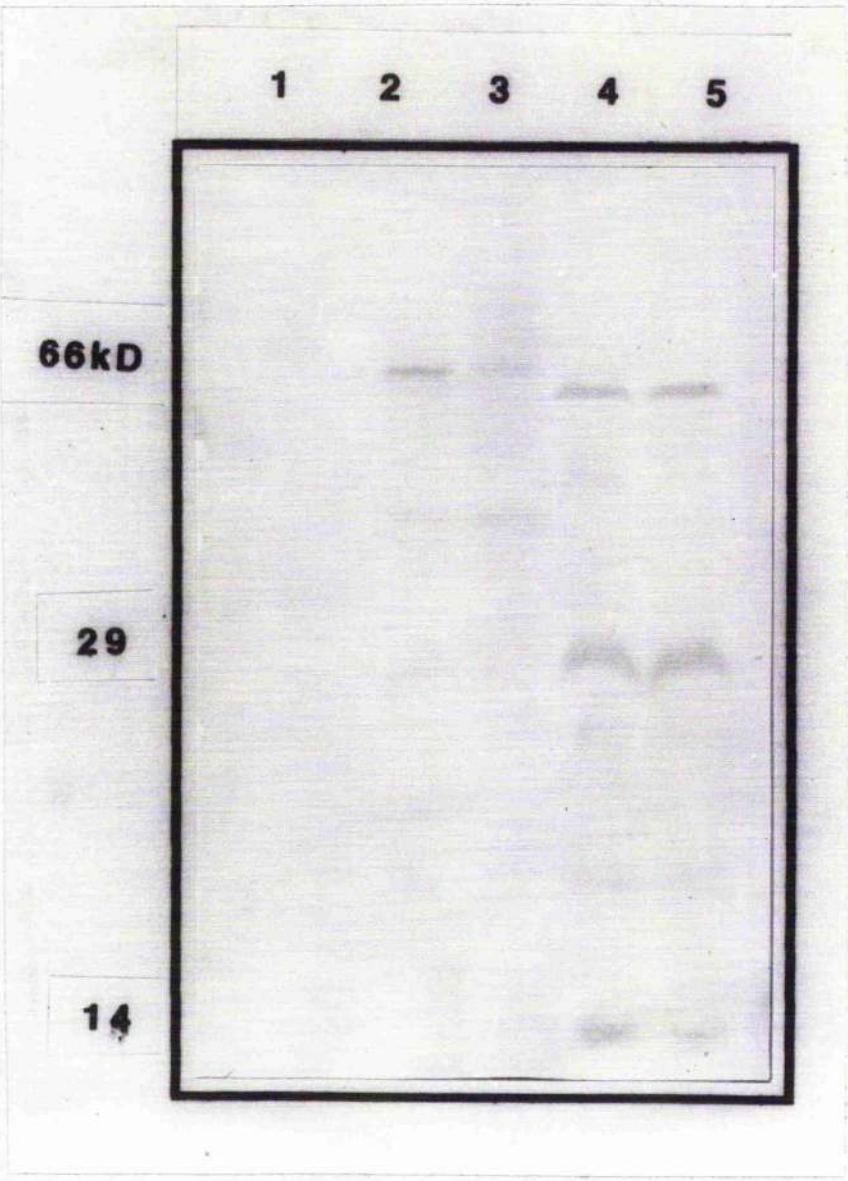


Figure 15 Western blot analysis of outer membrane proteins of *L. pneumophila* grown under different conditions and probed with anti-*L. pneumophila* polyclonal antibodies.

Lane 1 = whole amoebae; lane 2 = *L. pneumophila* grown on BCYE$^+$ at 30°C; lane 3 = *L. pneumophila* grown BCYE at 37°C; lane 4 = *L. pneumophila* grown in amoebae at 30°C; lane 5 = *L. pneumophila* grown in amoebae at 37°C.

3.4 Chemiluminescence in Acanthamoeba

This part of the study was designed first to investigate whether or not a detectable (by chemiluminescence) respiratory burst in Acanthamoeba is associated with the phagocytic uptake process and second, does legionella inhibit the respiratory burst? Should this prove to be the case, the mechanism of inhibition constitutes a further important question not addressed here.

Such respiratory bursts are a mark of the phagocytic process in professional phagocytes such as polymorphonuclear leukocytes in mammals. In this part of the work reported here, it was striking that the intensity of the CL response in amoebae, whatever the stimulus used, never approached that found with mammalian phagocytic cells. Always, the levels of CL detected were marginally above background, although these differences were ususally consistent. It appeared that in this CL study of legionellae-amoebae interactions, the methodology chosen was not the optimum in its sensitivity.

Heat-killed bacteria (*L. pneumophila* or *E. coli*) or latex beads were used as stimulants for phagocytosis and the response measured by lucigenin-dependent chemiluminescence. Latex beads served as an inert particle control, *E. coli* as a non-commensal 'normal' food source for amoebae, and legionella as the potentially invasive pathogen for the amoebae, able to avoid amoebal killing mechanisms under appropriate conditions. Our experiments involved determination of factors effecting chemiluminescence response of Acanthamoeba, such as amoeba cell concentration, particle nature and concentration, ratio of particles to amoebae, and incubation temperature. Chemiluminescent peak response produced by amoebae was mainly affected by amoeba concentration (Figure 17). The highest peak

response obtained was with 3-5 x10^6 amoebae in the reaction mixture, with viability being maintained at >95% as measured by dye exclusion. Higher numbers of amoebae were tested but viability fell markedly during the CL measuring period, invalidating the results. Background levels of CL with amoebae alone were between 0.6-0.9 mV. When two different stimulants (heat-killed *L. pneumophila* or latex beads) were compared and the amoeba cell concentration varied, *L pneumophila* gave a slightly higher response than latex at the higher amoeba concentrations (Figure 17). However, the peak responses were so small that the significance of any differences noted were questionable.

In attempts to increase the magnitude of the CL response, the effect of different forms of the stimulants, such as lysates of *L. pneumophila* and of *E. coli,* as well as latex beads were also tested (Figure 18). Fixed number of amoebae (10^6) were stimulated with lysates derived from approximately 10^7 particles as well as latex. Under these conditions, the highest response was produced by *L. pneumophila* lysate but again the CL responses were very low.

Because of the very meagre CL response of amoebae under all the conditions tested, when compared with the high responses in mammalian phagocytes, and the difficulty in interpretation of such low responses, this aspect of the work was not taken further.

Table 7. Lucigenin-dependent chemiluminescence response during phagocytosis in amoebae.

Amoeba (cell number)	Lucigenin (μM)	CL (mV) *E. coli*	*L. pneumo*	*Control
10^6	25	1.8	1.6	0.6
2 x 10^6	25	2.9	2.6	0.6
3x 10^6	25	5.3	4.9	0.6

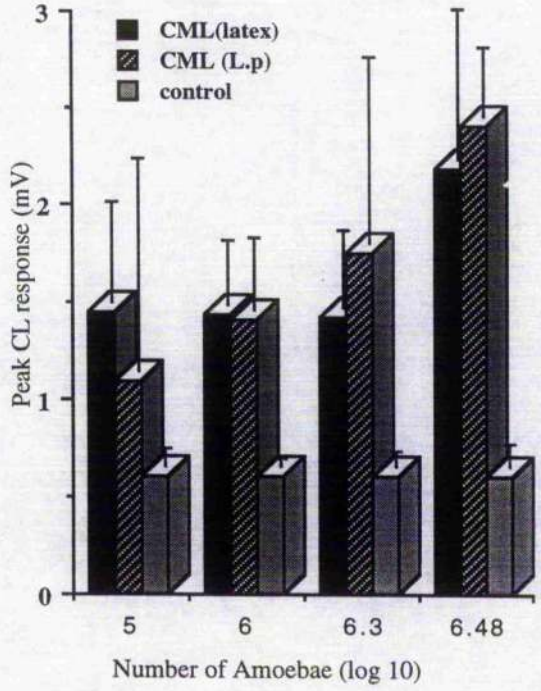
A. castellanii trophozoites were suspended in PBS at 30°C at the indicated cell concentration and 25 μM lucigenin was added to each tube. Chemiluminescence was then measured immediately after the addition of 10^9 particles (Latex beads, *L. pneumophila* whole cells or *E. coli* whole cells), using an LKB 1251 luminometer.

*Control, *A castellanii* + Lucigenin without stimulants

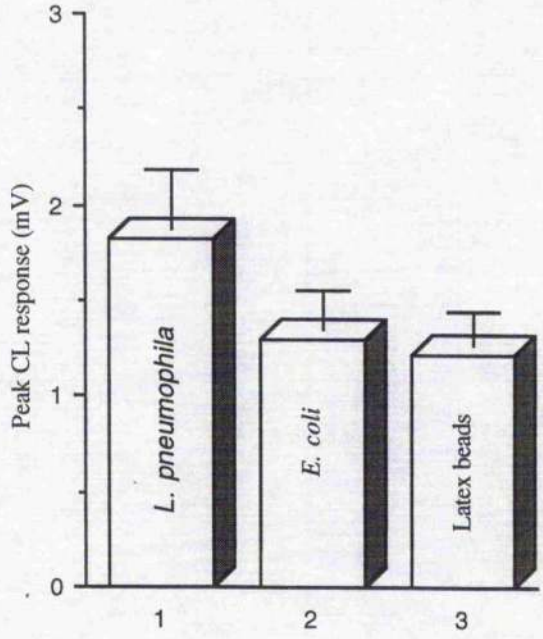
Figure 17. Lucigenin-dependent chemiluminescence response during phagocytosis by amoebae. *A. castellanii* trophozoites were suspended in PBS at 30°C at the indicated cell concentration and 25 µM lucigenin was added to each tube. Chemiluminescence was then measured in an LKB 1251 luminometer as the peak response in mV, after the addition of 10^8 particles.

Figure 18. Lucigenin-dependent chemiluminescence response during phagocytosis by amoebae. *A. castellanii* trophozoites (10^6) were suspended in PBS at 30°C, and 25 µM lucigenin was added to each tube. Chemiluminescence was then measured in an LKB 1251 luminometer as peak response in mV, after the addition of lysates from 10^8 *L. pneumophila*, *E. coli* or latex beads (10^8).

(Figure 17)
n=2



(Figure 18)
n=2



3.5 Phagosome-lysosome fusibility in Acanthamoeba, following phagocytosis of Legionellae

In order to understand the nature of events following uptake of Legionellae by Acanthamoebae, we examined the processes of phagocytosis and phagosome-lysosome fusion in Acanthamoeba infected with *L. pneumophila* or *E. coli.* Amoeba lysosomes were labeled with fluorescent marker (acridine orange), and fluorescence microscopy was used to follow transfer of the marker into the phagocytic vacuole, indicating phogosome-lysosome fusion.

When freshly labeled with fluorescent vital dye (acridine orange), amoebae lysosomes appeared as sharply defined red/orange inclusions (Fig. 20). Phago-lysosome formation was recognised by transfer of acridine orange into the phagosome containing *E. coli* (Fig. 23). In the absence of fusion, legionellae appeared as weakly fluorescent particles against a dark background (Fig. 22).

As shown in Table 3, *L. pneumophila* was taken up by phagocytosis more effectively than *E. coli* at the lower incubation temperature of 22°C, although neither bacterium was taken up at a high rate when compared to uptake at 37°C. The differences in uptake between the two bacteria were much less marked at 37°C, perhaps reflecting the fact that uptake rate is approaching a maximum level in both cases. Whatever the incubation temperature, phagosome-lysosome fusion was always inhibited in the case of *L. pneumophila* ($p<0.05$) when compared to *E. coli.*

Fluorescence micrographs of phagosome-lysosome fusion with *E. coli* and with latex beads are shown in Figures 23 and 24 respectively. Phase contrast (Fig. 21) and differential interference contrast (Nomarski) microscopy (data not shown) were used to check

specimens used in the fluorescence studies in order to confirm that bacteria were truly intracellular, and not simply surface located.

We attempted to confirm the above findings in an ultrastructural study using cytochemical staining for acid phosphatase, an enzyme marker for lysosomes. Preliminary results showed good resolution of intracellular legionella (Fig. 10 & 11) and there was good activity of acid phosphatase in the lysosomes of Acanthamoeba (data not shown). However, the interpretation of the results of cytochemical experiments where uptake of Legionella was involved was not possible because of relatively high levels of acid phosphatase in legionellae. This activity resulted in staining of both phagosomes and lysosomes, making it impossible to follow the process of phagosome-lysosome fusion and its inhibition.

Table 8 Fusion Index (FI) and Phagocytosis Index (PI) of *A. castellanii* during Phagocytosis.

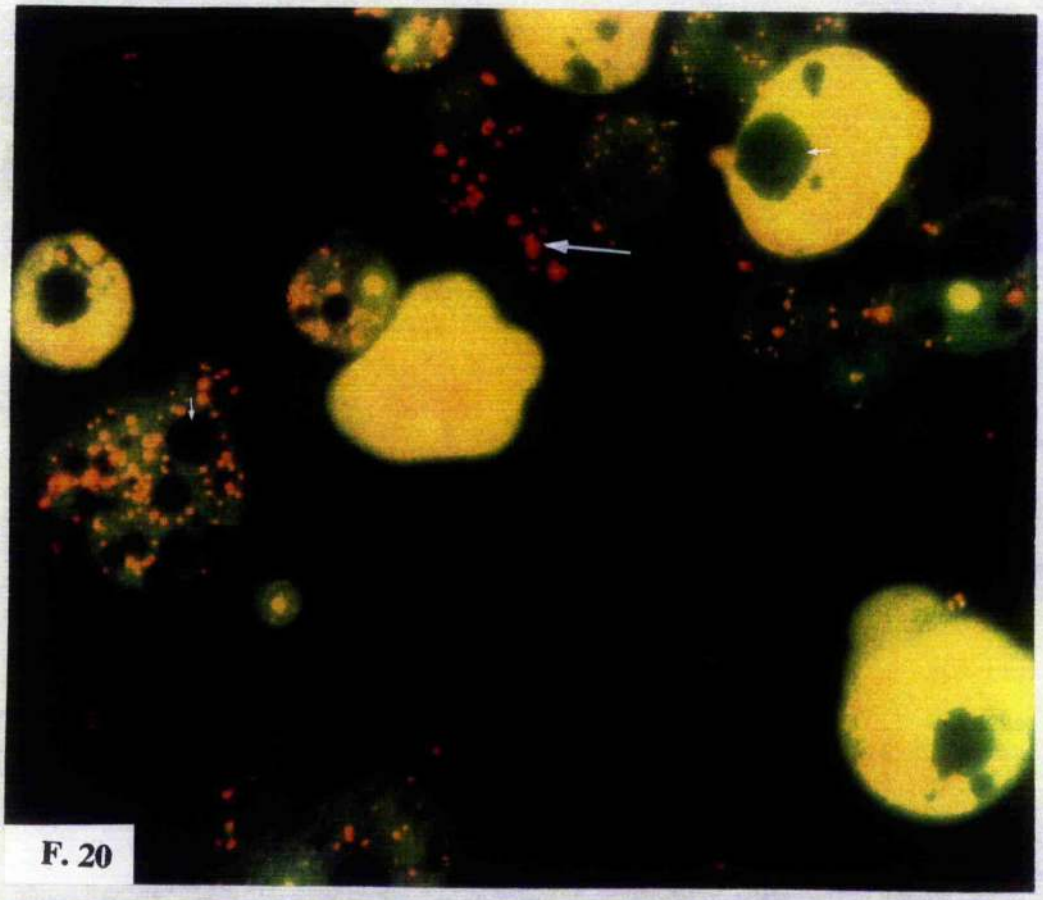
	Fusion index				Phagocytosis index			
	L. pneumophila		*E. coli*		*L. pneumophila*		*L. pneumophila*	
C°	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M
22	8.3	4.8	19.3	5.9	13.4	1.2	2.7	0.8
30	3.2	2.6	17.1	3.9	18.9	2.7	10.4	2.0
37	38.3	7.4	153	16.3	148.3	8.5	139.4	14.7

s.e.m. = standard error from mean (n=3)

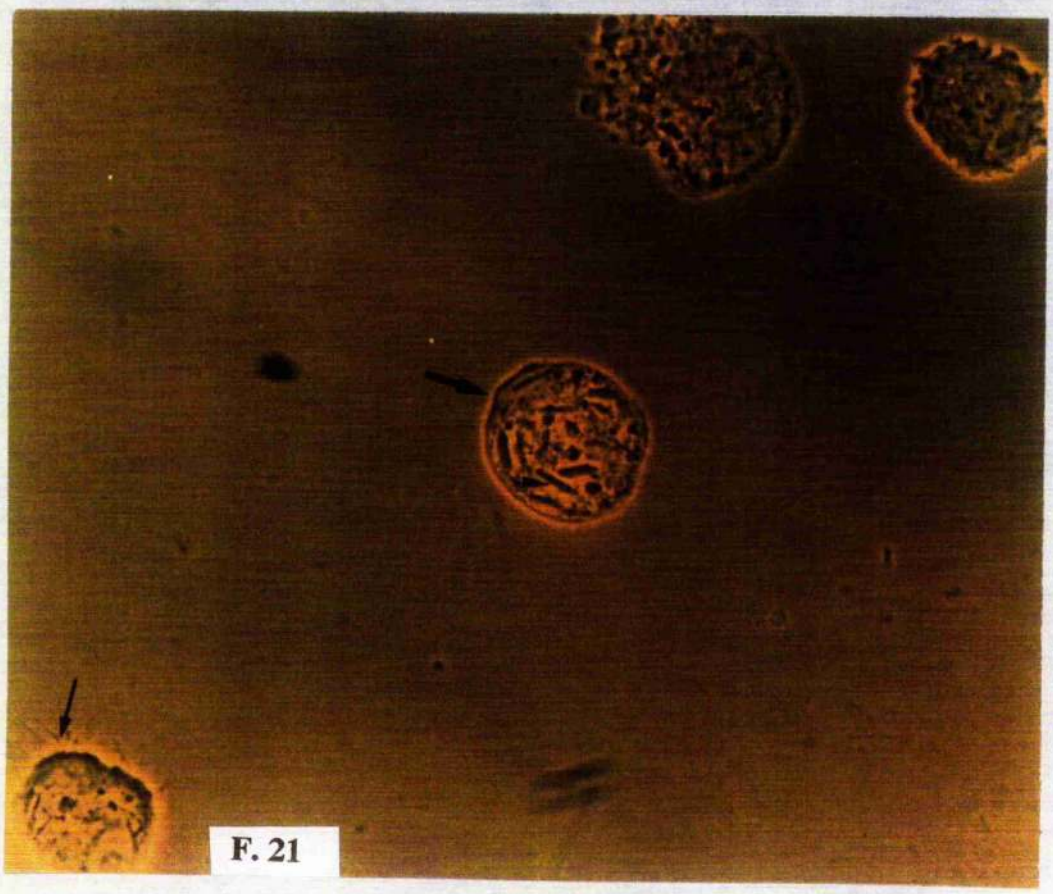
Fusion Index = Positive fusion % x Average number of fused phagosomes/cell. Positive fusion % = Fused phagosomes /100 cells. Phagocytic Index(PI) = Positive phagocytosis % x Average number of phagocytized particle/cell.

Figure 20. *A castellanii* trophozoites labeled with acridine orange. Lysosomes show as sharply defined red/orange inclusions. × 1100 photography; Leitz Orthoplan, Fujichrome professional 100D.
Thick arrows; amoeba lysosomes labeled with acridine orange.
Thin arrows; contractile vacuoles

Figure 21. *L. pneumophila* phagocytosed by *Acanthamoeba* trophozoites, Phase contrast × 750.
Leitz Orthoplan, Fujichrome professional 100D
Thick arrow: Acanthamoeba trophozoite.
Arrow head: intracellular legionella.



F. 20

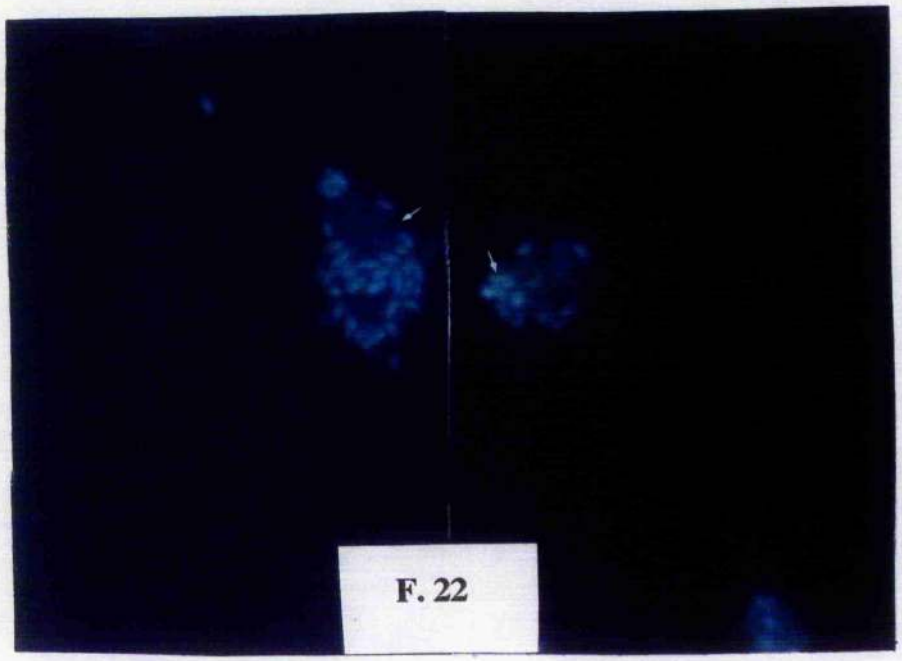


F. 21

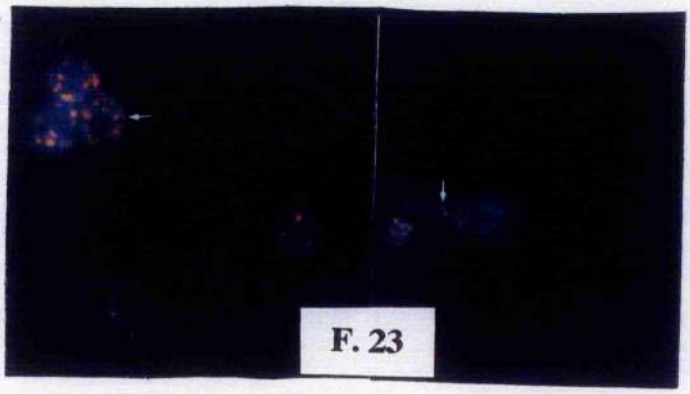
Figure 22. Intracellular *L pneumophila* in *A. castellanii,* stained with acridine orange × 400.
L. pneumophila cells showing as pale blue rods against a darker background in *A. castellanii.* Arrows; intracellular legionella.
Leitz Orthoplan. Fujichrome professional 100D

Figure 23. *E. coli* phagocytosed by *A. castellanii,* × 130
E. coli show as pink bodies against a blue background.
Arrows; intracellular organisms.
Leitz Orthoplan. Fujichrome professional 100D

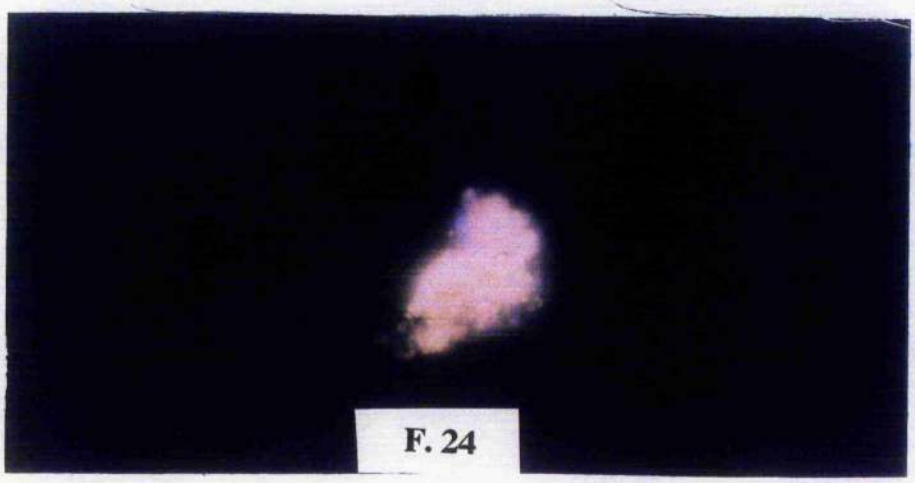
Figure 24. Fluorescein-labelled latex beads phagocytosed by *A. castellanii* × 400.
Trophozoite packed with phagocytosed latex beads, showing as pink bodies on a blue background.
Leitz Orthoplan. Fujichrome professional 100D



F. 22



F. 23

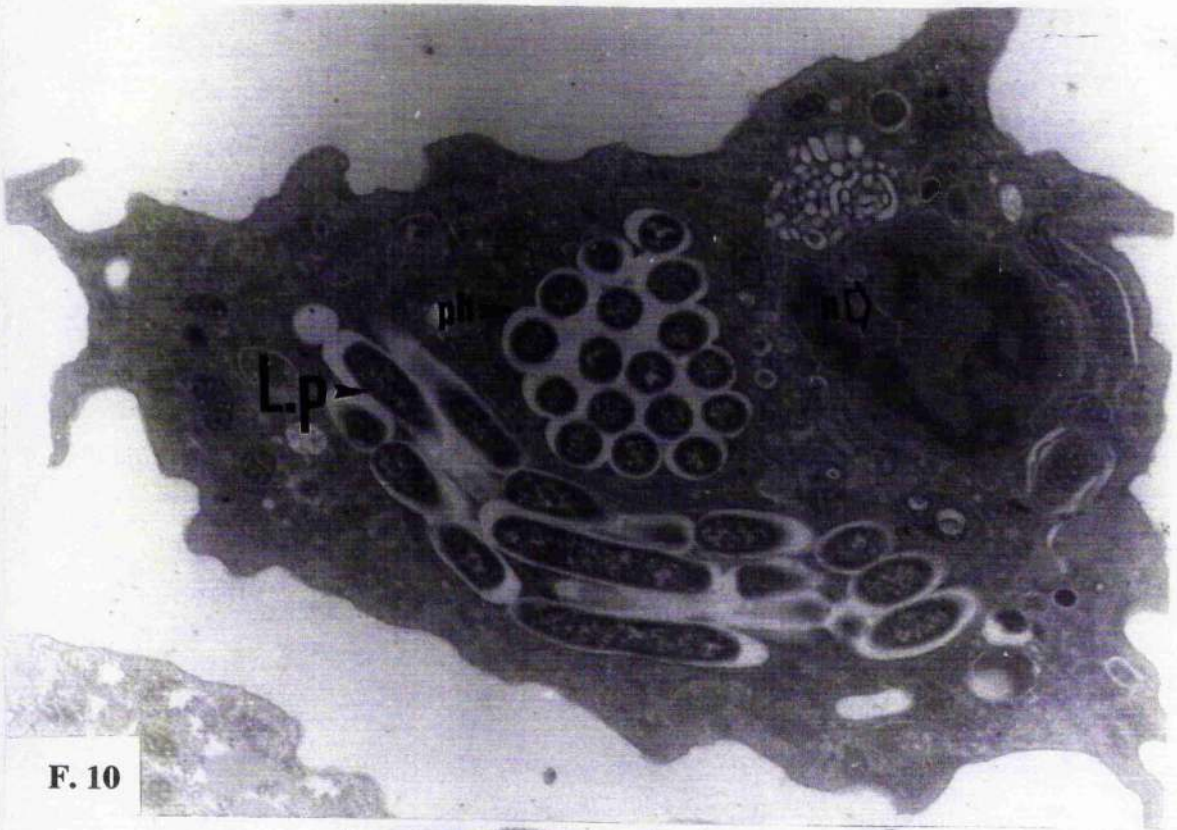


F. 24

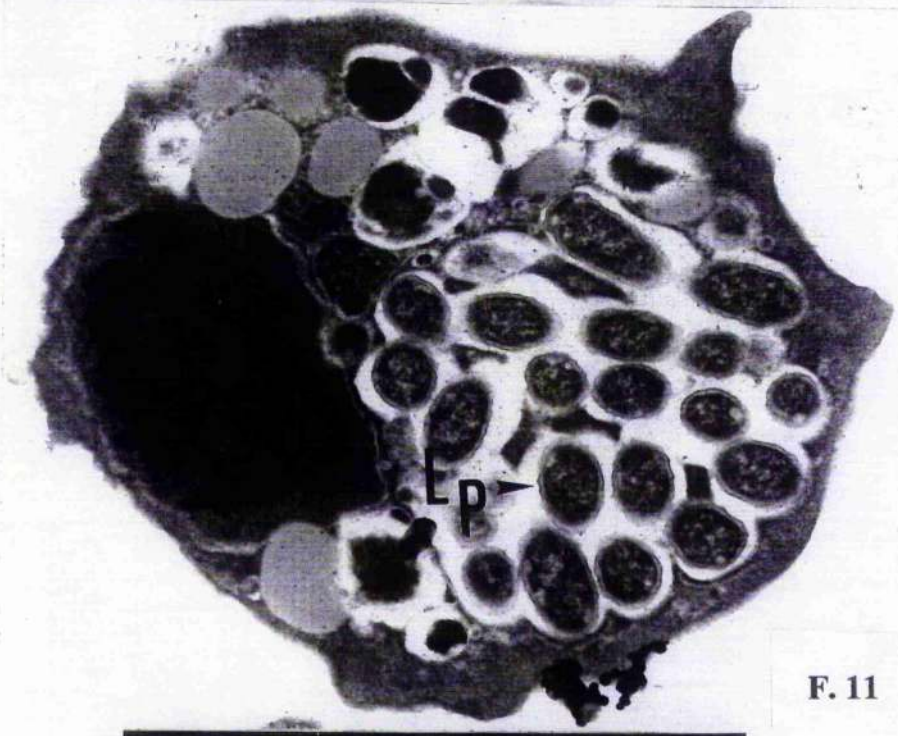
Figures 10 & 11 Transmission Electron Microscopy (TEM) of thin sections of *L. pneumophila* within *Acanthamoeba* vesicle, × 4400,

L. p = intracellular legionella, n = amoeba nucleus.

Ph = amoebae phagosome.



F. 10



F. 11

M4400 <==>1.7µm

U80-0000

3.6 Association of legionellae and amoebae in hospital water supplies

Associations between Legionellae and amoebae were investigated in hot and cold water supplies from 69 hospitals. The 138 samples fell into 4 major groups, based on the presence or absence of legionellae and of free living amoebae (Fig. 16). About 19% of hospital water samples were contaminated by both legionellae and amoebae. At least one genus of free-living amoeba or ciliate was found in 46% of the water samples (Figure 19). This observation is very significant from the public health point of view, because of the possible carriage of intracellular legionellae. Legionellae were not grown from 27% of samples in which free-living amoebae were isolated. Only 3% of the samples yielded legionellae, but not free-living amoebae. A fourth group contained neither legionellae nor amoebae.

The mean concentration of Fe (mg/L) and pH value for each of the 4 sample groups are shown in Fig. 41. Interestingly, the fourth group of water samples from which neither amoebae nor legionellae were isolated had the lowest mean values for Fe and pH, compared with others.

Tentative identification of free-living amoebae was based on morphological features and video film of both unidentified protozoa and authentic type cultures. They were then put into different categories according to these characters (Figure 19). About 54% of isolates were identified as Acanthamoebae, Hartmanellae, and Vahlkampfidae. Ciliates and other amoebae (45%) were categorised as unidentified protozoan isolates. Figures 25-37 show micrographs

taken from video-stills of different free-living amoebae isolated from hospital water supplies.

Each sample from the amoeba -positive, legionella-negative group was cultured on BCYE+ plates and also were subjected to PCR for *L. pneumophila* prior to incubation. No Legionellae were grown on BCYE+ agar and also no *L. pneumophila* DNA amplification products were detected on agarose gel following PCR (Fig. 38).

Each sample from the amoeba -positive, legionella-negative group was mixed with amoeba saline (1:1v/v) and enriched by addition of acid-washed cysts to 10^2-10^3/ml, generated from the same sample (see Materials and Methods). The enriched sample was then incubated at 30°C for one week before further testing. After incubation, and subsequent culture, *L. pneumophila* colonies were identified in 15% of the samples from this group (Table 9). Each legionella isolate was subjected to the Mercia slide agglutination test for serogroup identification. Of the 6 positive legionella samples, 4 were serogroup 1 and 2 samples were non-serogroup 1 (serogroup 2-12). Of these 2 samples, one agglutinated strongly whereas the other gave a very weak reaction. The same 6 samples were sent to the PHLS Legionella Reference Laboratory at Colindale, London, for serogrouping, strain identification by monoclonal antibody typing, and for restiction fragment length polymorphism analysis to identify genetic clones within the group. The samples which tested as serogroup 1 by the Mercia slide agglutination test were confirmed as serogroup 1 by the Colindale laboratory, and the 2 non-group 1 isolates were also grouped by Colindale as 4/10/12/14. Interestingly, the serogroup 1 samples were of different genetic clones, as evidenced by the differences in RFLP

banding patterns and monoclonal antibody typing (table 9). This result reinforces the conclusion that the legionellae came from different origins and did not arise through any cross-contamination between water samples during their handling within our laboratory.

These water samples yielding legionellae after the above enrichment and culture procedure, were again subjected to PCR to confirm the identity of legionellae. Amplification products (0.8 kb), characteristic of those expected of *L. pneumophila* (Figure 39), were obtained from the incubated enrichment mixture in all those samples which yielded legionellae by culture but tested negative for legionellae by both culture and by PCR before the enrichment step. The *L. pneumophila* control in Fig 39 (lane 9) was much weaker than either the DNA Gene Amp Kit control (lane 7) or the water samples (lane 1-6) probably because of the different way in which the DNA of water samples had been extracted (see Materials & Methods 2.7.5). In each positive sample, the PCR product was confirmed as *L. pneumophila* DNA by hybridization using southern blots with a specific oligonucleotide probe for *L. pneumophila* (Figure 40).

(Figure 16)

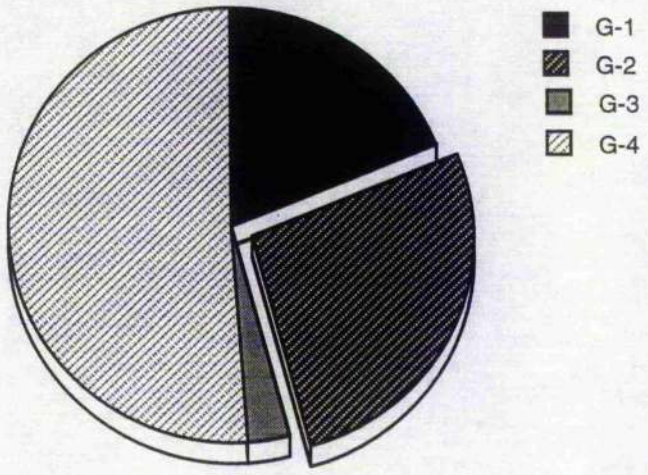


Figure 16 Grouping of hospital water samples (138 in total) with respect to the presence/absence of legionellae and amoebae.

G1, amoebae-positive, legionella-positive samples (19% of total).

G2, amoebae-positive, legionella-negative samples (27% of total).

G3, amoebae-negative, legionella-positive samples (3% of total).

G4, amoebae-negative, legionellae-negative samples (51% of total).

(Figure19)

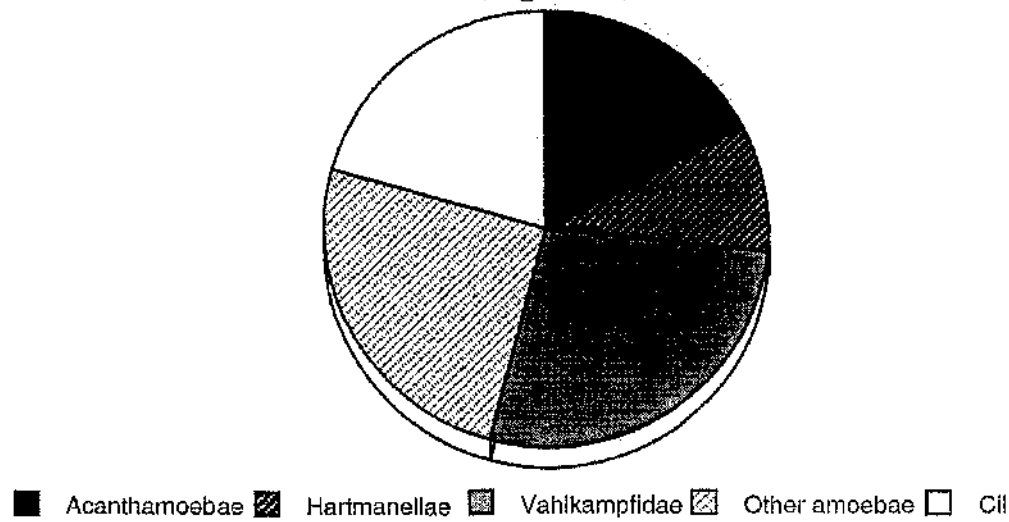


Figure 19 Relative abundance of different genera of free-living amoebae from hospital water samples.

Acanthamoebae (39 = 17%),

Hartmanellae (21 = 9%),

Vahlkampfidae (62 = 28%),

Other free-living amoebae (57 = 25%)

Ciliates (46 = 21%).

Note that some water samples contained more than one genus.

(Figure 41)

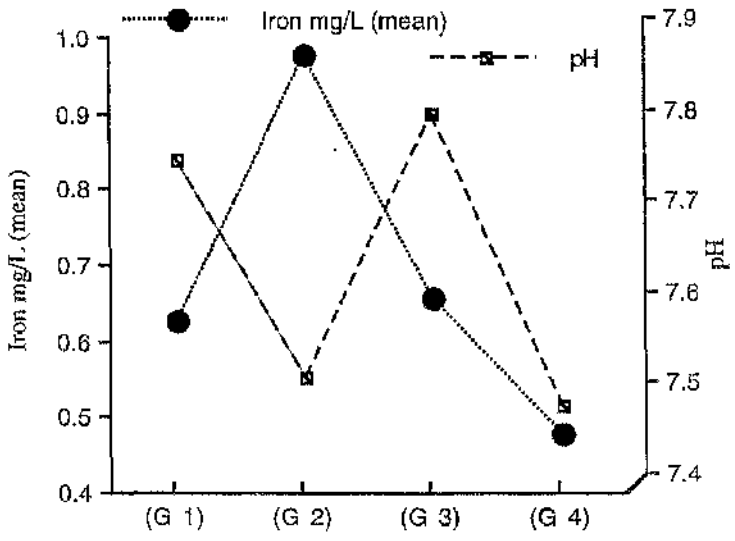


Figure 41 The mean Fe concentration (mg/L) and pH value of water samples containing amoebae or legionellae.

G1, amoebae-positive, legionellae-positive samples.

G2, amoebae-positive, legionellae-negative samples.

G3, amoebae-negative, legionellae-positive samples.

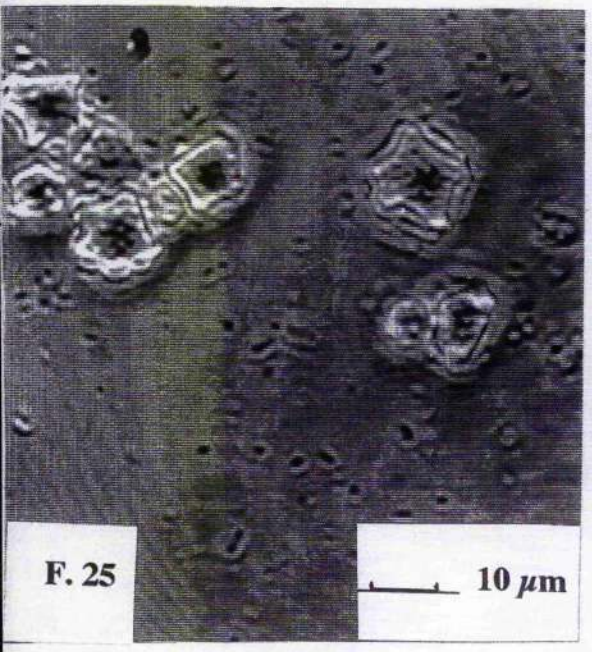
G4, amoebae-negative, legionellae-negative samples.

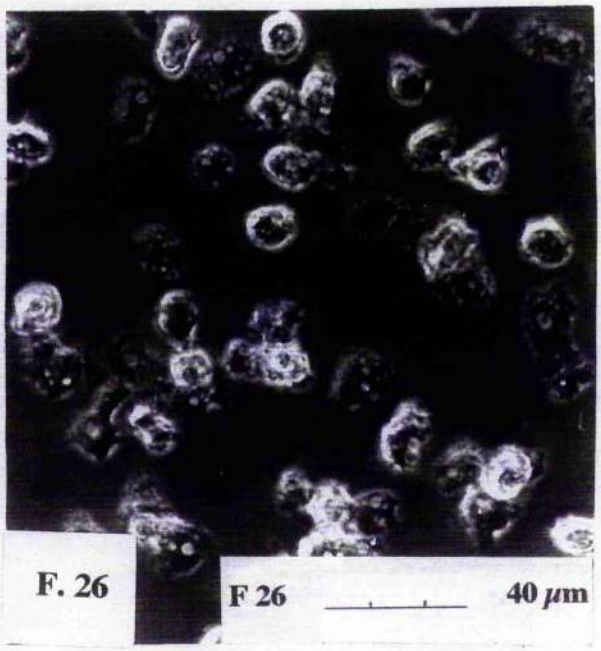
Figure 25. Water sample 74 (cold) showing Acanthamoeba cysts (video tape still)

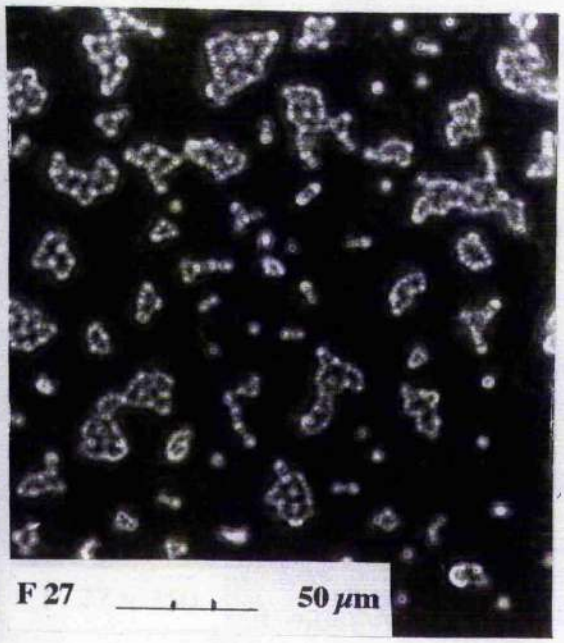
Figure 26. Water sample 74 (cold) showing Acanthamoeba trophozoites, some in pre-cyst stage.

Figure 27. Water sample 57 (cold) showin Vahlkampfia. cysts

Figure 28. Water sample 14 (cold) showing a mixture of valkampfids and Gymnamoebae.


F. 25
10 μm

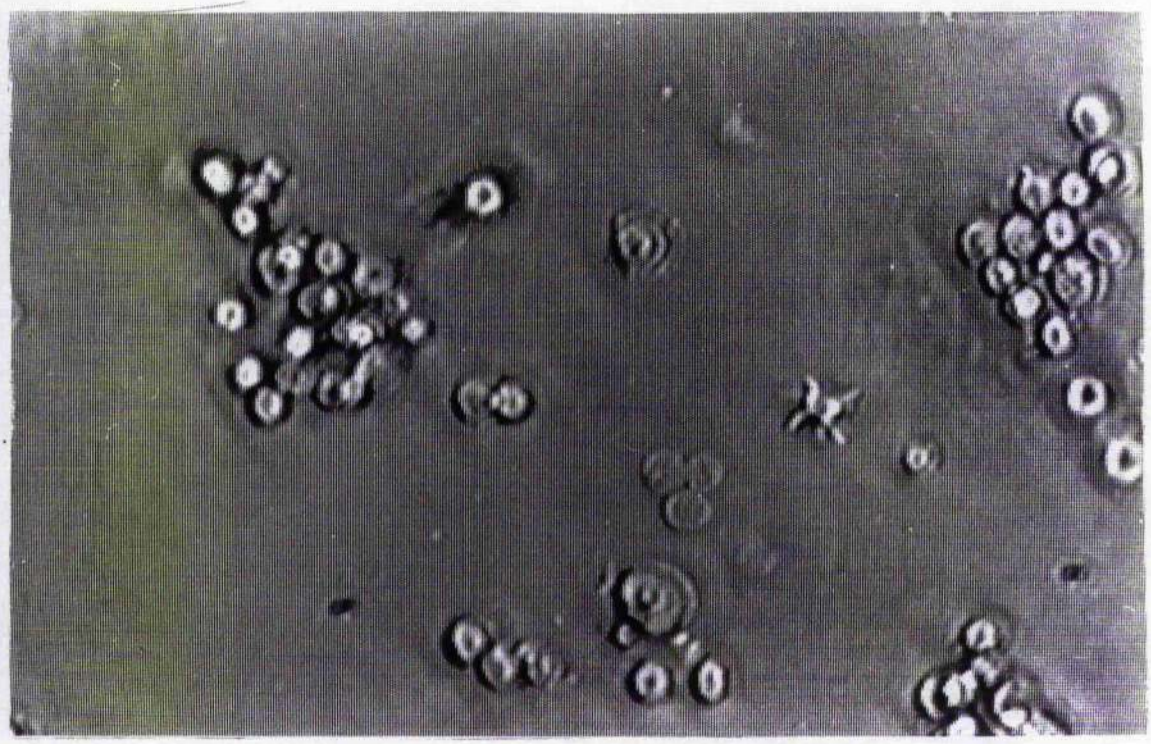

F. 26
F 26
40 μm


F 27
50 μm

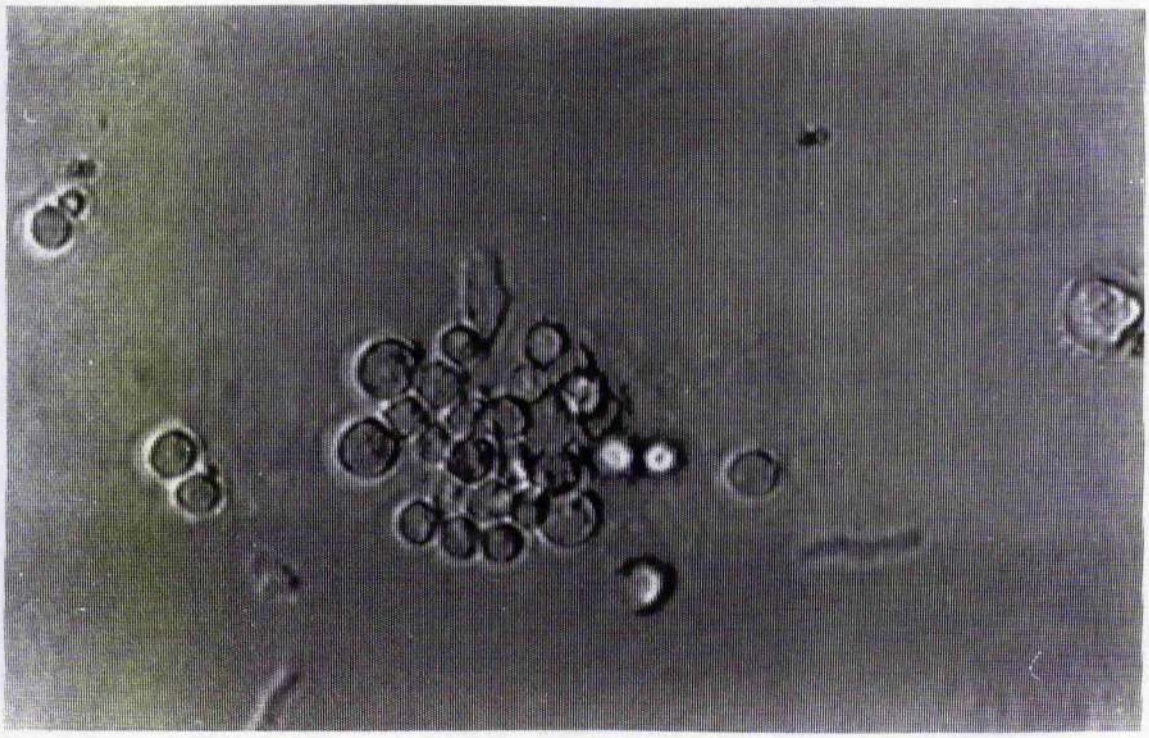

F 28
50 μm

Figure 29. Water sample 82 (hot) showing a mixture of amoebae including Hartmanella and Vahlkampfids

Figure 30. Water sample 16 (cold) showing a mixture of amoebae including Hartmanella



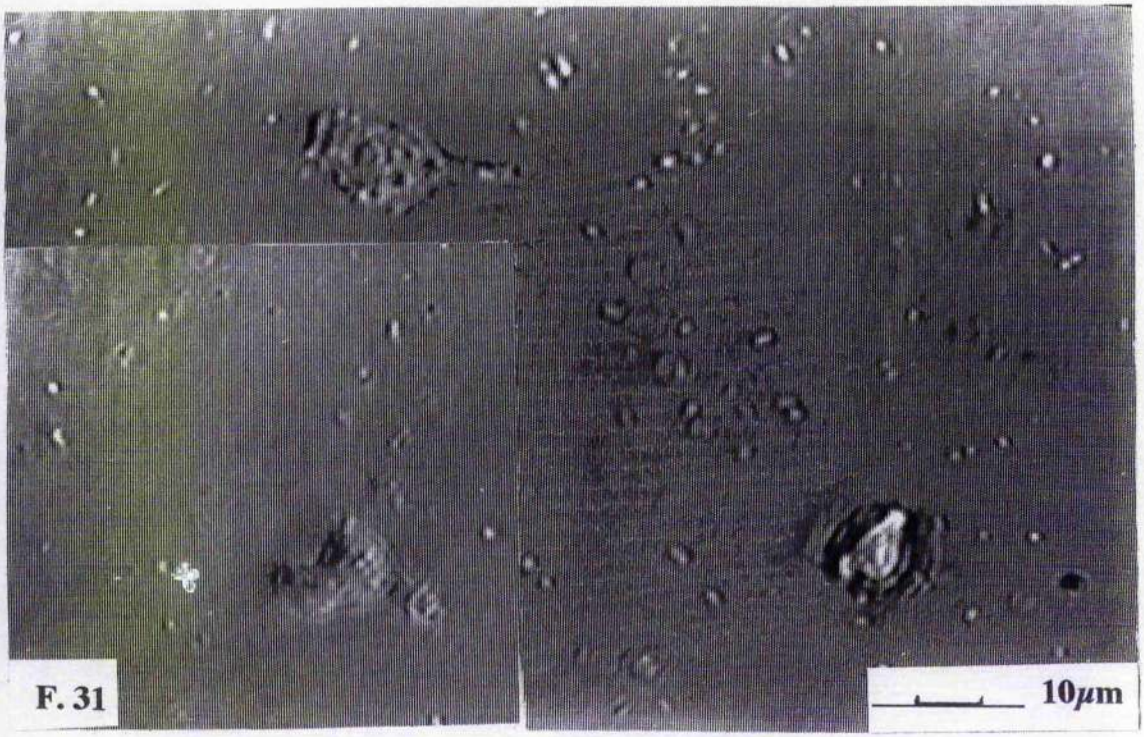
F. 29 10 μm



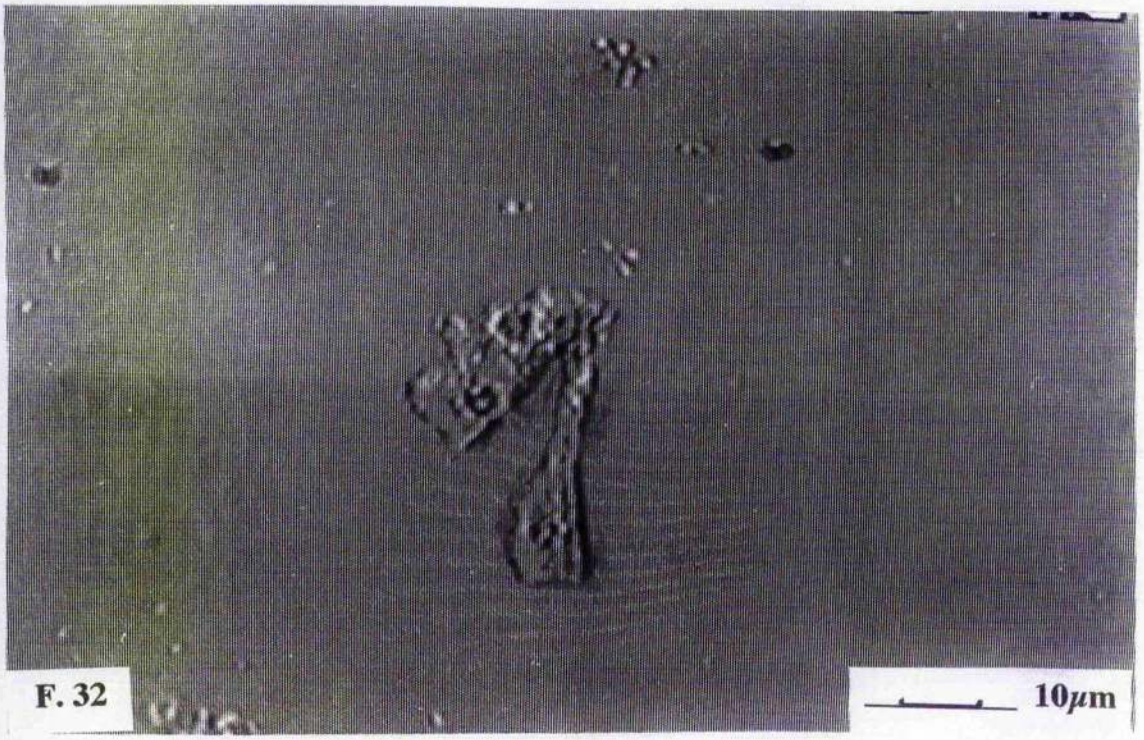
F. 30 10 μm

Figure 31. Water sample 74 (cold) showing a moeba spp,

Figure 32. Water sample 82 (hot) showing a mixed amoeba infection, which includes limax amoeba



F. 31

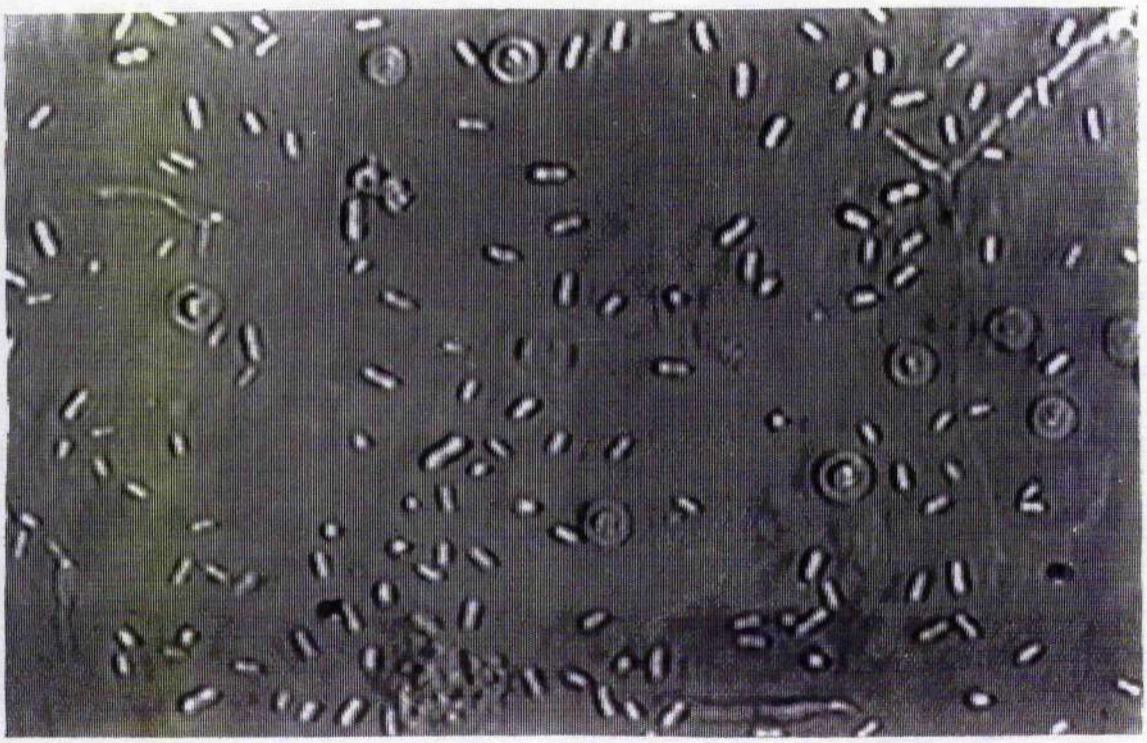


F. 32

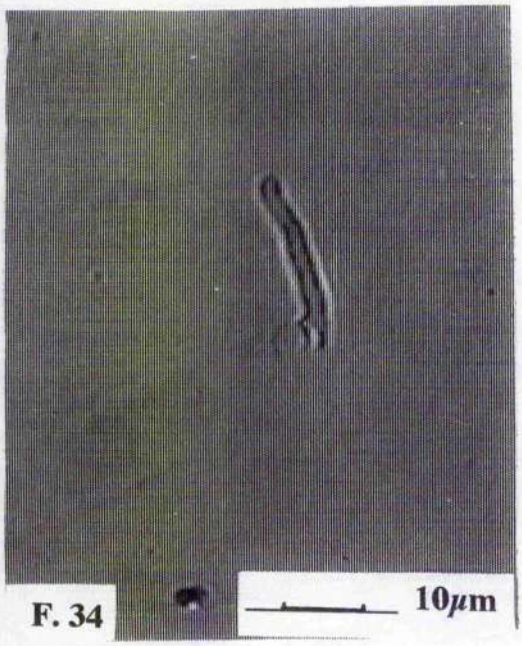
Figure 33. Water sample 47(hot) showing Ciliates and Gymnamoeba cysts

Figure 34. Water sample 5 (hot) containing Hartmanella

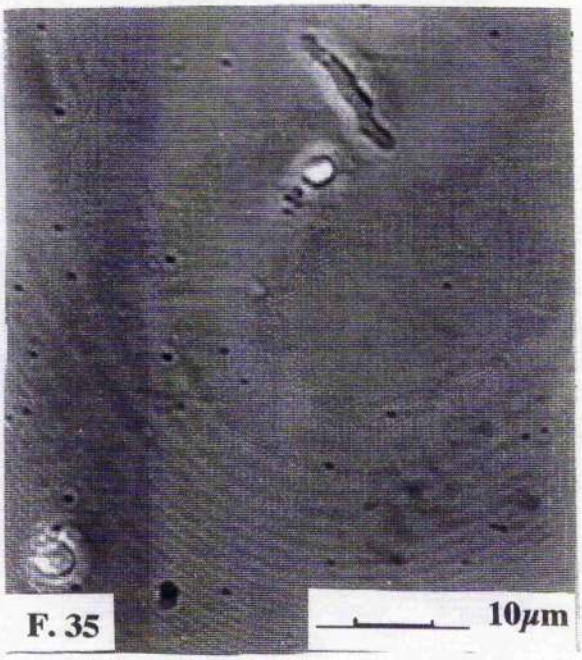
Figure 35. Water sample 79 (cold) containing Hartmanella



F. 33 10μm



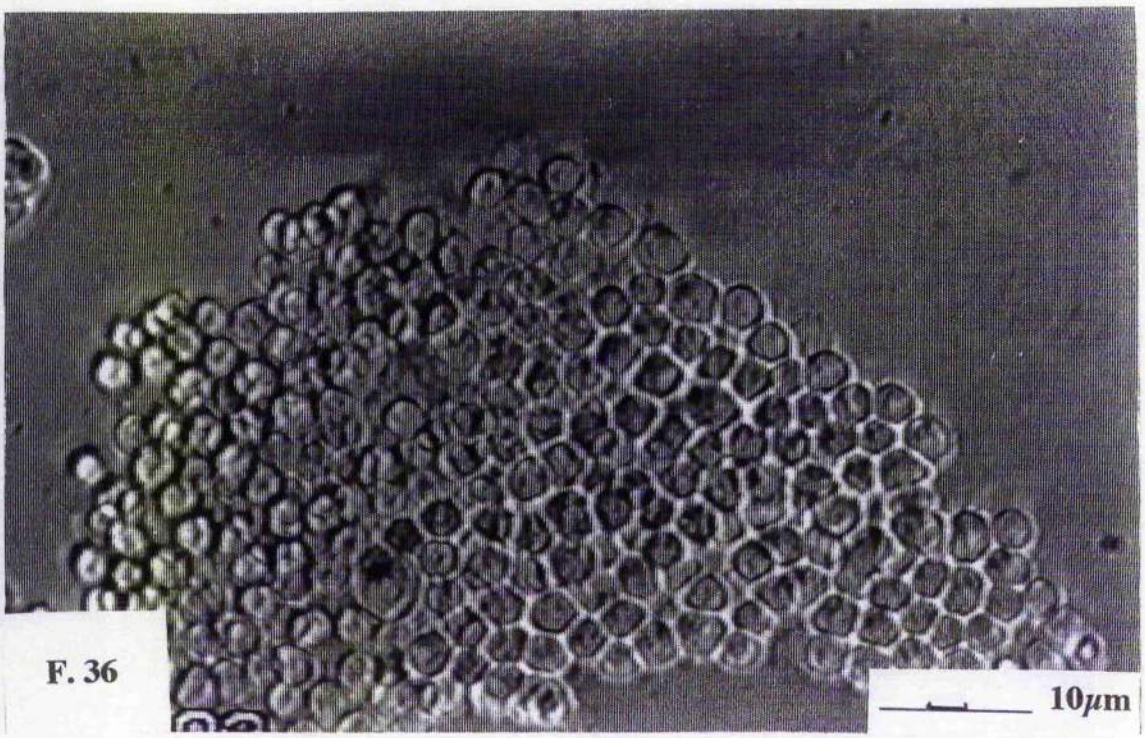
F. 34 10μm



F. 35 10μm

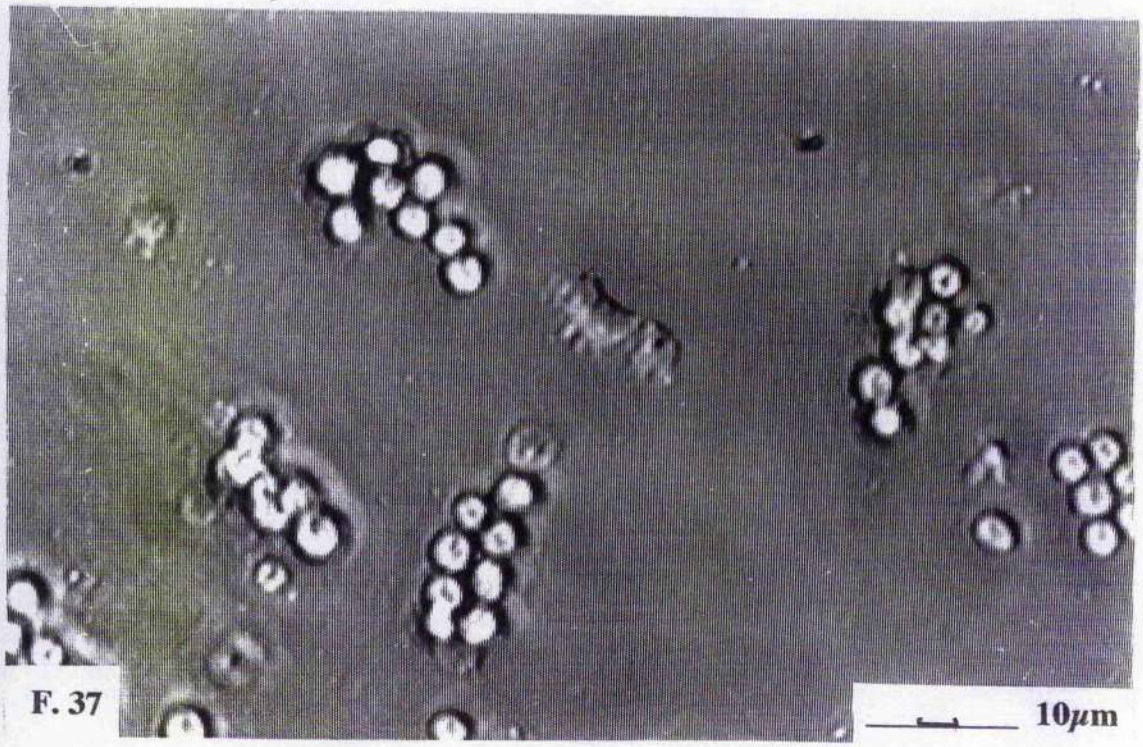
Figure 36. Water sample 37(cold) showing a mixture of amoeba cysts.

Figure 37. Water sample 6 (hot) showing a mixture of amoeba, including Valkampfid cysts and trophozoites.



F. 36

10μm



F. 37

10μm

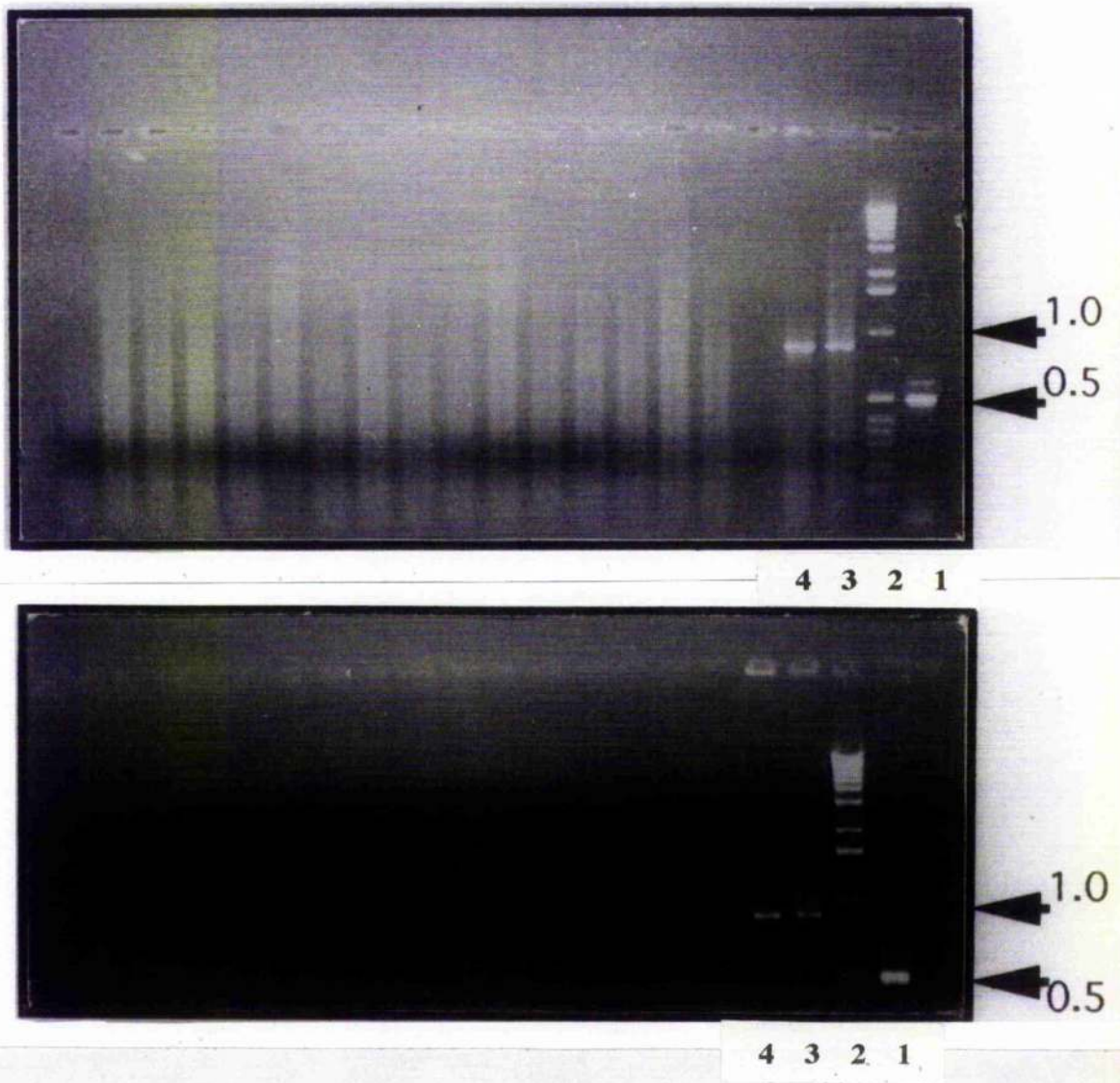


Figure 38. Agarose gel electrophoresis of polymerase chain reaction (PCR) products from Group 2 samples.

Water samples from Group 2 (before enrichment and incubation), positive for free-living amoebae and negative for Legionellae were subjected to PCR. No amplification of legionella DNA was detected in any of the samples. Controls from right to left: Lane 1 = Gene Amp Kit, lane 2 = DNA ladder (Kb), lane 3 = *L.pneumophila* 1 and lane 4 = *L.pneumophila* 6.

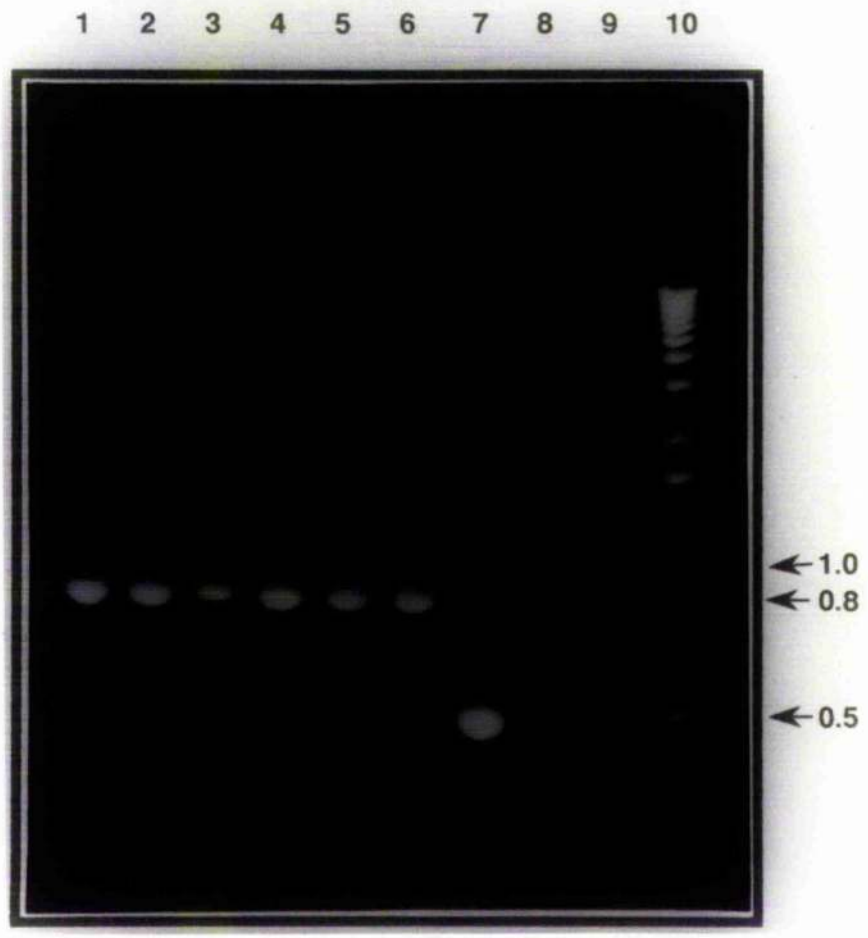


Figure 39. Agarose gel electrophoresis of Group 2 water samples, initially negative for legionella but positive by PCR after enrichment and incubation.

Water samples which were positive for free-living amoebae were incubated in AS at 30°C for 1 week before subjecting them to PCR. Lane 1 = sample 6 (hot water); lane 2 = sample 82 (hot water); lane 3 = sample 55 (cold water); lane 4 = sample 56 (cold water); lane 5 = sample 43 (hot water); lane 6 = sample 12 (cold water); lane 7 = Gene Amp Kit enzyme control; lane 8 = distilled water control; lane 9 = *L. pneumophila*1 DNA control; lane 10 = DNA ladder (Kb).

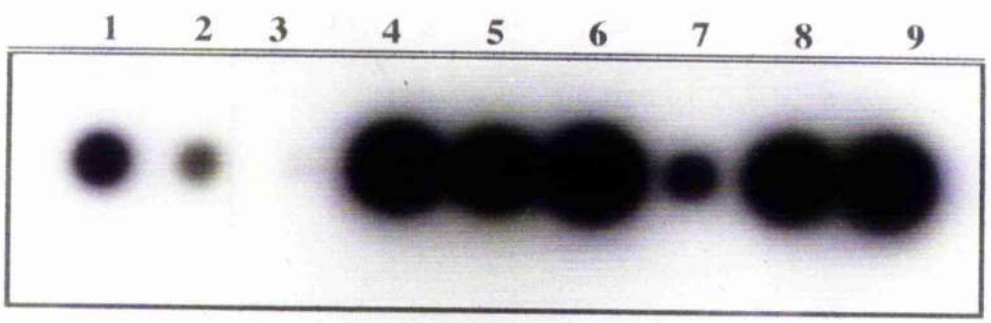


Figure 40. Dot blots of amplified DNA products shown in Fig. 39. probed with γ–^{32}P labeled DNA probe specific for *L. pneumophila.*

Lanes exhibiting an amplification product showed positive hybridization with the *L. pneumophila* radiolabeled probe. Lane 1 *L. pneumophila* 1, Lane 2 *L. pneumophila* 6, Lane 3 distilled water , Lanes 4-9 are PCR products from samples 6 (hot water), 82 (hot water), 55 (cold water), 56 (cold water), 43 (hot water), and 12 (cold water).

Table 9 Analysis of hospital water samples for legionellae and amoebae: summary of results.

Sample number	initial culture	amoebae genera*	post enrichment culture	Legionella agglutination test	serogroup	RFLP§	PCR**	monoclonal antibody typing
6 (hot)	-	H V A m	+	1	1	15	+	Benidorm
12(cold)	-	H V	+	1	1	27	+	Olgal Oxford
43 (hot)	-	H V	+	2-12(PA^+)	4/10/12/14	13	+	Not done
55 (cold)	-	H V	+	1	1	NRP	+	Belingham
56 (cold)	-	H V A m	+	2-12(PA^-)	4/10/12/14	13	+	Not done
82 (hot)	-	H V	+	1	1	15	+	Belingham

*H= Hartmanella, V= Vahlkampfidae, A=Acanthamoebae

m= Mixed free-living amoebae (including unidentified spp.)

§ = restriction fragment length polymorphism analysis

NRP = non-readable pattern

** Verified by hybridization (see Figs. 39, 40)

PA^+: strong agglutination

PA^-: weak agglutination

4- DISCUSSION

4.1 Intracellular multiplication

This study showed that Legionellae can infect and multiply within protozoa in an aquatic environment. Previous work has shown that Legionella was unable to multiply on extracellular products or lysed amoeba cells (Fields *et al.* 1984). We have shown that incubation temperature has a marked influence on the outcome of the association between Legionella with Acanthamoeba. The fact that increased multiplication of *L. pneumophila* occurred when coculture were incubated at 37°C may have been due to the compromised condition of protozoa. However, surviving protozoa did continue to multiply for a period of 10 days. Because incubation at 37°C will eventually destroy the amoeba cells, it is debatable whether their death was the result of elevated growth temperature, infection by Legionella, or combination of both. The results of this study showed that *L. pneumophila* was readily taken into amoeba and this protozoan could provide an intracellular environment conducive to multiplication of the bacteria.

When Acanthamoebawere incubated with Legionellathe number of bacteria taken up by the amoebaeincreased over the period of the 4h experiment, whereas similar experiments with *E. coli* showed a fall in bacterial numbers with time. This confirmed the findings Weekers *et al.* (1993) , who showed that *Escherchia coli* and *Klebsiella erogenous* served as excellent food sources for amoebae. Newsome , (1985) reported some cytotoxic effects of *L. pneumophila* when cocultivated with amoeba. When Legionella and Acanthamoeba cells were cocultivated in saline, some amoeba cells showed to degeneracy distracted during the experimental period (especially at long incubation time) which suggested a cytotoxic effect. This cytotoxic effect was not observed when the two

organisms were cocultivated in amoeba culture medium or even in saline at shorter incubation times. Previous studies have already suggested the production of a cytotoxic factor by *L. pneumophila* (Friedman *et al.* 1980). The uptake of bacteria by amoebae is a two-sided process and usually is affected by both the physiological conditions of both the host cell (Amoeba) and the target cell. The optimal temperature for legionella growth *in vitro* is 36± 1°C(Thacker *et al.*,1981), therefore increasing the incubation temperature to 37°C may stimulate legionellae to enter the amoeba host cell actively. Legionellae fail to grow in the absence of acanthamoeba, but growth of Legionella in co-culture with Acanthamoeba is enhanced at higher temperature. The maximum rate of Legionella growth was obtained at 37°C. Although amoebae were quite healthy at 25°C, the Legionella growth rate was decreased at this temperature when compared to 30°C and 37°C. The majority of the amoeba trophozoites encysted or rounded, especially at higher temperature, during the experimental period. Increases in numbers of intracellular bacteria were evidence in transmission electron micrographs of specimens taken during growth at 37°C.

Rowbotham (1980) first showed that the trophozoites of free-living amoebae can support the intracellular replication of *L. pneumophila* under laboratory conditions. We have confirmed this finding by demonstrating the intracellular growth of *L. pneumophila* in *A. castellanii* trophozoites. The ability of free-living amoebae to support the growth of *L. pneumophila* has led to the suggestion that this bacterium is not free-living in aquatic environments. Although the precise role of free-living amoebae in the ecology of legionellae has yet to be firmly established, laboratory observations on the biology of Legionellae and its association with free-living amoeba suggest that amoeba may play an essential role in bacterial

multiplication. Free-living amoebae are commonly found in soil and aquatic environments (Page, 1967; Morris *et al.* 1979). They have been isolated together with Legionellae from different aquatic sources (Barbaree *et al.* 1987).

Many genera of free-living amoebae are able to form cysts that are resistant to extremes of temperature, desiccation and disinfection (De Jonckheere & Van de Voorde 1976). Acanthamoeba cysts containing *L. pneumophila* were observed during trophozoites cocultures experiments (Anand *et al.*, 1983) , and this phenomenon was suggested as a way by which the bacterium could colonize new environments throughout domestic water supplies. In our study about 90% of infected trophozoites (with *L. pneumophila*) rounded up or encysted during the experiments (Biddick *et al.* 1984).

Some coliforms and other pathogenic bacteria survive within protozoa after chlorination of water samples (King *et al.* 1989). Kilvington *et al.* (1990) showed that cysts of *A. polyphaga* can contain viable *L. pneumophila* , which are thereby protected from chlorine disinfection. Recovery of Legionella from cysts exposed to 50 mg/L free chlorine suggest an important possible role for free-living amoebae in the ecology of the bacterium. Free chlorine at 0.40 mg/L can kill free-living Legionellae (Domingue *et al.* 1990). Cysts and cyst-like structures can support the growth or at least survival of Legionellae, as well as protection them from high level of chlorine. Therefore, the rounded up amoebae or cysts containing motile Legionella, which we observed in the cocultures probably play essential role in transmission of these pathogens to man and may, under appropriate conditions lead to legionellosis.

4.2 Effects of Cytoskeletal and metabolic inhibitors

L. pneumophila is considered a facultative intracellular pathogen because it will grow on artificial media (Mc Dade *et al.* 1977) as well as in cell culture. It multiplied rapidly when coincubated with axenic *A. castellanii* in amoeba saline but was unable to increase in number without amoebae. It is apparent that bacterial multiplication in co-culture with amoebae was dependent upon an intracellular environment and not on soluble nutrients secreted by amoebae (king *et al.* 1991). Transmission electron microscopy confirmed that increase of bacterial numbers was due to intracellular multiplication of legionella in amoeba.

Cytoskeletal and metabolic inhibitors have been useful in studying bacterial invasion of different host cells, due to the conserved nature of eukaryotic cell biology. Cytochalasin D is an inhibitor of actin filament assembly involved in plasma membrane invagination during phagocytosis (Cooper 1987). Cytochalasins have been used extensively in studying microfilament-dependent phagocytosis of bacterial pathogens in non-professional and professional phagocytic cells (Elliott &Winn, 1986).

They reported that *L. pneumophila* failed to grow when phagocytic activity of alveolar macrophages was inhibited with cytochalasin D. The use of Cytochalasin B and D has demonstrated that actin filaments also play a significant role in amoeba locomotion and phagocytosis (Ravdin *et al.* 1985). Methylamine is an inhibitor of transglutaminase, a plasma membrane enzyme involved in the aggregation of ligand receptor complex, and is required for receptor-mediated pinocytosis in fibroblasts and macrophages (Kaplan & Keogh, 1981). This inhibitor has been used to inhibit adsorptive pinocytosis of Semliki Forest virusis in BHK-21 cells and infection of McCoy cells by *Chlamydia trachomatis* (Soderlund & Kihlström,

1983). Previous studies have demonstrated that *L. pneumophila* do not actively penetrate or passively enter macrophages (Van oss, 1978), but are ingested by a type of microfilament-dependent phagocytosis which can be inhibited with Cytochalasin D (Elliott *et al.* 1986, King *et al.* 1991). There are several reports of the use of cytochalasins to inhibit actin polymerization in amoebae(Ravdin *et al.* 1985). Cytochalasin D has been used at a concentrations of up to 50 μg ml to study the phagocytosis of Legionella by Hartmanella (King *et al.* 1991). Howevere, in their study, King and colleagues found that the growth of Legionellae was inhibited only in the presence of high concentrations of Cytochalasin D (50 μg/ml) which apparently killed the amoeba.

We used different concentrations (1μg and 0.5μg per ml) of Cytochalasin D in the present study. Legionella uptake by Acanthamoeba and its growth in coculture were not affected by these concentrations of cytoskeletal inhibitors. Methylamine at concentrations of 100 mM and 50 mM inhibited multiplication of *L. pneumophila* in *A. castelanii*. The pH values in these assays were not sufficiently extreme to affect the intracellular growth of *L. pneumophila*. Legionellae persisted in assay medium containing Cytochalasin D (1μg/ml) or 100 mM of methylamine, and multiplied extracellularly in YE broth containing the same inhibitors.

These findings indicate that the above inhibitors are not toxic to *L. pneumophila* at the concentrations used and had no effect on bacterial multiplication under favourable conditions. These findings also indicate that intracellular multiplication of *L. pneumophila* in Acanthamoeba occurs in part by a microfilament-independent process, such as pinocytosis. The results of this study were in keeping with the previous study on *Hartmanella vermiformis* (King *et al.* 1991). It would not be surprising to

find that *L. pneumophila* have developed a more efficient mechanism, i.e., a single event, for the invasion of amoebaeas a means of ensuring their survival in the environment (Fields *et al.* 1984).

4.3 Cell envelope composition properties of amoebae-grown legionellae

This study has shown that intra-amoeba-growth conditions have an abstruse effect on the LPS production by *L. pneumophila*. These findings agreed with the concurrent study by Barker *et al.*(1993) who, in an extension of this work, reported that intra-amoeba-grown Legionellae recovered from the amoeba host, produced relatively large amounts of a monounsaturated, straight-chain, 18-carbon fatty acid. The significance of this change in fatty acid profile is not obvious, but clearly shows the presence of mechanisms in the bacterium which recognise and possibly adapt to the change to intra-amoebal growth conditions. For example, *L. pneumophila* tend to form large aggregates of cells when growing within amoebae. Such phenotypic changes in amoeba-grown Legionella may reflect stress factors *in vivo* such as nutrient limitations, osmolarity, ionic composition and pH.

There appear to be changes in cell surface hydrophobicity or inter -bacterium adherence associated with *in vivo* growth. While these may be related to altered fatty acyl characteristics within the outer membrane, there is no supporting evidence available as yet. Enhanced adhesion manifested by cell clumping does not appear to reflect a simple change from smooth to rough LPS type, since intra-amoebae grown legionellae display a smooth type LPS profile (see Fig.1, lanes 5 & 6). Interestingly, they also have high molecular weight doublet bands in gels stained for LPS which are not

evident in similarly processed BCYE-grown cells. What molecular species these bands represent is not known, but they may constitute an additional lipo-oligosaccharide (LOS) which is related to cell hydrophobicity.

Intra-amoeba-grown *L. pneumophila* had approx. 10 bands resolved in the LPS ladder, whereas BCYE grown cells had 5-8 bands. We have also shown that the LPS profiles of intra-amoeba-grown *L. pneumophila* depend to some extent upon incubation temperature. Nolte *et al.* (1986) have shown that the majority of serogroup 1 strains have 7-10 repeating units, although as many as 40 discrete bands could be resolved on some gels. Gaby & Horwitz (1985) and Barker *et al.* (1993) also found that *L. pneumophila* serogroup 1 LPS resolved into 10 bands which corresponds to the finding of the present study. However, to attach significance to these differences in banding patterns reported in different studies is probably folly. Differences observed between samples within a single study on the same gel are more likely to be significant. For example, there appears to be a basic difference in LPS/LOS between bacteria grown in amoebae at 22°C and those grown on BCYE^{+} at the same temperature (see Fig.11, lanes 1 and 2). The lack of any ladder pattern signifying a smooth LPS in the amoeba grown cells at 22°C correlates with their increased susceptibility to intra-cellular killing at this temperature when compared to bacteria growing intracellularly at 30°C or 37°C.

The predominant 28 kDa protein band in the outer membrane profiles corresponds to the major porin in this species (MOMP)(Ehret *et al.* 1986). It was expressed in both agar-grown or intra-amoeba-grown *L. pneumophila* and expression was affected by growth temperature, with evidence of reduced expression at 30°C, both in agar-grown and amoeba-grown cells (compare lanes 6 & 7, 8 & 9, Fig.12).

In our study, a striking feature of intra-amoeba-grown Legionellae was the presence of a 15 kDa OMP. This protein has previously been assumed to be a novel outer membrane protein induced under intra-amoeba conditions (Barker *et al.* 1993). It was therefore surprising to us that amoeba membrane preparations from uninfected Acanthamoeba trophozoites showed a 15 kDa membrane protein band.

Thus, there exists the possibility that the 15 kDa component we observed in the intra-amoeba grown bacteria represented an adsorbed amoeba protein rather than a true bacterial outer membrane component. Furthermore, a strain of *L. pneumophila* other than serogroup 1, also expressed the 15 klDa protein under intra-amoeba-growth conditions (Barker *et al.* 1993).

Western blot analysis with anti-*L. pneumophila* polyclonal antibodies recognised a 15 kDa protein present in intra-amoeba grown Legionellae. In contrast, these antibodies did not react with the previously noted 15 kDa protein in Acanthamoeba lysates. It seems that although these 2 components have similar molecular weights, they appear to differ in antigenicity and therefore in identity. Further investigations would be required to confirm this preliminary conclusion. The appearance of new surface components in *in vivo* grown cells, such as the 15 kDa protein identified in this study, may fulfil functions such as inhibition of phagosome-lysosome fusion, or facilitate the escape from the phagosome of the bacterium, as is the case for Listeria, and its protein toxin Listeriolysin (Camilli *et al.*, 1993). Another possibility is that new surface proteins expressed *in vivo* may be involved in trafficking the bacteria into a safe endocytic compartment, where the bacteria can replicate yet be protected from the intracellular killing mechanisms of the amoebae. It has been

proposed, based on electron microscopy evidence, that such a safe compartment in the case of Legionellae is the endoplasmic reticulum (Fields *et al.*, 1993). Thus the uptake of the bacteria may occur by a receptor mediated mechanism which directs the ingested bacteria to the endoplasmic reticulum, analogous to the uptake of transferrin by eukaryotic cells. Other studies have shown that protein synthesis is required for uptake of *L. pneumophila* by amoebae, but not by macrophages (Kwaik, 1994).

4.4 Measuring superoxide production in Acanthamoebae

Phagocytosis is the process whereby certain cells convey solid objects from the external milieu to their interior, and then subject those objects, which are surrounded by an envelope of plasma membrane (the phagosome), to chemical and enzymatic attack . During phagocytosis, or upon appropriate stimulation of the plasma membrane by surface-active agents [e.g. digitonin and phorbol myristate acetate (PMA)], phagocytic leukocytes produce substantial quantities of superoxides (O^{-}_{2}) and hydrogen peroxide (H_2O_2) in the so-called oxidative burst. These substances may subsequently interact to produce hydroxyl radicals (OH.) and singlet oxygen (O^{-}_{2}) detected by CL (Badway *et al.* 1980) and constitute components of the killing mechanisms for ingested microbes.

The results of present study indicate that *A. castellanii,* an alternative professional phagocytic cell, generates low-level chemiluminescence following phagocytosis of *E. coli, L. pneumophila* and latex beads, however, this chemiluminescence response was barely detectable above background levels. Lloyd & Edwards (1979) using a single photon counter to detect the CL response in Acanthamoebae, confirmed that the response is very low compared to that found in

mammalian phagocytes. Which mechanisms operate in Acanthamoeba in killing phagocytosed bacteria is not known in detail. Clearly these are defective in the case of Legionella, particularly at temperatures above 30°C, when intracellular bacterial numbers increase considerably. Escape from the phagosome before fusion with the lysosome is used by other intracellular pathogenic bacteria to gain a sheltered environment in which multiplication can proceed with impunity. Legionella appears to operate a similar mechanism in that phagosome-lysosome fusion is inhibited, and bacterial multiplication occurs in the phagosome (see later).

When different stimulants were used, no clear differences were observed, although at higher amoeba numbers, which generally increased the CL, the bacteria tended to induce a higher peak response than the inert particles. Different stimulants such as zymosan-activated particles (ZAP), phorbol myristate acetate (PMA), and formyl-methionyl-leucyl-phenylalanine (*F*MLP) have all been used successfully in studies with mammalian polymorphonuclear leukocytes , but none of these techniques appear to have any utility in studies of phagocytosis in amoebae.

Davies *et al.* (1991) showed that *Acanthamoebae* display an increased level of lucigenin-dependent CL response (0.9mV) following phagocytosis of latex particles, and concluded that although the levels of CL were very low they were significant. The highest CL response in our present study was higher (1-3 mV) than that recorded by Davies and colleagues, but in our opinion, the levels of CL recorded are so low and near to background levels as to be of dubious significance.

From all of the parameters studied in this investigation, it appears that although lucigenin-dependent CL in Acanthamoeba is detectable upon phagocytosis, it is not comparable with the CL response in mammalian

professional phagocytic cells like polymorphonuclear leukocytes. It is too weak to be accurately measured by luminometry. The LKB luminometer is more accurate at higher levels of photon emission. Indeed, according to Davies *et al.*, (1991) the luminometer is more accurate at higher levels of photon emission. The level of photon CL emission by amoebae, on a per cell basis, is some 200-fold lower than that observed in mammalian neutrophils.

The possible factors that contribute to low CL response may include;

(i) The physiological state of the Acanthamoebae. It is likely that amoebae carry out phagocytosis much less frequently when cultured in chemically defined liquid medium.

(ii) Inhibition of the oxidative burst via a cytotoxin produced *in vivo* by the bacteria

(iii) The LKB luminometer used in this study was designed to study CL in mammalian phagocytes. These cells are capable of producing CL signals of >100 mV compared with levels of 3-4 mV for *Acanthamoebae*.

In order to clarify these results and also to further investigate whether or not pathogens such as *L. pneumophila* affect the generation of superoxide and other respiratory burst enzymes in amoebae, a more sensitive method of measurement is required. For example, specific enzyme assays have been used which measure the level of the oxidative burst by oxidation-reduction coupled reactions using components of the respiratory chain such as cytochrome C (Brooks & Schneider 1985).

The low levels of CL induced by both *E. coli* and latex beads as well as *L. pneumophila* suggest that they are not due to production by Legionella of a specific cytotoxin such as the low molecular weight heat-stable toxin reported by Friedman *et al* (1980).

4.5 Phagosome-lysosome fusion in amoebae following uptaking of legionellae

The data provided here show some aspects of the events following internalisation of pathogenic and non-pathogenic bacteria, as well as an inert particle in the form of latex beads, by *A. castellanii*. This amoeba is capable of internalising a wide variety of particle types including inert latex beads, bacteria , erythrocytes, and yeasts (Allen & Dawidowicz, 1990).The binding of a particle to the extracellular surface of the plasma membrane can be considered as the initial event in phagocytosis. The purpose of our study was to elucidate the events which are involved in the uptake, survival, and under some circumstances multiplication of legionellae in amoebae. An essential element of the study was to compare these events with those which follow uptake of *E. coli* by Acanthamoeba, since this bacterium can be used as a particulate food source in this amoeba.

It appears from our results that both types of bacteria were taken up very effectively at 37°C, whereas when the incubation temperature was lowered to 22°C, the legionellae were engulfed and internalised more effectively than *E coli.* This may reflect the fact that at 22°C, the process of phagocytosis is running at relatively low efficiency, and Legionellae have a mechanism for promoting their own uptake, whereas *E coli* does not. When the temperature is raised to 37°C, the process of phagocytosis is running at very high efficiency, and hence any marginal advantage which was displayed by Legionella at 22°C disappears because of the relatively large increase in the rate of the process at the higher temperature. The Mip protein or an analogue may be involved in enhanced uptake by amoebae and it would be interesting to compare the rates of uptake of *mip* mutants with wild type cells in Acanthamoeba.

Phagosome-lysosome fusion (PLF) has been studied mostly in mammalian cells using thorotrast, thorium dioxide, and acid phosphatase . Fluorescent markers such as acridine orange and fluorescein isothiocyanate have also been used for PLF assessment (Kielian, 1986).

Using a modification of the previously described method (Ishibashi & Yamashita , 1982),, we studied the events following uptake of Legionella by its amoeba host cell (Acanthamoebae). *L. pneumophila* has been reported to inhibit phagosome-lysosome fusion in monocytes (Horwitz, 1983); it also inhibits acidification of its phagosome in these cells (Horwitz & Maxfield 1984). Our study shows that the strategies used by Legionellae to survive within Amoebae are comparable, at least in some respects, with those which operate in the survival and multiplication of Legionellae within monocytes.

The acridine orange assay of PLF has been described as a simple and rapid assay compared with those involving the use of other lysosomal markers. It also enables detailed and reproducible rate studies (Kielian, 1986).There is a discussion concerning the usefulness of the acridine orange technique to test PLF by Mor & Goren, (1987). However, by using adequate controls and taking into account non-specific fusion events, this technique can provide useful data in PLF studies. In our experiments with acridine orange staining, results were not always easy to interpret because of the lack of consistent availability of good fluorescence microscopy facilities.

Here, we examined phagocytosis and phagosome-lysosome fusion in Acanthamoebae infected with *L. pneumophila* and *E. coli.* Legionellae were efficiently phagocytosed by Acanthamoeba as were *E coli,* but fusion of legionella-containing phagosomes with lysosomes was greatly impaired. The basis for the inhibition of the membrane fusion event associated with

phagolysosome formation is not understood. In other organisms, phagosomal membrane modification (and destruction) is achieved, for example in Listeria, by production of the membrane lysing toxin Listeriolysin under the conditions of pH and Fe^{+} limitation experienced in the phagosome. Whether a similar mechanism operates in Legionellae, possibly involving the membrane active lysin - Legiolysin, or alternatively a phospholipase or protease has not been investigated. Suitable mutants deficient in these extracellular products could be generated and compared in behaviour with wild type isogenic cells. A further possible component worthy of investigation is the LPS of Legionella. This molecule has a propensity for solubilising in membranes, with resultant modification of the behaviour of the target membrane. The ability of cells with different LPS types to inhibit phogosome-lysosome fusion could be studied experimentally in our system.

It is unquestionable that electron microscopy (EM) with cells labeled by electron opaque markers is the most useful methodology to distinguish specific fusions, although EM sometimes produces alterations in the sample that could interfere with observation.

We attempted to label the amoeba lysosome by virtue of its acid phosphatase activity, and to study the ultra structure of PLF. Preliminary results showed that Acanthamoebae had good acid phosphatase activity located in vesicles presumed to be lysosomes. Unfortunately, *L. pneumophila* also produces a wide range of extracellular enzymes including an acid phophatase (Saha *et al.* 1985), Because of this obstacle, it was not possible for EM results to be interpreted clearly. We were unable to obtain an acid phosphatase minus mutant which would have made the

technique more useful. Further studies essentially at ultrastructural level, using such mutants are required to support the present conclusion.

4.6 Legionella-amoeba interactions in hospital water supplies

Free-living amoebae were isolated from 46% of the hospital water samples, most of which were contaminated with more than one genus of amoeba. Morphological characters and movement behaviour, recorded on video tape, were used to tentatively identify protozoa to genus level. In 25% of the samples, both free-living amoebae were observed and legionella cultured. Free-living amoebae were absent from only 3% of the samples from which legionellae were cultured. These Legionellae were probably planktonic forms and derived from biofilm on the inner surface of the water pipes. Outwith hospital water supplies, legionellae have frequently been found in association with amoebae (Barbaree *et al.* 1987), and other protozoa in public water supplies, as well as with blue-green algae in habitats lacking free-living amoebae (Tison *et al.* 1980; Fields *et al.* 1984).

In our study, *L. pneumophila* was isolated from hospital water samples which were initially negative for *Legionellae* by standard culture methods, but which contained free-living amoebae. Only after enrichment of the protozoa and subsequent incubation were legionellae cultured from such samples. The likely explanation for this result is that legionellae are sequestered in some of the amoeba cysts, and are not recoverable from these by the normal laboratory culture methods used to screen for legionellae. Only after germination of the cysts does an opportunity arise for legionellae to recover and then become susceptible to recovery by normal laboratory culture techniques. There are many unanswered

questions which arise from these results. What proportion of amoebae cysts is infected with Legionellaein the original water samples; do the cysts fail to germinate in BCYE+ medium, or if they do, do the legionellae they contain not recover into a culturable form? Is there a requirement for a low nutrient step (incubation in AS for 1 week) in order for the legionellae to recover to a culturable form? Examples of the influence of culture medium and bacterium;protozoan ratio on the outcome of the interaction between bacteria and amoebae come from recently published work by Lebbadi *et al* (1995). In this work, nutrient conditions and bacteria:protozoa ratio during co-cultivation of *Bacillus licheniformis* and *Naegleria fowleri* influenced the production of amoebicin and the consequent lysis or survival of the amoebae. While there is no evidence of amoebicins being produced by legionellae (as yet), the nutrient conditions and bacterial numbers in relation to host amoebae may influence the outcome of the association between legionella and acanthamoeba, in terms of whether viable and culturable bacteria are released upon germination of cysts and their subsequent growth.

Subjecting water samples to laboratory culture methods such as plating on BCYE+ for Legionella is the detection method most widely used, but it may not be sensitive enough. Rowbotham (1983) suggested the use of amoebae to improve recovery of legionellae from clinical specimens and he successfully cultured *L. pneumophila* after adding *Acanthamoeba polyphaga* to lung lavage specimens. Fields *et al.* (1984) found that addition of *Tetrahymena pyriformis* to environmental specimens served as an enrichment method that improved isolation of legionella from the specimens. Such studies lend support to the procedure

we adopted of incubating water samples containing free-living amoebae to improve the sensitivity of culture for Legionellae isolation.

Water sample enrichment with amoebae and subsequent incubation enabled us to recovery *L. pneumophila* from specimens which were initially negative by laboratory culture, and identified reservoirs of potential infection that were not recognised during epidemiologic investigations with routine procedures.

These observations may indicate that legionellae increase in numbers to culturable levels by replicating within amoebae. Alternatively, there may be a change in the physiological state of intracellular legionellae from a non-culturable to a culturable form following growth in low nutrient medium in association with amoebae.

The universal presence of Hartmanellae and Vahlkampfidae in the Group 2 samples which yielded legionellae shows that the association between legionella and the amoeba is not strictly species restricted and that aquatic Legionellae were adapted to replication within autochthonous amoebae. This is borne out by the reported recovery of an epidemic strain of legionella by co-culture with *Hartmanella spp.* from potable water sites (Breiman , 1990).

Given the common occurrence of free-living amoebae in water systems, incubation (with prior autochthonous amoebae enrichment) seems a simpler and more efficient strategy than the addition of exogenous amoebae (Rowbotham, 1983). However, this exogenous "addition" method may be useful for recovering legionellae in samples which do not harbour native amoebae. Incubating water samples increased the sensitivity of culture for Legionellae in our study. No additional materials or equipment beyond those used for conventional culture were needed. The

presence of amoebae should indicate that the sample requires enrichment and incubation before reculturing for Legionellae.

Amplification of specific DNA sequences by the polymerase chain reaction (PCR) in association with the use of gene probes has been used for detection of Legionellae in environmental water samples (Mahbubani *et al.* 1990; Bej *et al.* 1991; Maiwald *et al.* 1994; Hay *et al.* 1995) in sewage and ocean water (Palmer *et al.* 1993) and in clinical samples (Jaulhac *et al.* 1992). The method used in the present study specifically detects *L. pneumophila* by amplification of a fragment of DNA of unknown function and has a reported sensitivity equivalent to 35 cfu detected by colony count (Starnbach *et al.* 1989). In our hands, the sensitivity of this PCR method was in the region of 5 cfu (results not included).

Using the primer set and the detection probe described by Starnbach *et al.* (1989), DNA of *L. pneumophila* was detected in 6 of 38 water samples containing free-living amoebae, previously recorded as negative by direct culture onto supplemented media. This system significantly detects *L. pneumophila* as opposed to that of Jaulhac *et al.* (1992), when DNA of all *L. pneumophila* strains (1-14) and also *L micdadei*, *L. bozemanii* and *L. anisa* were detected by these primer sets and detection probe. The legionellae isolated from the Group 2 samples were of diverse origins, as evidenced by their serogroup identity, MAb typing and RFLP profiles. These results were confirmation that the recovered legionellae did not arise by cross-contamination during sample preparation, but were of diverse origin. These results have very considerable significance in the infection control protocols used for monitoring the presence and eradication of legionellae after hospital outbreaks. Cleaning of water systems involves sampling before and after biocide treatment to confirm freedom of

legionellae after such treatment. If the treatment serves to induce encystment of amoebae, and encystment involves sequestration of legionellae to non-culturable forms, as shown in our study, then monitoring procedures need to be modified to include an enrichment and incubation step. Initial culture of the water sample with its own amoebae followed by the PCR method of Starnbach would appear the best and most sensitive procedure at the present time. These findings are the first reported detection of initially non-culturable legionellae by autochthonous enrichment amoebae from environmental samples, confirmed by PCR. This approach has recently been used with laboratory specimens (Hay *et al.* 1994, Sanden *et al.*, 1992).

In summary, amoebae are commonly are found in rivers, lakes, and drinking water reservoirs, and may act as a protected niche for pathogenic bacteria. They can protect them from chlorination and other changing environmental conditions, such as desiccation, osmotic stress and high temperatures (encystment). Free-living amoebae are common environmental organisms (Page, 1967) and an integral part of natural and man-made water systems where they are not easily controlled or eradicated. Their role in the maintenance of human disease such as legionellosis and their role in the survival and distribution of other pathogenic bacteria such as Listeria has recently been acknowledged. Associations between amoebae and legionellaein hospital water systems is of particular concern because of the resistance of amoebal cysts to chlorination procedures and the subsequent release of sequestered legionellae from surviving cysts upon subsequent germination. Such released organisms pose a very real threat to patients, many of whom have their immune system compromised either by infection or by specific drug therapy.

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6- APPENDICES

Appendix 1

BUFFERS AND MEDIA FOR ACANTHAMOEBA

Amoeba saline (Page's modified Neff's amoeba saline)(Page, 1976).

Solution A:

NaCl	1.2 g
$MgSO_4.7H_2O$	0.04g
Na_2HPO_4	1.36g
KH_2PO_4	1.36g
d/w	100 ml

Solution B:

$CaCl_2, H_2O$	0.04g
d/w	100 ml

Amoeba Saline:

Solution A	10 ml
Solution B	10 ml
d/w	980 ml

pH adjusted to 6.9 with N KOH.

Modified Neff's amoeba saline (As) (Page, 1988).

Separate stock solution of each component were made by dissolving in 100 ml of glass-distilled water.

NaCl	1.20 g
$MgSO_4.7H_2O$	0.04 g
$CaCl_2.2H_2O$	0.04 g
Na_2HPO_4	1.42 g
KH_2PO_4	1.36 g

The final dilution of saline was prepared by adding 10 ml of each stock solution to enough glass-distilled water to make 1 litre.

Proteose peptone Yeast extract Glucose (PYG) broth (equivalent to the PYG broth of Drozanski) for the axenic cultivation of *Acanthamoeba*

Proteose peptone (Oxoid)	15 g
Yeast extract powder (Oxoid)	5 g
D- glucose	10g
$FeSO_4$	3 mg
Amoebae saline (AS)	1 litre

Sterilised by autoclaving, 115°C for 15 min, final pH 6.6

Amoeba non-nutrient agar (Amoeba solid media) (Page, 1988):

Agar (Oxoid No.1)	15 g
Amoeba saline	1 litre

* Heat-killed *E. coli* was used as solid food for Amoeba on non-nutrient agar.

Phosphate- Buffered Saline (Dulbecco A)

Phosphate- buffered saline tablets (Dulbecco A, pH 7.3 Oxoid) were used. Each tablet was added to 100 ml of distilled water and the solution was autoclaved at 121 °C for 15 min.

Appendix 2

Buffer Charcoal Yeast extract (BCYE+) agar

ACES buffer	10.0 g
d/w	500 ml

ACES buffer (N-2-acetamido-2-aminoethane-sulphonic acid) (Sigma)

Dissolved by heating in a water bath at 45 - 50°C.

The above solution was mixed with :

KOH 1.0 N	40 ml
d/w	440 ml

Then the following ingredients were Added:

Activated charcoal	2.0 g
yeast extract	10.0 g
Agar (Difco or Oxoid)	17.0g

Activated charcoal (acid washed with Phosphoric and sulphuric acid) available throughout Sigma Ltd.)

Dissolved by boiling, then autoclaved for 15 min at 121°C. Cooled to 50°C, followed by aseptically adding of:

L-cysteine-HCl.H_2O solution (0.4 g in 10 ml of distilled water, sterilised by filtration).

Ferric pyrophosphate solution (0.25 g in 10 ml of distilled water , sterilised by filtration).

If the colour of Ferric pyrophosphate solution changes from green to yellow or brown, it is not usable. Ferric pyrophosphate, should not be heated over 60°C; a 50°C water bath is satisfactory. The pH of the final solid medium should be 6.9$\pm$ 0.05 at room temperature.

Since reagents vary, each laboratory must determine the amount of KOH required. Do this by holding the bulk medium at 50°C while pouring one plate and checking its pH. When necessary, adjust the bulk medium with either 1.0 N KOH or 1.0 N HCl. Note that the pK_{α} of ACES buffer is influenced by temperature (0.02/°C); consequently, this must be considered with all pH determinations. Dispense 20 ml portions of the complete medium into 10x100 mm plastic Petri dishes'; swirl the medium between pouring plates to keep charcoal particles suspended.

Yeast extract Broth (YB):

YB (Ristroph *et al.* 1980);

Yeast : extract (Oxoid)	10 g
L-cysteine-HCl.H_2O	0.4 g
Ferric Pyrophosphate	0.25 g
d/w	1000 ml

The ingredients were all added to the distilled water and then membrane filter sterilised (0.45 μm, Millipore Corp.,). The pH was adjusted to 6.9.

Appendix 3

Preservation of cultures

1-L. pneumophila was preserved on glass beads as described previously (Harrison *et al.* 1988). Briefly, embroidery beads (2mm Ellis & Farrier Ltd) were soaked in 5% HCl overnight and then washed repeatedly in tap water until the pH was that of the tap water . After the final rinse in distilled water the beads were dried overnight in a drying cabinet. Glass beads (20-30) were placed in a suitable screw-cap container, loosely capped, and sterilised by autoclaving.

Growth from a BCYE^{+} plate, incubated for 48-72h, was emulsified in about 1 ml of Glycerol broth to give a dense bacterial suspension. Legionella suspension was added to a vial which was then gently shaken to ensure that the beads were thoroughly coated. Excess fluid was withdrawn from the base of the vial with a Pasteure pipette. The beads were sloped in the vials to facilitate their removal after freezing. The vials were stored at -70°C for long term preservation.

To avoid the repeated thawing and freezing of the stored culture, the vial containing beads was kept frozen throughout recovery procedure. Sterile forceps were used to remove a single bead from the vial. The bead was then placed on to a BCYE plate and a flamed wire-loop was used to roll it over the surface of the agar. The vial containing the remaining beads was immediately replaced in the -70°C freezer. *Escherchia coli* (C118) was obtained from R. Aitken, Laboratory of Microbiology, University of Glasgow (personal communication).

Preparation of glycerol broth (Harrison & Taylor, 1988).

Oxoid nutrient broth No. 2	5 g
Glycerol	Glycerol

d/w	170 ml

The Glycerol broth was prepared by dissolving 5 g Oxoid Nutrient Broth No.2 in 170 ml distilled water and then 30 ml glycerol was added. The broth was dispensed into 2ml volumes, sterilised by autoclaving, and stored at room temperature until required.

Freeze-Drying (Lyophilisation)

L. pneumophila was maintained in a dried state in small evacuated glass vials. The following procedures were applied for lyophilisation:

Into small vials or ampoules of neutral glass were inserted tiny pieces of whatman No.1 filter paper on which was typed necessary information about organism. The ampoule was then closed by having a cotton wool plug inserted, and sterilised by autoclaving.

L. pneumophila were grown on BCYE medium for 48 hours.

Into a sterile test tube or bijou bottle was placed 0.5 ml of the suspending fluid. The commonest fluid used is MISC desiccans or 2% dried milk in H_2O.

Misc-desiccans:

30% (W/V) glucose	1 Vol.
Sterile inactivated serum (horse)	3 Vols.

A heavy suspension of the culture was made in the suspending fluid.

Using a Pasteur pipette with a long capillary, 2-3 drops of the bacterial suspension were added to each ampoule . Once the organism had been added to the ampoules, a gauze hood was placed over each tubethey were put into the centrifuge head of machine.

Machine used was the Edwards 5 PS. freeze drying machine; this consisted of a centrifuge head contained inside a bell-jar which could be

evacuated. From this bell-jar is a duct leading to a chamber containing a desiccant i.e., phosphorous pentoxide. The bell-jar was evacuated by an oil operated vacuum pump. A Pirani gauge measured the vacuum inside the bell-jar.

The metal tray of the chamber was charged with phosphorus pentoxide.

Bell-jar was placed over the centrifuge head and the air inlet was closed; the centrifuge was then switched on, spun for 10 min without a vacuum, then spun with a vacuum for 1 hour, followed by vacuum only over night.

The pump was switched off, the air inlet was opened to release the vacuum and the ampoules were removed.

The hoods were replaced with sterile cotton-wool plugs (non-absorbent).

The stems of the ampoules were drawn out to form a thin capillary neck. (This can be done using a fan-tail Bunsen burner, or the constricting machine).

The drying tray of the centrifuge was replenished with fresh phosphorus pentoxide. The air release valve was closed and the rotory pump switched on . The ampoules were left in the vacum for 18-20 hours (the moisture content showed less than 1%).

The ampoules were sealed off with a gas cross fire burner, while still on the vacum manifold.

The ampoules were tested for vacuum by glow discharge, and kept at 4 °C until needed.

2- *Acanthamoebae* were cryopreserved by the method previously described (Kilvington & White, 1991). Briefly, log-phase cultures of axenic trophozoites were harvested by centrifugation at 500 x*g* (MSE

centrifuge) for 5 min at room temperature. The cell pellet was resuspended to a concentration of 10^6 trophozoites /ml in fresh Amoeba broth. Trophozoite counts were made using a modified Fuchs Rosenthal haemocytometer chamber. Volumes of 0.5 ml were placed into 1.2 ml screw capped polypropylene ampoules measuring 41mm x 12 mm (Gibco, NUNC, Middlesex, England). An equal volume of Amoeba broth containing 10% dimethylsulphoxide (DMSO) was added to each ampoule and the contents mixed by inversion. Immediately, the ampoules were placed free-standing into the bottom of a -20°C freezer for 60 min before transferring to a -70°C freezer for a further 60 min. Following cooling at -70°C, the ampoules were then plunged into liquid nitrogen for long-term storage.

Amoebae cells were recovered by removing an ampoule from the liquid nitrogen and placing it into an enclosed container under an operating class 2 cabinet at room temperature. After 3 min, the ampoule was placed in a 37°C circulating water bath for rapid thawing. Axenic cultures were inoculated into 2 ml of pre-warmed PYG amoeba broth (Page, 1988), and incubated at 30°C. After 1 hour, trophozoites were transferred to 25 cm^2 tissue culture flasks containing fresh amoeba broth and incubated at 30°C for a further 24h. The medium was changed and amoeba cultures were incubated for 3-5 days at 30°C to form monolayer cell cultures in the tissue culture flasks.

Appendix 4

SDS-PAGE (SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS)

(A) Chemicals and reagents

1 Acrylamide/Bis acrylamide

Acrylamide	29.2g
N, N-methylene bis Acrylamide	0.8 g

Distilled water (Volume made up to 100 ml).

2 Lower gel buffer (1.5 M Tris-HCl pH 8.8)

Tris HCl	18.1g
SDS	0.4 g
d/w	50.0 ml

The pH was adjusted to 8.8 with 1N HCl and the final volume made up to 100 ml with distilled water. The buffer was filtered through Whatman No. 1 and stored at 4°C.

3 Upper gel buffer (0.5 M Tris-HCl pH 6.8)

Tris HCl	6.0 g
SDS	0.4 g
d/w	50.0 ml

The pH was adjusted to 6.8 with 1 N HCl and the final volume made up to 100 ml with distilled water. Buffer was filtered through Whatman No.1 and stored at 4°C.

4 TEMED (Undiluted stock)

5 Ammonium persulphate solution (APS)

A 10% solution was made up freshly (50 mg in 0.5 ml distilled water).

6 Solubilizing Buffer (Sample buffer)

Glycerol	10 ml
2- mercaptoethanol	5 ml
SDS	3 g
Bromophenol blue	0.01 g

1 in 8 dilution of (Upper buffer)to 100 ml

7 Running Buffer (pH 8.3)

Tris HCl	3.03 g
Glycine	14.4
SDS	1.0 g
d/w	1000ml

8 Staining Solution

Coomassie blue R 250	1.525 g
Methanol 50% (v/v)	454 ml
Glacial acetic acid	46 ml

9 Destaining Solution

Methanol	50 ml
glacial acetic Acid	75 ml
d/w	875 ml

B Slab gel preparations

Two 1.5 mm thick gels 200x 200 mmfor protean II, Bio Rad).

1 Separating (lower) gel (volume 60 ml)

	Acrylamide (%)			
	7.5	10	12	15
Acrylamide/ Bis (30%)	4	15	20	30 ml
Lower gel buffer (pH 8.8)	15	15	15	15 ml
d/w	29	24.1	20.1	14.1 ml
SDS 10%	600	600	600	600 μl
APS 10%	150	300	300	300 μl
TEMED	15	30	30	30 μl

For LPS; 4M urea (0.24 g/ml) was dissolved in Acrylamide plus buffer and SDS solution and volume made up to 60 ml with distilled water and degassed under vacuum for 20 min. The APS and TEMED were then added and mixture poured into gel casts. Separating gels were carefully layered with 0.1% SDS in distilled water to ensure a smooth interface at the gel surface and allowed to polymerise overnight.

2 Stacking (upper) gel (volume 20 ml)

	Acrylamide (4%)
Acrylamide/ Bis (30%)	2.6 ml
Upper gel buffer (pH 6.8)	5.0 ml
d/w	12.2 ml
SDS (10%)	200 μl
APS (10%)	100 μl
TEMED	20 μl

Gel mixture was degassed for 20 min under vacuum before adding APS and TEMED. Polymerised separating gels were rinsed with distilled water and then carefully layered with stacking gel solution and comb of appropriate size. The gels were polymerised for 1-2 h and used immediately.

2-Western blotting

1 Electroblotting buffer

Tris HCl	3.03 g
Glycine	14.4 g
Methanol 20% in d/w	1000 ml

2 Blot developing substrate solution (prepared fresh before use).

3, 3'- diaminobenzidine (DAB)*	0.05 g
Cobalt chloride 1% (w/v) in d/w	2.00 ml
PBS pH 7.4	98.0 ml
Hydrogen peroxide 30%	0.1 ml

* DAB is a carcinogen! handle with due care.

3 Ponceau-S solution.

Ponceau-S	0.5 g
Glacial acetic acid	1.0 ml
d/w	100 ml

Appendix 5

Protein concentration

Protein concentrations were determined by either the method of Lowry *et al.* (1951) or its modification for membrane proteins as described by Markwell (1978), with graded concentrations of bovine serum albumin as control:

Reagent A:	Na_2CO_3	2g
	NaOH	0.4g
	Sodium Tartrate	0.16
	SDS	1g
	d/w	100ml

Reagent B:

$CuSO_4$	4g
d/w	100 ml

Reagent C: 1 part of reagent B was added to 100 part of reagent A

Folin-Ciocaltev reagent, was diluted 1:1

Bovine Serum Albumin (BSA) was prepared at 1 mg/ml; the following concentrations were prepared as standards: 500μg ml^{-1}, 400μg ml^{-1}, 300μg ml^{-1}, 200μg ml^{-1}, 100μg ml^{-1}, 50μg ml^{-1}.

0.1 ml (100 μl) of each sample was diluted with 900μl of distilled water (total 1 ml).

Assay method:

1 ml of diluted sample (or standard concentration of BSA) was added to 3 ml of reagent C. The tubes were incubated at room temperature for 30 min, 0.3 ml of FCR was then added and incubated for another 45 minutes at room temperature.

Measurements of absorbance at 660 nm were made with a Shimadzu, PR-1 Graphicord spectrophotometer (Shimadzu Coperation, Kyoto, Japan) in its auto calculation computation mode for generation of standard concentration curve readings, and thereby sample protein concentrations.

Appendix 6

Buffer and reagent for Cytochemistry and Transmission electron microscopy

1 Tris / Maleate Buffer;

The 0.2 M stock solution was prepared by dissolving 24.2 g Tris and 23.2 g Maleic acid in 1 litre of distilled water. To 50 ml of the stock solution x ml of 0.1 M NaOH was added and diluted to 200 ml with distilled water.

pH	x	pH	x
5.2	14	6.6	85
5.4	22	6.8	90
5.6	31	7.0	96
5.8	41	7.2	102
6.0	52	7.4	108
6.2	63	7.6	116
6.4	74	7.8	127

2 Sodium Cacodylate Buffer

To 50 ml of 0.2 M Sodium Cacodylate x ml of 0.1 N HCl was added and diluted to 200 ml with distilled water.

pH	x	pH	x
5.8	70	6.8	27
6.0	59	6.8	19
6.2	48	7	13
6.4	37	7.2	8

3 Cacodylate Buffer Fixative

0.2 M Sodium cacodylate	50 ml
2.5 % glutaraldehyde	10 ml
d/w	40 ml

The pH was checked with 0.2 M HCl (pH 7.4), before adding distilled water.

4 Rinse Buffer

0.2 M Sodium Cacodylate	50 ml
d/w	50 ml
Sucrose	2 g
	100 ml

pH was adjusted to 7.4

5 Sodium acetate-Acetic acid Buffer (0.2 M):

0.2 M Sodium acetate	70 ml
0.2 M Acetic acid	30 ml
	100 ml

pH 5.0

6 Acetate Buffer (0.05 M):

0.2M Acetate Buffer	10 ml
d/w	40 ml
	50 ml

7 Sucrose Buffer (washing Buffer):

0.2 M Tris / maleate buffer (pH 5.2)	12.5 ml
Sucrose	4.2 g
d/w	37.5 ml
	50 ml

8 Incubation Medium for Acid phosphatase staining:

0.2 M Acetate Buffer (pH 5.0)	10 ml
0.1 M Sodium ß-glycerophosphate	4 ml
0.02 M Lead nitrate	6 ml
Sucrose	4.2 g
d/w	30 ml

*The buffer and distilled water were divided between two measuring cylinders. Then the substrate solution (0.1 M Sodium ß-glycerophosphate) was added to one, and lead nitrate solution to another. The contents of two cylinders were then gradually poured into a beaker with rapid stirring. The medium was prepared immediately before use.

Appendix 7

Anti-fading solution

Mowiol 4-88 (Hoechst) 2.4 g was added to 6 grams (w/w) of glycerol, stirred to mix. Then 6 ml of distilled water was added and the mixture was left for several hours at room temperature. 12 ml of 0.2 M Tris ($_{pH}$ 8.5) was added, and heated to 50°C for 10 min with occasional mixing. After the Mowiol dissolves , clarify by centrifugation at 500g for 15 min. For fluorescence detection, add (Tricthylenediamine) 1,4 - diazobicyclo -[2.2.2.]-octane (DABCO) Fluka AG, Buchs SG Switzerland, to 2.5% to reduce fading. Aliquot in air-tight containers and stored at -20°C. A small drop of mounting medium was added to the specimen. The mounting medium will set overnight. For immediate observation of samples the coverslip was secured to the slide by placing a small drop of nail polish at the edges of the coverslip.

Preparation of glycerol mounting medium:

Buffered saline pH 8.5	1 part
Glycerol	9 part

Buffered saline: Ten parts M/15 K_2HPO_4 (1.161 g /100 ml 0.85% NaCl) plus one part M/15 KH_2PO_4 (0.907 g/100 ml 0.85% NaCl).

Appendix 8

Legionella oligonucleotides preparation and purification

Primers (LEG 1 19 mer, LEG 2 19 mer, LEG 3 25 mer) were synthesised by the Biochemistry Department (University of Glasgow) as a solution in 0.88 M ammonium hydroxide (NH4OH) and 3M Sodium acetate (NaOAc). Oligonucleotides were purified by the standard ethanol precipitation method (Darbre, 1988)). Absolute ethanol was used to precipitate oligonucleotides. Briefly, 1.2 ml of absolute ethanol was added to the samples and mixed well; a lightly turbid suspension was formed. The suspensions containing oligonucleotides were placed in the oven for about 30 minutes and alowed to dry. Sodium acetate 0.3M (pH. 6.0) was prepared and pre-cooled to -20°C. Then 200 μl of pre-cooled sodium acetate was added to each sample,mixed well and then 600 μl of absolute ethanol (pre-coolled to -20°C) was added and mixed well. The solution of oligonucleotides was then kept at -70°C for about 15 min. The samples were centrifuged 10000 xg for 10 minutes at 4°C. The supernatant was then removed with a sterile glass Pasteure pipette, and the pellet was mixed with 1 ml of pre-cooled (-20°C) absolute ethanol, and kept at -70°C for 15 min. The samples were re-centrifuged for 15 min, and the ethanol was removed from the microfuge tube carefully as previously described. The oligonucleotides were dried in an oven and stored at -20°C. The purified oligonucleotides were reconstituted with pyrogen-free sterile water (for irrigation Baxter Healthcare Ltd., Lot 94D26B26) prior to using for PCR.

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