Rapid diagnosis of **Legionella** infections and identification of **Legionella** species isolated from man and the environment.

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Preface

This thesis is the original work of the author. He wishes to acknowledge Mr. R. Wait, Porton Down, Salisbury for the mass spectrometry of samples produced during the course of this work. In addition it is recognised that the DNA homology in this thesis was the work of Dr. P.A.D. Grimont, Institut Pasteur, France.

Kenneth Mitchell

Dedication

I dedicate this thesis to my mother and family for their relentless support over the last three years.

"The great tragedy of Science - the slaying of a beautiful hypothesis by an ugly fact."

"Aphorisms and Reflections"

Thomas Henry Huxley.

Acknowledgements

Foremost, I would like to express my sincere thanks to Dr. R.J. Fallon for his guidance, encouragement and supervision during this work.

My special thanks are also due to Professor J.H. Freer for his untiring patience and valuable advice, to technicians of the Department of Microbiology, University of Glasgow and M.L.S.O.'s of the Department of Laboratory Medicine and the Regional Virus Laboratory for their assistance in the use of equipment, to members of staff of the Divisions of Bacterial Metabolism and Pathology, PHLS CAMR, Porton Down, Salisbury for mass spectrometry of samples, helpful suggestions and discussions.

Thanks are also due to Dr. R.M. McKinney, CDC in Atlanta, Georgia, Professor F.J. Fehrenbach, Berlin, Germany, and Miss A. Williams, PHLS CAMR for their kind gifts of antisera without which, much comparative work would not have been possible.

This research was made possible by the Scottish Home and Health Department who provided my grant for materials and funds to attend PHLS CAMR, Porton Down, Salisbury, a training workshop and a S.A.B. symposium. I express my thanks to the S.H.H.D.

Finally, I would like to thank Dr. R.J. Fallon and Professor J.H. Freer for their reading and constructive criticism of this manuscript.

SUMMARY

Four immunisation protocols were compared for their ability to produce avid polyclonal antisera against L.pneumophila so that Legionella urinary antigen, if excreted from patients with L.D., could be detected in an ELISA.

Results from this work indicate that one of the four protocols examined produces avid capture antibody in 75% of NZW rabbits immunised, which can be collected on the 35th day after the priming immunisation. This was before the end point of the protocol. The capture antibodies (IgG) produced by this method were highly specific for Legionella antigen with no cross reactions recorded with the many urines collected from patients with no evidence of Legionella infections. The sensitivity of the test equalled that of an RIA (Dupont) for the detection of Legionella antigen but this kit is licensed only for sale in the U.S.A.. The reagents for the ELISA test have the advantage of having an indefinite shelf life compared with that of the RIA. Also shown is an urinary antigen excretion profile from a patient throughout his illness where capture antibody produced during this work enabled a diagnosis of Legionnaires' disease to be made before culture or serological methods provided any evidence of the cause of the patient's pneumonia. Other animal protocols tested were less successful in the production of avid capture antibody.

One urine collected from a patient with L.D. shown to contain urinary antigen by ELISA, was concentrated and then analysed by SDS-PAGE with a lipopolysaccharide profile (LPS) produced. Further attempts to establish the antigen in other ELISA-"positive" urines to be LPS by the same procedure were unsuccessful. Immunoblotting of concentrated urinary components in antigen and non-antigen containing urine to identify the antigen detected by avid capture antibody was unsuccessful. However, a proteinase-K digest of L.pneumophila SG 1 cells was electrophoresed in SDS-PAGE and after transfer to nitrocellulose, stained with 4 different avid IgG preparations "effective" in the ELISA and produced smooth banding patterns typical of LPS in each case. Two bands at 80 and 60kD were detected on the same immunoblots, although their importance is unclear.

A reproducible system for growth, extraction and analysis of both FA and ubiquinones from the Legionellaceae is described.

A comprehensive "library" of FA profiles is presented.

The need for expensive HPLC equipment to analyse complex mixtures of ubiquinones may not be necessary in view of the reproducible results obtained from 36 Legionella species by the method of thin-layer chromatography using high performance reversed-phase plates. Some Legionella species could be differentiated as they produced consistent

differences in FA and ubiquinone profiles. When results of FA and ubiquinones from type strains were compared with the FA and ubiquinone profiles of some environmental and patient isolates, it was found that in most cases, these were similar.

Finally, as a result of investigative work to identify a number of legionellae whose identities beyond family level were unknown, a probable new subspecies of L.quinlivanii is reported.

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Mitchell, K. and Fallon, R.J. (1990) "The determination of ubiquinone profiles by reversed-phase high-performance thin-layer chromatography as an aid to the speciation of Legionellaceae". J.Gen.Microbiol., 136, 2035-2041.

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List of Abbreviations

$A_{660/595/492/280}$	Absorbance of a solution in a cell of 1cm light path at 660/595/280nm
BCYE	Buffered charcoal yeast extract medium
CAb	Capture antibody (in ELISA)
CIE	Crossed immuno-electrophoresis
dH_2O	Distilled water
DNase	Deoxyribonuclease
DEAE	Diethylaminoethyl cellulose
DPM	Disintegrations per minute
EDTA	Ethylene-diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
FA	Fatty acid
FAB	Fast atom bombardment
GLC/GC	Gas-liquid chromatography
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
IFAT	Indirect immunofluorescent antibody test
IgA/G/M	Immunoglobulin A/G/M
IU	International units
LAL	Limulus amoebocyte lysate
LD	Legionnaires' disease
LPS	Lipopolysaccharide
PBS	Phosphate-buffered saline
RIA	Radio-immunosorbent assay
RNase	Ribonuclease
RFLP	Restriction fragment length polymorphisms
RPTLC	Reversed-phase thin-layer chromatography
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SG	Serogroup
TLC	Thin-layer chromatography
TSE	Tris-saline EDTA
UTI	Urinary tract infection

INTRODUCTION

The Family Legionellaceae

1.1 Historical Background

Since the isolation of a rod-shaped Gram-negative bacterium, from the lungs of a patient who died of "Legionnaires'" disease (L.D.) in the original outbreak at the 58th Annual Convention of the American Legion in Philadelphia in July of 1976, similar organisms have been isolated from both patients and from environmental specimens in many parts of the world. This originally unidentified bacterium, which came to be known as the Legionnaires' disease bacterium (LDB), was discovered after intensive research (McDade et al. 1977), which included efforts to isolate rickettsial forms by intraperitonial inoculation of guinea pigs followed by the transfer of infectious extracts to the yolk sac of developing chick embryos. No rickettsial forms were seen but a Gram-negative bacillus, difficult to grow on ordinary laboratory culture media was revealed, and this organism came to be known as Legionella pneumophila serogroup (SG) 1.

Unclassified organisms isolated in previous years were grown on enriched culture medium (McDade et al. 1979) and resembled the "Philadelphia" bacterium. One of the two strains identified came from a sudden, short outbreak of febrile disease among staff and visitors of a health department in Pontiac, Michigan, in 1968. Now classed as the "Pontiac strain" it was identified in 1977 after its

recovery from the stored frozen lung tissue of guinea pigs. These animals had been placed in various parts of the departmental building to allow their exposure to aerosols, later to be traced to their source, the evaporative condenser of the air conditioning system. The strain was also recovered from the cooling water itself (Glick et al., 1978, Kaufmann et al., 1981).

Similarly the other strain named "Olda" was isolated by guinea pig inoculation from the blood of a patient with a febrile respiratory illness (McDade et al., 1979).

1.2 Nomenclature and Taxonomy of Legionella species

At the first International Symposium on Legionnaires' disease, Atlanta, USA, in November 1978 a number of techniques, most prominently deoxyribonucleic acid (DNA) homology, in addition to phenotypic and serological characteristics (Brenner et al., 1979) enabled this newly recognised pathogen to be designated as a new family (Legionellaceae), genus (Legionella) after the Philadelphia victims and species (pneumophila), after the affinity of the bacteria for the lung. Serological diversity within the species had already been recognised by the time the nomenclature was established (McKinney et al., 1979).

Since then, many species and serotypes have been discovered. At the time of writing this thesis, these number 42 species (including 7 proposed and 6 possible new species) with L.pneumophila represented by 15 serogroups (including

one proposed new unnumbered serogroup) and L.bozemanii, L.longbeacheae, L.feeleii, and L. hackeliae each represented by two serogroups (see table 1, Materials and Methods). Legionellae are rod-shaped being 0.3-0.9um in diameter and 2-6um long. Filamentous forms up to 90um may be found after growth on agar media, are less common in yolk sac material, and very rarely observed in human or guinea pig lung tissue (Weaver and Feeley, 1979, Chandler et al., 1979).

Electron microscopical examination reveals filamentous nucleoids, ribosomes and vacuoles thought to represent poly beta-hydroxybutyrate granules (Chandler et al., 1979). Cells are enclosed by an envelope composed of two three-layered unit membranes; a peptidoglycan layer has been demonstrated for L.pneumophila, and for L.micdadei (Flesher et al., 1979, Pasculle, Myerowitz and Rinaldo, 1979, Gress et al., 1980).

Electron microscopy shows the cells to have a rugose surface with fimbriae present and also a single polar or sub-polar flagellum (Rogers et al., 1980).

Legionellae are not acid-fast by the Ziehl-Neelsen staining procedure for mycobacterium (Hebert et al., 1980) but L.micdadei may appear acid-fast in tissue preparations when stained by methods which use weak acids as decolorising agents (Pasculle et al., 1979).

1.3 Clinical features of pneumonia caused by L.pneumophila

Legionnaires' disease (L.D.) is a potentially fatal pneumonia which in 96% of all cases is caused by

L.pneumophila SG 1. The initial symptoms usually consist of malaise and headache, followed within 24 h by the sudden onset of high non-remittent fever. In some patients the onset is more gradual. Gastro-intestinal symptoms may also appear in the early stages of the illness. Nausea, vomiting, abdominal pain and diarrhoea may occur.

On the second or third day of illness a dry cough begins producing small amounts of mucoid sputum.

High fever continues until the institution of antimicrobial therapy. Spontaneous resolution of the infection may begin on the eighth to tenth day.

Confusion and disorientation are present in some patients with their occurrence indicating some degree of toxic encephalopathy (Bartlett et al., 1986).

1.4 Radiographic Features

Radiography can be of use to complement other diagnostic measures in the identification of L.D. The radiographic appearance is variable, but commonly patchy lung infiltrates are seen initially. A characteristic feature of the pneumonia is that it may become extensive involving more than one lobe of the lung.

The resolution of radiographic infiltrates often lags behind clinical recovery. Complete resolution usually requires many months, even in previously healthy patients (Bartlett et al., 1986).

1.5 Pontiac Fever

This is the non-pneumonic form of *Legionella* respiratory infection (Glick *et al.*, 1978) and was identified in the influenza-like epidemic in the County Health Department building in Pontiac, Michigan.

A *Legionella* infection may either manifest itself as a *Legionella* pneumonia, with a low attack rate and high mortality or as a Pontiac fever, often with a high attack rate and no mortality.

The reason for the manifestation of one or other forms of infection is not clear. *L.pneumophila*, causing both types of illness, has shown no difference in virulence in animal models (Huebner *et al.*, 1984).

Examination reveals a fever greater than 39.5°C, and only modest respiratory symptoms. The illness usually resolves within 2-5 days after the initial incubation period of 36-48 h. The clinical diagnosis is retrospective by serological testing, but *Legionella pneumophila* antigen has been reported in patients' with Pontiac fever (Freidman *et al.*, 1987) and treatment is symptomatic as the disease is self-limiting.

1.6 Legionella and other respiratory pathogens

Four patients, in a total of seven described from one institution, died. These patients had a severe pneumonia and a dual infection with *L.pneumonia* SG 1 and *L.micdadei*. Most of these seven patients were markedly immunocompromised

(Muder et al., 1983, 1984). Also reported are mixed infections with L.pneumophila and viruses (Helms et al., 1984), Mycobacterium tuberculosis (Milder and Rough, 1982), Mycoplasma pneumoniae (Helms et al., 1984), Coxiella burnetii (Domaradzki et al., 1984), Klebsiella species (Kirby et al., 1980) and Streptococcus pneumoniae (Macfarlane et al., 1982). A mixed infection should be suspected if a patient is not improving as expected.

Important clues in the differentiation of Legionella pneumonia from other pneumonias include high fever, unexplained confusion, multi-system involvement (eg. central nervous system, gastro-intestinal systems, liver, renal, heart and skeletal muscle), sparse mucoid sputum with scanty pus cells on staining and no predominant pathogens on culture. There is also a lack of response to beta-lactam and aminoglycoside antibiotics. Community-acquired pneumonias that can produce a clinical picture similar to Legionella infection, include Q fever, psittacosis and pneumococcal infection especially when complicated by bacteraemia. In addition, the awareness of cyclic epidemics (eg. Mycoplasma pneumoniae every 3-5 years) and seasonal variations, should be borne in mind.

1.7 Pathology

When the lungs are examined macroscopically, a multifocal pneumonia with a tendency for coalescence of lesions, is most common. The exudates contain large amounts

of fibrin, producing a granular white appearance after fixation with formaldehyde solution.

Microscopically, there is an abundant inflammatory exudate in the distal airspaces, alveoli and bronchioles. The exudate is composed of a mixture of polymorphonuclear leukocytes (PMNL) and macrophages, with one or other predominating in some cases. This appearance is not confined to Legionella pneumonias. Legionella bacteraemia has been demonstrated (Edelstein et al., 1979) and may be more common in severely ill patients, although this has never been demonstrated in our laboratory.

1.8 Patient management

This is divided between antibiotic therapy and equally important general supportive therapies.

1.8.1 Antimicrobial Therapy

In-vitro, Legionella species are susceptible to a variety of antimicrobial agents using available methods to determine minimal inhibitory concentrations (MIC's). *In-vitro*, Legionella are most sensitive to rifampicin and in guinea pigs (lowest MIC of 0.03㎍g/L) tested against these organisms (Ristuccia et al., 1984) but they are sensitive *in-vitro* to erthromycin (MIC of about 0.2㎍g/L), aminoglycosides, sulfamethoxazole-trimethoprim, chloramphenicol, cefotixin, doxycycline (the most active of the tetracyclines), and minocycline (Thornsberry et al., 1978). Almost all Legionella species produce beta-lactamase

and other enzymes capable of hydrolysing beta-lactam substrates.

However, these *in-vitro* studies have uncertain relevance to the treatment of human infection as, *in-vivo*, the legionellae are intracellular pathogens. Many of the bacteria can survive and multiply in pulmonary macrophages.

Clinical experience indicates that intravenous erythromycin therapy is associated with the lowest mortality and it is, at present, the recommended antibiotic for treating Legionella pneumonia. Use of rifampicin, together with erythromycin, is recommended in patients with Legionella pneumonia who are critically ill or immunosuppressed and the earlier the diagnosis can be established, the sooner specific treatment can be commenced. Importantly, the response of severely ill patients to these antibiotics is rapid, but these antibiotics are different from those normally administered when pneumonia is diagnosed and a Legionella infection is not suspected.

1.8.2 General supportive measures

Patients may require intensive care. The two most important factors affecting the outcome include the state of the patient's health before the infection and the therapy given for the infection. The prognosis for previously healthy individuals is good and the fatality rate low (10-15%). Predisposing factors including smoking, age (where infection is greatest in the 40-70 years old bracket),

and prior chronic diseases raises the fatality rate and can be as high as 70-80 % in immuno-suppressed patients.

1.9 Environmental sources of infection and modes of transmission

The occurrence of L.D. cases have been mainly sporadic and the source of these infections have not been identified. However, investigations into outbreaks of L.D. and Pontiac fever indicated that aerosols were responsible for infection and many different sources of these have been identified.

Recent outbreaks of L.D. in England include London where several outbreaks have been reported. In January 1989 there were 33 confirmed cases of Legionella infection with 5 deaths recorded. Infection in these cases were linked to cooling towers at two sites in Piccadilly (House of Commons; Employment Committee (session 1989-'90) 1st Report L.D.: Further Developments). The other outbreak, discussed in the same report, occurred in April 1989 with the Imperial College and the Science Museum both being implicated as the source of the outbreak where 5 fatalities were recorded. Legal proceedings are underway. Another outbreak, again described in the same report was at Nottingham University and resulted in 2 deaths. This time the outbreak was associated with the hot-water system. Finally, again in the London area, an investigation in the Spring of 1988 into an outbreak where of 55 people who fell ill, 3 died, was linked with the retirement of one man at the BBC headquarters who

was responsible for the maintenance of the building's cooling towers. This provoked anger as it was due entirely to "a period of confusion" after the man's retirement where the "safety arrangements were appalling" (House of Commons; Employment Committee (session 1989-1990); 1st Report: Legionnaires' disease at the BBC). In Scotland, an outbreak was recorded in November 1985 where a number of hospital-associated cases of L.D. were reported, the result of aerosol infection originating from the cooling tower of Glasgow Royal Infirmary (Timbury et al., 1988). Also the largest outbreak of Pontiac fever outside North America and the first ever attributable to L.micdadei occurred between 31st December 1987 and 4th January 1988 at Lochgoilhead on the West coast of Scotland, where about 200 individuals who were visitors to a hotel and leisure complex developed symptoms consistent with Pontiac fever. L.micdadei was isolated from a whirlpool spa and antibodies to L.micdadei were found in 180 individuals (Goldberg et al., 1989) who had visited the complex whereas no antibodies were found in 31 local residents who had not visited the complex. Legionella urinary antigen was detected in one individual (S.Toma, communication).

In other outbreaks, cooling towers for wet air-conditioning systems (Dondero et al., 1980), piped water systems in hospitals and hotels, particularly hot-water circuits, cooling systems employed for industrial purposes,

spas and whirlpools have been identified as the sites of origin of infection. One unusual source was identified when a new species, L.feeleii (WO-44C), was isolated from cutting oil at an automobile plant in Ontario, Canada, after a number of workers reported ill with symptoms of Pontiac fever (Herwaldt et al., 1984). Other sources include natural and potable water supplies, so that the ubiquity of this family of organisms must be recognised. As Legionella species occur in many aquatic habitats it is interesting to note that outbreaks are more limited than perhaps would be expected. It is not understood why a Legionella infection may result in L.D. or in Pontiac fever. However, in either infection there must be a reservoir of bacteria where amplifying factors allow legionellae to grow from low to high concentration. There must be a mechanism for dissemination of legionellae, which must be virulent for humans and finally these organisms must be inoculated at an appropriate site on a host susceptible to a Legionella infection.

1.10 Laboratory diagnosis

1.10.1 Clinical Material

In patients with a suspected Legionella infection, specimens are taken to demonstrate bacterial antigen or antibody. Clinical material for examination may be sputum, tracheal and bronchial aspirates or washings, pleural exudate, lung tissues, blood for culture, urine and serum

taken in life or at post mortem.

The rate of recovery of Legionella species from sputum of patients by the use of selective media is quite high and should be attempted routinely, assuming sufficient sputum can be obtained. This is often not the case. Tracheal aspirates are claimed to be the optimal source for the recovery of Legionella species (Zuravleff et al., 1983). However, bronchial washings obtained by bronchoscopy, when this is warranted on clinical grounds, are also valuable. Pleural fluid has enabled early isolation of L.pneumophila (Dumoff, 1979) but the recovery of legionellae is very low. Lung biopsies are not usually required but may be taken from seriously ill patients. Although the various manifestations of legionellosis are not due to spread via the blood, bacteraemia has been demonstrated.

The low recovery rates of Legionella are probably due in part to the lack of reliable liquid culture media and better results are said to be obtained by lysing and centrifuging blood and inoculating the deposit onto BCYE. This method may also recover intracellular organisms in monocytes and macrophages.

Berdal et al. (1979) first demonstrated the potential use of enzyme-linked immunosorbent assay (ELISA) to detect the soluble antigens of L.pneumophila in patient's specimens (e.g. urine and sputum). The majority of subsequent published data has been presented in a series of papers by

Kohler and colleagues. The first of these (Kohler et al., 1981) reported the use of a solid-phase radioimmunoassay to detect Legionella antigen in urine. The specificity of this test was investigated by Kohler and colleagues and found to be almost 100%. In a later publication (Sathapatayavongs et al., 1982) antigen was detected in 83% of urine specimens where these were taken retrospectively from 39 L.D. patients during an outbreak. In these studies, one of the most important findings was the demonstration of urinary antigen in most patients 1-3 days after onset of symptoms and that this excretion of antigen may persist for many weeks. The RIA was adapted, first as an ELISA (Sathapatayavongs et al., 1982) and then as a latex agglutination (Sathapatayavongs et al., 1983). The ELISA took longer to perform but had comparable specificity and sensitivity as the RIA. The latex agglutination, although simple to perform, was not as specific or sensitive as either the ELISA or RIA. The value of a specific test for the detection of soluble Legionella antigens in urine is important in the early diagnosis of Legionnaires' disease. Also, patients infected with L.pneumophila other than SG 1 may excrete urinary antigen which can be detected by ELISA or RIA (Tang et al., 1989) Finally, other species of Legionella may give rise to antigenuria (Tang and Toma, 1986).

Serum is routinely examined for the development of specific antibodies. These do not appear until at least

eight days after the onset of illness and their appearance may be delayed for many weeks (Bartlett et al., 1986).

1.10.2 Bacterial culture

For the investigation of clinical material this method is very sensitive and specific (Edelstein et al., 1980, 1984, Zuravleff 1983). It is the definitive indication of infection. Pinpoint-size colonies appear on buffered-charcoal yeast extract (BCYE) agar in 2-3 days on primary culture, the colony diameter reaching 3-4 mm after 5-7 days of incubation at 36 +/- 1°C.

Colonies are usually greyish-white but may appear green, yellow (unpublished observation) or pink on microscopical examination with young colonies having a characteristic "cut-glass" appearance when observed under a plate microscope in all except one species (L.moravica).

Legionellae do not grow on standard blood agar, nutrient broth or other commonly employed laboratory culture media. Mueller-Hinton agar supplemented with 1 % haemoglobin and 1 % Isovitalex (MH-IH) was first used by Weaver for cultivation of L.pneumophila (Feeley et al., 1978) but better media are now available. Charcoal yeast extract (CYE) agar was subsequently developed and found superior to Feeley-Gorman agar and the addition of ACES buffer (Sigma) greatly improved its performance as did the addition of alpha-ketoglutarate (Pasculle et al., 1980). More recently, the addition of 1% (w/v) BSA has been reported to increase

the success of isolation of L.bozemanii and L.micdadei from clinical specimens (Morrill et al., 1990). In all of these media, soluble ferric pyrophosphate replaces haemoglobin and L-cysteine-HCl replaces Isovitalex as growth factors for legionellae.

1.10.3 Serological detection of Legionella antigen

The direct fluorescent antibody (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. It is used both to determine whether legionellae are present in clinical and environmental samples and as a rapid presumptive test for the identification of legionellae (Cherry et al., 1978, Cherry and McKinney, 1979). The use of the indirect fluorescent antibody test (IFA) to detect Legionella antigens has the advantages that the specific antisera is used at a very high dilution with the result that any cross reactions are almost eliminated, a problem sometimes encountered with DFA (Orrison et al., 1983).

When compared with culture, DFA is reported to be the more sensitive as only 15 % of DFA-positive environmental samples proved culture-positive for Legionella (Fliermans et al., 1981).

An alternative to DFA is a commercially available DNA probe (Gen-probe) for the detection of Legionella species

and this has been evaluated (Edelstein, 1986., Pasculle et al., 1989) and deemed a satisfactory replacement for DFA with a specificity of up to 99.2% and a sensitivity of 68.9 to 69.2%. This has obvious value for laboratories with limited DFA capability.

Legionellae share a number of immunologically cross-reactive flagellar antigens (Thomason et al., 1979) but this property is not used as a diagnostic test as flagella are rarely seen in isolates from respiratory tract infections.

1.10.4 Detection of antibodies to Legionella

The indirect fluorescent (IFA) antibody test for the detection of antibodies in patients' sera was first developed by Wilkinson et al., (1979) who used heat-killed bacterial suspensions as antigen. Later, Taylor et al., (1979) reported the successful use of formalin-treated Legionella-infected yolk sac of the embryonated hen's eggs (FYSA) as an alternative. Both antigens are in wide use but most laboratories in the U.K. use FYSA to aid in the diagnosis of legionellosis.

A four-fold or greater rise in titre to 128 in paired acute and convalescent sera in a clinically suspicious case is considered definitive evidence of infection with L.pneumophila SG 1. With other serogroups and species this is still a tentative indication of infection. In a single serum sample from a patient with suspected L.D., titres of 256 or more are considered "presumptive" evidence of

infection. Unfortunately, IFA reagents are not commercially available for all serotypes and species of Legionella and "in-house" manufactured reagents may differ widely between laboratories.

Despite the many new species of Legionellaceae being identified (Brenner 1984, Brenner et al., 1985), the organism most commonly responsible for the severe pneumonia is still L.pneumophila SG 1. The factors responsible for the virulence of L.pneumophila SG 1 are presently under investigation.

Other tests for the detection of antibodies to legionellae include the microagglutination test which was developed by Harrison and Taylor (1982), who claimed it to have sensitivity equivalent to that of the IFA test. An ELISA which utilises soluble or EDTA-extracted antigen from Legionella species adsorbed to polystyrene microtitre plates has been described (Farshy et al., 1978, Wreghitt et al., 1982, Zuravleff et al., 1983) as has an indirect haemagglutination test with the antigens adsorbed to turkey erythrocytes (Yonke et al., 1981).

1.10.5 Detection of urinary antigen by ELISA and RIA

The study of urine as an antigen reservoir has provided evidence of soluble or particulate microbial antigens being excreted during many infections (Coonrod, 1983).

Both polysaccharide and protein antigens have been detected in urine by immunological methods for different

infections including bacterial, viral and parasitic diseases (e.g. H.influenzae type b, bovine Leukaemia virus in cattle and humans infected with Schistosoma mansoni). Detection of a thermostable antigen in the urine of Legionnaires' disease patients by ELISA, radioimmunoassay (RIA) or latex agglutination has been an important advance. An enzyme-linked immunospecific assay "sandwich" technique was developed for detecting soluble antigen from the Legionnaires' disease bacterium (L.pneumophila) (Berdal et al., 1979). With this technique, antigen was detected in urine specimens from guinea pigs inoculated intraperitoneally with heat-killed L.pneumophila and in urine specimens from three of four patients who attended the American Legion Convention in Philadelphia in 1976. A fifth patient, who was a dubious seroconverter, was negative (Berdal et al., 1979). Goat antiserum used in the test, was diluted but unfractionated and the test sensitivity was such as to detect as little as 3 pg of LPS/protein complex. These early results were published without delay because of the potential usefulness of the test, even though only four patients were examined.

Interest in the rapid diagnosis of Legionnaires' disease by urinary antigen detection grew and a number of laboratories became active, notably Kohler and his associates, in an effort to consolidate this non-invasive and simple test. Initially, RIA was more sensitive, but

doubling of the IgG conjugate (IgG-horse radish peroxidase) concentrations from 5 to 10 ug/ml raised the sensitivity of the ELISA to that approaching the RIA test (Sathapatayavongs et al., 1982). Both ELISA and RIA (Sathapatayavongs et al., 1983) revealed that of 47 patients with L.D. tested, antigen was detected in 39. In the case of ELISA visual analysis of the test plate gave 35 clear positive results, the remaining four requiring spectrophotometric verification. Another 178 urine specimens from patients with non-Legionella associated pulmonary infections proved to be negative. Conclusions drawn were that the ELISA was a rapid, sensitive and specific means for the rapid diagnosis of L.D. that can be performed in clinical laboratories unwilling or unable to use radio-isotopes.

The antiserum used by Sathapatayavongs et al. (1983) was not directly compared with that used by Berdal et al. (1979) and was obtained using a different immunisation schedule consisting of multiple inoculations over a fifteen month period with a "many-month" rest period. This extended period, it was claimed, was the reason for a more sensitive antiserum when compared to Berdal's antiserum taken 6-7 weeks post immunisation. This claim is only speculative though as a direct comparison with Berdal's antiserum which was obtained in six weeks was never performed. Serendipitous animal immunoresponsiveness alone

could have played a major role in the length of time for a working antiserum to be obtained and sensitivity of the antiserum could be due to other factors.

Another survey of clinical specimens of urine and serum from an outbreak in Burlington, Vermont, compared the sensitivity of RIA with that of DFA and culture (Kohler et al., 1984). Twenty-six (76%) of 34 patients had detectable urinary antigen. Antigen was detected by RIA (a test similar to ELISA except that the IgG conjugate was labelled with ^{125}I instead of an enzyme) in 14 (93%) of the 15 patients with positive cultures and 12 (63%) of the 19 with serological but not cultural evidence of infection (Kohler et al., 1981). It would appear that when RIA or ELISA examination of urine is used together with DFA it is possible to make a diagnosis in most cases of clinically suspected pneumonias due to L.pneumophila SG 1.

Monoclonal antibodies (IgG), to L.pneumophila SG 1 specific antigen have been bound to horseradish peroxidase and used as detecting antibody in an indirect ELISA allowing serogroup identification to sub-typing level after well-coating with fractionated rabbit anti-L.pneumophila SG 1 antiserum as capture antibody. It was noted (Bibb et al., 1984) that by a combination of mouse monoclonals conjugated to horseradish peroxidase and rabbit antisera raised to "Knoxville-1" and "Olda" used as capture antibody that the rabbit antisera raised to "Knoxville-1" gave the more

sensitive ELISA system for urinary antigen detection. These antisera were tested using specimens from three cases of legionellosis confirmed by culture of the organism from respiratory secretions or lung tissue.

To enable an ELISA test to be used in the diagnostic laboratory for the detection of antigenuria, the actual onset and duration of the excretion of urinary antigen itself must be investigated.

A study designed to determine whether antigen is excreted by patients with Legionnaires' disease early enough after the onset of symptoms to be useful for making therapeutic decisions and whether antigen excretion ends when successful treatment is concluded was carried out by Kohler et al. (1984). Specific antigen was detected by ELISA in the urine of 14 (86%) of 16 patients with Legionnaires' disease during days 1 to 3 after the onset of symptoms, 33 (80%) of 41 during days 4 to 7, 25 (89%) of 28 during days 8 to 14 and 11 of 11 patients after day 14. Antigen excretion persisted for 42 days or longer in 15 of the 16 patients. Conclusions drawn were that antigen can be detected early after symptoms thus allowing meaningful therapeutic decisions to be made, but that prolonged antigen excretion (the longest documented duration was 326 days) may negate the diagnostic value of urinary antigen detection for relapsing or recurrent L.pneumophila pneumonia.

Preparation of the immunogen for raising rabbit

antisera differed between studies (Berdal et al., 1979, Kohler et al., 1983, Kohler et al., 1985). These workers used autoclaved or boiled standardised suspensions of whole cells of L.pneumophila SG 1, and mixed them with equal volumes of Freund's adjuvant for vaccination of rabbits. Formalin-treated whole cells of L.pneumophila SG 1 can also be used to produce a suitable antiserum (McKinney et al., 1979, Tang and Toma, 1986,). Again, in other studies (Conlan and Ashworth, 1986, Williams and Featherstone, 1988) saline extracts from whole cells of L.pneumophila SG 1 were successful immunogens, although, difficulties in raising an avid and specific antiserum routinely have been acknowledged (Samuel et al., 1990, Fehrenbach et al., 1986, Sathapatayavongs et al., 1982).

Antisera to L.pneumophila SG 1 may be of use in the detection of urinary antigen produced by serogroups of L.pneumophila other than SG 1 (Kohler et al., 1985), and this has recently been documented (Tang et al., 1989) where the first Canadian case of L.pneumophila serogroup 12 causing Legionnaires' disease in a 52 year-old male was identified using a "broad-spectrum" capture antibody in an ELISA to detect urinary antigen. Also a solid phase radioimmunoassay was developed to detect antigens of L.pneumophila SG 4. Antigen in the urine of two patients, one infected with the Leiden-1 strain (SG 10) and another with a L.pneumophila SG 1 infection was detected using the

antiserum to SG 4. Urinary antigen in patients with SG 1 and Leiden-1 Legionella infections may therefore contain components serologically cross-reactive to L.pneumophila SG 4.

Hence, a number of reports have been published since 1979 about the application of the rapid techniques for demonstrating Legionella antigens in body fluids, using monoclonal or polyclonal antibodies produced in the rabbit and other animals against legionellae. These assays differ individually in methodology, specificity, sensitivity and may react to a range of individual serogroups or subtypes. The number of specimens investigated ranged from a few cases to a large group of patients. However, antigen assays of the ELISA and RIA type have not been widely used. The major drawback with these tests is the preparation of suitable working antiserum for antigen capture (the binding of antigen to a solid phase). Kohler and Sathapatayavongs (1983) reported that of six rabbits inoculated only one produced an avid antiserum. The difficulty of producing large quantities of suitable antiserum has prevented the establishment of this as a routine diagnostic test in most laboratories.

There must be no doubt though, that the diagnostic panel of tests used by reference laboratories for diagnosis of legionellosis should include, in addition to their conventional diagnostic tests, ELISA or RIA procedures for

urinary antigen detection.

1.11 Characterisation of urinary antigen

A number of Legionella antigens have been identified and described in the literature.

A species-specific antigen in L.pneumophila was identified by a monoclonal antibody in enzyme-linked immunosorbent and immunofluorescent assays of serogroups 1 through to 8 (Gosting et al., 1984). The species-specific antigen was a heat-stable protein and the molecular weight of the major band was 29,000 kD by immunoblot analysis. In direct immunofluorescence assays, the antigen was cryptic or only partially exposed by treating with detergent and EDTA (Gosting et al., 1984). Antigens of six serogroups of L.pneumophila were compared in two dimensional (crossed) immunoelectrophoresis using rabbit antisera to serogroups 1,2,3 and 4. The close relationship between the serogroups was shown by the fact that 27 of the 31 antigens demonstrated were common (Joly and Kenny, 1982). However, distinctive group-specific antigens with slow electrophoretic mobility were observed for serogroups 1, 2, 3, and 4. When intact serogroup 1 organisms were extracted with EDTA, the group-specific antigen was recovered in a virtually pure form. The group-specific antigen was pronase resistant, heat-stable, ampiphilic and had a surface location, all of which suggest lipopolysaccharide (Joly and Kenny, 1982). Since urinary antigen has been detected by

ELISA with antiserum raised to boiled or autoclaved cells (Berdal et al., 1979, Kohler et al., 1983. Kohler et al., 1985), the excreted antigen may be heat-stable lipopolysaccharide (LPS). Most surface protein epitopes would be denatured by this method of antigen preparation. A method described by Conlan and Ashworth (1986) involved the treatment of a saline extract of the "Corby " strain of L.pneumophila SG 1 with deoxyribonuclease-1, ribonuclease-1 and pronase and was utilised for the preparation of immunogen for raising antiserum for an ELISA to detect urinary antigen in patients with L.pneumophila SG 1 infections (Williams and Featherstone, 1988). This proved to be an effective way of obtaining a working antiserum and showed that the urinary antigen was resistant to each of these enzymes, providing further evidence that the antigen found in urine is indeed LPS, possibly in a low molecular weight form. Microbial polysaccharide antigens up to 70kD have been reported in urine (Coonrod, 1983). This may be the size exclusion limit beyond which particles greater than 70kD are not excreted through the kidneys.

1.12 Other techniques for urinary antigen detection

As previously noted, latex agglutination and reverse passive haemagglutination tests have also been described for the rapid diagnosis of Legionnaires' disease by detecting antigen in human urine (Sathapatayavongs et al., 1982, Mangiafico et al., 1981 and Tang et al., 1982). Latex

agglutination is a simple, rapid and inexpensive method for the diagnosis of infections but again relies on the availability of avid and specific antiserum. These authors have previously published data on working ELISA and RIA diagnostic procedures. When compared with RIA and ELISA using serial dilutions of a single antigen-containing urine, latex agglutination was 16-fold less sensitive (Sathapatyavongs et al., 1982). Reverse passive haemagglutination (RPHA) has been used successfully in the past for detection or assay of both tetanus toxin and staphylococcal enterotoxin B in culture filtrates and in food samples. RPHA has not been directly compared with RIA or ELISA, but has been quantitatively evolved to detect as little as 200 pg of total antigen (Mangiafico et al. 1981).

None of these alternative methods has yet been routinely applied in diagnosis of Legionella infection.

1.13 Definition of Legionella species

The generally accepted classification of the family Legionellaceae is based on the work of Brenner and colleagues (1984). These workers take the definition of a "genetic" species as a group of strains whose DNAs are 70% or more related at optimal reassociation conditions; 55% or more related at stringent conditions and have 6% or less divergence in their related sequences. The identification of legionellae outwith genetic means is an exhaustive task and includes the observation of colonial morphology; with

convex, round-edged colonies and a "cut-glass" appearance under a plate microscope; by microscopy after Gram staining or flagellar staining; testing for nutritional requirements; biochemical testing for catalase; this being positive for all legionellae when carried out according to the Howie Code of Practice (1978); oxidase which varies considerably within the family; gelatin liquefaction with most species producing a gelatinase in BCYE where the agar is substituted with 3% gelatin; nitrate reduction negative in all legionellae enabling them to be distinguished from members of the Enterobacteriaceae; hippurate hydrolysis allows L.pneumophila to be distinguished from other legionellae (except L.feeleii [+/-]); beta-lactamase production positive with most legionellae and easily seen with a chromogenic cephalosporin (Nitrocefin); urease production all legionellae are reported to be urease negative; browning of tyrosine-supplemented medium varies within the family; and colony autofluorescence which, when seen can be observed as as blue/white, red or yellow (unpublished observation), the colour being species dependent.

These phenotypic characteristics can be supplemented by analysis of whole cell fatty acids by gas-liquid chromatography (GLC), by the analysis of the composition of their ubiquinones and by using serological methods.

1.13.1 GLC of whole cell fatty acid methyl esters (FAMES) in identification

The use of GLC in clinical and diagnostic bacteriology laboratories has increased significantly in recent years. This is most probably as a result of the vast technical advances in column manufacturing resulting in consistency of the highest order particularly with "capillary-type" columns. GLC is a simple and powerful method for the separation and quantitation of volatile organic compounds by partitioning these components between a mobile carrier gas, and a liquid stationary phase bound to an open tubular column. Separated components are detected and quantified using a detector (FID) and recorded on a computer integrator. In the analysis of metabolic products from bacterial growth and chemical components of bacterial cells, GLC has provided useful information for rapid identification of bacteria assigning them to genus or even to species level e.g. Legionellaceae have a very complex nonhydroxy FA profile compared with the simple FA profile found in the genus Bordetella and where Bordetella pertussis (FA content is unaffected by phase variation) may be differentiated from B.bronchiseptica (Kawai and Moribayashi, 1982).

Members of the genus Legionella contain large amounts of branched-chain fatty acids released from the cell envelope with both quantitative and compositional differences reported between some species. Reported

quantitative differences in cellular fatty acids within a species are small (2-4 %) e.g. variations in the amount of dl-cis 9,10 methylhexadecanoate (17:0$^{\Delta}$) in the Pontiac and Philadelphia-2, Philadelphia-3, and Philadelphia-4 strains (Moss et al. 1977, Moss and Dees, 1979). Other reported quantitative differences in environmental and control strains of Legionella pneumophila (Philadelphia-1 and Dallas-1E/ species 1) (Selander et al., 1985) fall within this range. No major differences in cellular fatty acid composition have been shown between serogroups of L.pneumophila or within a serogroup for either environmental or patient strains (Moyer et al., 1984).
Bacterial FA's analysed and identified by GLC should have their identity confirmed through structural analyses by mass spectrometry.

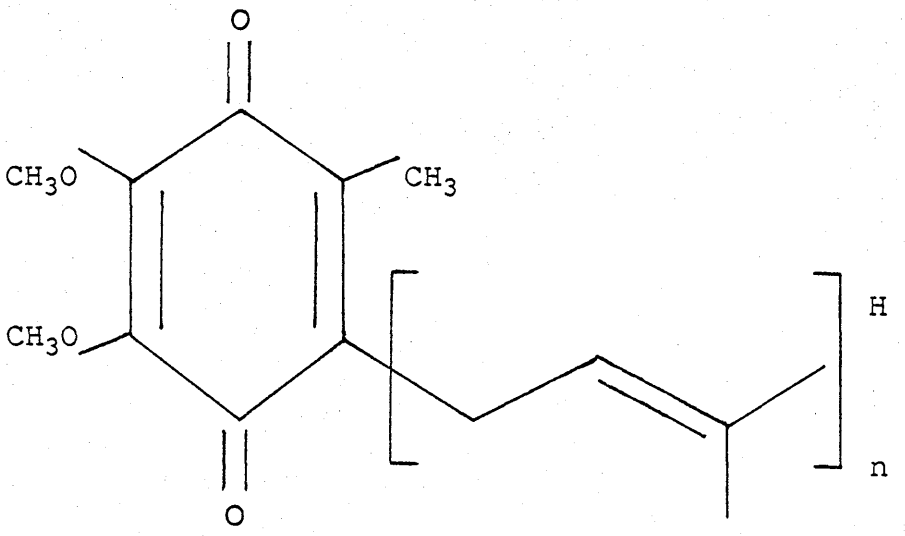
Epidemiological studies may be enhanced by the identification of new species as well as known species by their characteristic fatty acid methyl ester (FAME) profile (Moyer et al., 1984). There are many published chromatograms and discussions of saponified and methylated fatty acids (Edwards and Feltham, 1983, Mayberry 1981, 1984) of the more commonly found Legionella species. More recent publications report unidentified species as a result of combined gas-liquid chromatographic, genetic and serological tests (Lindquist et al., 1988, Thacker et al., 1988 and Gorman et al., 1985). The role of GLC is therefore contributory to

the identification of Legionella species and also to help classify new isolates. The analysis of cellular fatty acids of all known Legionella reference strains to investigate the possibility of identifying isolates from man and the environment to species level is investigated in this thesis.

1.13.2 Reversed-phase thin layer chromatography of ubiquinones

The Legionellaceae possess an unusual pattern of respiratory quinones, which differs between the few species previously investigated in this way and may be used in identification (Wait, 1988). This is more fully investigated in this thesis. Isoprenoid quinones (2,3-dimethoxy-5-methyl-6-multi-prenyl-1,4 benzoquinones (figure 1) are found in bacterial plasma membranes where they are the electron acceptors and donators of the oxidative phosporylation process in the Tricarboxylic acid cycle. The more common classes of quinone are the menaquinones and the ubiquinones. Only ubiquinones are present in detectable quantities in the Legionellaceae and using reversed-phase thin layer chromatography (RPTLC) and mass spectrometry, Karr et al., 1982 identified Q-11 (where the number refers to the length of the carbon chain linked to carbon-6 on the benzoquinone ring), Q-12 and Q-13. These observations were extended (Collins and Gilbart, 1983), and the presence of Q-14 and Q-15 was demonstrated as well as information of ubiquinone content in 5 species;

Figure 1 Structure of ubiquinone (Q-n)



L.dumoffii, L.gormanii, L.jordanis, L.micdadei and L.pneumophila by high performance liquid chromatography (HPLC, Gilbart and Collins, 1985). HPLC is not always available and reversed-phase thin-layer chromatography (RPTLC) was investigated as an alternative technique to provide a complete library of ubiquinone profiles for all known legionellae (Mitchell and Fallon, 1990). Ubiquinone profiles, by this method, together with the GLC of fatty acids of the Legionellaceae have been compiled in this thesis to compare these with both patient and environmental Legionella isolates. To confirm the presence and/or identity of ubiquinones in preparations made from cells and used for either RPTLC or HPLC, samples should be analysed by mass spectrometry.

1.13.3 Identification of Legionellae by Serological Methods

The use of biochemical reactions, GLC and isoprenoid quinone profiles can confirm that an isolate is a Legionella and sometimes to which species it belongs, but DNA hybridisation studies are needed for a definitive identification where this may also be supplemented by restriction enzyme digestion of cellular DNA to determine the restriction fragment patterns (Saunders et al., 1990). As these techniques are not available in most laboratories, a presumptive identification is usually made using serological methods. Legionellae can be subdivided into serogroups by their reaction with hyperimmune rabbit

antisera containing antibodies directed against the heat-stable somatic lipopolysaccharide (LPS) or "O" antigens. Slide agglutinating antisera have also been used for the rapid identification of legionellae (Wilkinson and Fikes, 1980). However, many species and serogroups within the family Legionellaceae have antigens in common and cross-reactions are seen and therefore the serological identification of legionellae is not always simple. However, the identification and detection of L.pneumophila is now possible in most laboratories with the availability of monoclonals to a species-specific antigen (e.g. Genetic system, U.S.A.) and also the detection of L.pneumophila SG 1 also using a monoclonal (ICN ImmunoBiologicals Ltd.) to a unique antigen common only to all strains of this serogroup.

1.14 Aims of Research

Legionnaires' disease, a potentially lethal pneumonia and hence the more serious form of Legionellosis, is caused in 96% of cases by, L.pneumophila SG 1. Rapid diagnostic techniques are necessary for the prompt recognition and treatment of infection. Thus two aims of this project were:

a) to produce specific antiserum to L.pneumophila SG 1 which will bind urinary antigen to plastic microtitre trays used in the ELISA test thus enabling a valuable service to be offered to Scotland and for the possible distribution of this antiserum to other laboratories for their use.

b) to investigate and characterise the heat stable antigen detectable in urine from most patients in the early stages of L.D..

Species Recognition

Speciation of isolates where phenotypic observations leave doubt or serological cross-reactions have occurred may be supported by other investigations including a variety of analytical techniques. Further aims were:

c) to develop a library of fatty acid methyl ester (FAME) profiles of Legionella species by capillary gas-liquid chromatography.

d) to develop a library of ubiquinone (Co-enzyme Q) profiles of Legionella species by the separation and analysis of these components by high performance reversed-phase thin-layer chromatography.

e) To compare the results obtained from the libraries with a number of environmental and patient isolates.

MATERIALS AND METHODS

2.1 Bacterial strains

The following *Legionella* strains obtained from the -70°C stocks of frozen bacterial suspensions at the Department of Laboratory Medicine at Ruchill Hospital are recognised reference strains. The strain numbers are given together with the original source of each culture in table 1.

Other strains used in these studies are of environmental origin obtained mainly through the Wolfson Laboratory or isolated from patients and are designated by laboratory assigned numbers. These are :

86/35784	(*L.quinlivanii*-subspecies)
87/31871	*L.pneumophila*
88/1058	*L.micdadei*-responsible for the Lochgoilhead outbreak - February 1988 (Goldberg *et al.*, 1989)
88/24485	*L.anisa* (patient isolate)
88/42783	*L.pneumophila* SG 12
88/42784	*L.pneumophila* SG 1 (Olda)
88/42785	*L.pneumophila* SG 6
88/42787	*L.pneumophila* SG 1 (Olda)
88/42788	*L.pneumophila* SG 6
88/42791	*L.pneumophila* SG 1 (Bellingham strain)
88/42869	*L.longbeachae* SG 2
88/46768	*L.pneumophila* (patient isolate)
ML-76	Serovar of *L.spiritensis* (Harrison *et al.*, 1988) (Colindale)
P185	(*L.pneumophila* strain, SG 9 ?)
89/03289	*L.pneumophila* SG 5
89/13077	*L.hackeliae* SG 1
89/20794	*L.quateriensis*
89/29805	*L.pneumophila* SG 5
89/42151	*L.pneumophila* SG 10

Table 1 Recognised Legionella strains

SPECIES	SEROGROUP	STRAIN	SOURCE
L.adelaidensis	-	F/636	IMVS
L.anisa	-	CH-47-C-1	CDC
L.birminghamensis	-	1407-AL-H	CDC
L.bozemanii	1	WIGA	CDC
L.bozemanii	2	Toronto-3	CDC
L.brunensis	-	444-1	CDC
L.cherrii	-	ORW	CDC
L.cincinnatiensis	-	70-OH-H	CDC
L.dumoffii	-	TEX-KL	CDC
L.erytha	-	SE-32A-C8	CDC
L.feeleii	1	WO-44-C3	CDC
L.feeleii	2	691-W1-H	CDC
"L.geestiae"	-	1308	CAMR
L.gormanii	-	LS-13	CDC
L.gratiana	-	ATCC 49413	Lyon
L.hackeliae	1	Lansing-2	CDC
L.hackeliae	2	798-PA-H	CDC
L.israelensis	-	Bercovier-4	CDC
L.jamestowniensis	-	JA-26-G1-E2	CDC
L.jordanis	-	BL-540	CDC
"L.londoniensis"	-	1224	CAMR
L.longbeachae	1	Longbeach-4	CDC
L.longbeachae	2	Tucker-1	CDC
L.macheachernii	-	PX-1-G2-E2	CDC
L.micdadei	-	TATLOCK	CDC
L.moravica	-	316-36	CDC
"L.nautarum"	-	1477	CAMR
L.oakridgensis	-	OR-10	CDC
L.parisiensis	-	PF-209C-C2	CDC
L.pneumophila	1	(Camperdown)	NCTC 12098
L.pneumophila	1	(Knoxville-1)	CDC
L.pneumophila	1	(Olda 1a) 2129	CAMR
L.pneumophila	1	(Olda 1c) 2143	CAMR
L.pneumophila	1	(Philadelphia-1)	CDC
L.pneumophila	1	(Pontiac-1)	NCTC 11191
L.pneumophila	1	(Bellingham-type, Hastings strain)	Nott. PHL
L.pneumophila	1	(Corby)	RH
L.pneumophila	2	Togus-1	CDC
L.pneumophila	3	Bloomington-2	CDC
L.pneumophila	4	Los-Angeles-1	CDC
L.pneumophila	4	Leiden-3	CDC
L.pneumophila	5	Cambridge-2	Camb.PHL
L.pneumophila	5	Dallas-1E	CDC
L.pneumophila	5	MICU-B	Columbia
L.pneumophila	5	U7W	Columbia
L.pneumophila	5	U8W	Columbia
L.pneumophila	6	Oxford-1	CDC
L.pneumophila	7	Chicago-8	CAMR

Table 1 cont.

SPECIES	SEROGROUP	STRAIN	SOURCE
L.pneumophila	8	Concord-3	CDC
L.pneumophila	9	IN-23-G1-C2	CDC
L.pneumophila	10	Leiden-1	CDC
L.pneumophila	11	797-PA-H	CDC
L.pneumophila	12	570-CO-H	CDC
L.pneumophila	13	Seattle-1	CDC
L.pneumophila	14	1169-MN-H	CDC
L.pneumophila	-	Lansing 3	CDC
"L.quateriensis"		1335	CAMR
L.quinlivanii		1442-AUS-E	CDC
L.quinlivanii		1448-AUS-E	CDC
L.quinlivanii		1449-AUS-E	CDC
L.quinlivanii		1450-AUS-E	CDC
L.quinlivanii		1451-AUS-E	CDC
L.quinlivanii		1452-AUS-E	CDC
L.quinlivanii		2359-AUS-E	CDC
L.rubrilucens		1267	CAMR
L.sainthelensi		Mt.St.Helens-4	CDC
L.santicrucis		SC-63-C7	CDC
L.spiritensis		Mt.St.Helens-9	CDC
L.steigerwaltii		SC-18-CD	CDC
L.tucsonensis		1087-AZ-H	CDC
L.wadsworthii		81-716A	CAMR
"L.worsleiensis"		1347	CAMR
A 36*		-	IMVS
B 86*		-	IMVS
C 449*		-	IMVS
D 500*		-	IMVS
D 532*		-	IMVS
E 594*		-	IMVS

KEY

"..."	: Proposed new species.
*	: Possible new species as described in Wilkinson et al., (1990)
IMVS	: Institute of Medical and Veterinary Science, Adelaide, South Australia.
CDC	: Centers for Disease Control, Atlanta, Georgia.
CAMR	: Centre for Applied Microbiological Research, Porton Down, Salisbury.
RH	: Dept. of Laboratory Medicine, Ruchill Hospital.
Camb.PHL	: Cambridge Public Health Laboratory.
Nott. PHL	: Nottingham Public Health Laboratory.
Columbia	: Wm. Jennings Bryan Dorn Veteran's Hospital, Columbia, USA.

2.2 Culture media

BCYE

All except three *Legionella* strains (*L.geestiae*, *L.adelaidensis* and 86/35784) were grown on a solid charcoal yeast extract medium (Feeley *et al.*,1978) modified by the incorporation of N-2 acetamido-2-amino-ethanosulfonic acid (ACES) buffer. To 10 g of ACES buffer (Sigma), 900 ml of distilled water at 50°C was added. Further additions were; 2.8 g of KOH pellets (BDH), 1.5 g of Norit A activated charcoal (Serva), 10 g of yeast extract (Difco) and 17 g of agar (Bacto). Distilled water was added to make up the volume to 1 l and the mixture stirred and boiled to dissolve the soluble components and the pH was then adjusted to 6.85-6.95 with KOH pellets if required. The medium was autoclaved at 121°C for 15 min and allowed to cool to 56°C before the additions of filter-sterilised (0.20 um) alpha-ketoglutarate (10 ml of 10% w/v), L-cysteine HCl as a source of sulphur (10 ml of 4% w/v), and ferric pyrophosphate as a source of soluble Fe++ ions (10 ml of 2.5% w/v) per litre and the medium poured.

"Oxoid" BCYE

One environmental strain No. 86/35784 failed to grow on in-house BCYE but a commercial BCYE (Oxoid) did support its growth. Also after passaging, the growth of *L.geestiae* and *L.adelaidensis* was not supported by in-house BCYE and were subsequently grown on Oxoid BCYE.

Briefly, 2.5 g of Legionella CYE Agar Base was suspended in 90 ml of distilled water and gently boiled to dissolve completely. Sterilisation was by autoclaving at 121°C for 15 min. After cooling to 50°C, one vial of Legionella BCYE Supplement Code SR110 reconstituted with 10 ml of sterile distilled water, was added aseptically, and after gentle mixing, plates were poured.

Columbia blood agar

Columbia Agar Base (39g, Oxoid) was suspended in 1 l of distilled water containing 8% (w/v) NaCl and boiled until the contents dissolved. After sterilisation by autoclaving at 121°C for 15 min., the medium was cooled to 50°C, sterile 5% (v/v) defibrinated horse blood was added and after gentle mixing, plates were poured.

2.3 Growth conditions

All Legionella strains were incubated at 37°C +/- 1°C in CO_2 jars on solid media in an atmosphere of air (unless stated) for 48-72 h except where whole-cell fatty acid analyses were carried out when the incubation time was standardised to 96 h.

2.4 Maintenance of cultures

Stock cultures were held as frozen bacterial suspensions at -70°C in peptone water (see Appendix IV). However for day to day use all Legionella strains were inoculated onto BCYE slopes incubated for 24-48 h at 37°C and then stored at 4°C until required. These BCYE slope cultures were passaged every 4-6 months to ensure continued

viability, inoculated onto a blood agar plate to ensure purity and examined by Gram-stain to show the presence of weakly staining Gram-negative rod-shaped bacteria. All plates were also examined under a plate microscope for a characteristic "cut-glass" appearance (Dournon, 1988) which all Legionellae with the exception of L.moravica exhibit.

2.5 Production of rabbit antisera for the detection of urinary antigen

2.5.1 Method of McKinney et al., (1979)

Legionella pneumophila SG 1 (Knoxville-1) were grown on BCYE medium, harvested in 0.85% NaCl (w/v) containing 0.5% (v/v) formalin and fixed overnight at 37°C. The cell suspension was then centrifuged at 1350 g and resuspended in 0.1% (v/v) formol saline to 40 International Units of turbidity or approximately 4 x 10^9 bacterial cells/ml. Two strains of rabbit were inoculated, young adult New Zealand Whites and Half Lops reared from crossing a New Zealand White male and a French Lop-Eared female in our own animal house. Following a pre-immunisation bleed of 10 ml the first injection consisted of 2 ml equal volumes of cell suspension and Freund complete adjuvant (Difco Bacto Adjuvant, Difco Labs. Detroit, Mich.) administered intracutaneously into the sub-scapular region (i.e. 1 ml under each scapula). After 21 days, injection of a mixture of 2 ml of equal volumes of cell suspension and Freund's incomplete adjuvant (Difco) was given intramuscularly into the hind quarters. Seven days

later, approximately 50 ml of blood was taken from the marginal ear vein and 2 ml of cell suspension was injected intravenously. This procedure was repeated after 7 days. The rabbits were exsanguinated under anaesthesia 7 days later.

2.5.2 Method of Kohler et al., (1981)

The Knoxville-1 strain of L.pneumophila SG 1 was inoculated onto BCYE and incubated at 37°C in a 3% CO_2 atmosphere in sealed CO_2 jars. After 7 days incubation the organisms were scraped from the agar, resuspended in phosphate-buffered saline pH 7.2 and autoclaved at 101°C for 1 h. The cells were removed by centrifugation and resuspended in 10 ml of phosphate-buffered saline per 0.5 ml of packed cells, repacked by centrifugation at 1350 g for 10 min and left at 4°C for 14 days. For immunisation the packed organisms were resuspended and 1 ml mixed with an equal volume of Freund's complete adjuvant (Difco). After taking a pre-immunisation blood sample the immunogen was injected subscapularly and intramuscularly (0.5ml per site) into the hindquarters of the rabbit. Subsequent injections of the same inoculum and volume but mixed with incomplete Freund adjuvant (Difco) were given twice weekly for 2 weeks then once weekly for 2 weeks. Antiserum (10-20ml) was taken 14 and 21 days after the sixth injection and tested by IFA and ELISA.

2.5.3 Method of Tang and Toma (1986)

The RH strain (Bellingham-type) of L.pneumophila SG 1

was grown on BCYE at 37°C for 48 h in a 3% CO_2 atmosphere in sealed containers and harvested in 1% (v/v) formalin in PBS pH 7.4. After 18 h at 22°C, the cells were washed by centrifugation three times in PBS, and the packed cells adjusted to a density of 40 IU in 0.1% (v/v) formalin in PBS. A sample of pre-immune blood (15 ml) was taken before the immunisation of a New Zealand White rabbit with intradermal injections of 1 ml of antigen into 12 dorsal sites. After 30 days, 1 ml of antigen in was injected intramuscularly at one site. Then, monthly intravenous injections of 1 ml were begun 7 days later and continued for 8 months. Blood samples (10ml) were taken at monthly intervals 14 days after each monthly intravenous injection and tested by IFA and ELISA.

2.5.4 Method of Conlan and Ashworth (1986)

Antisera to serogroup antigen of L.pneumophila SG 1 was obtained by immunising New Zealand White rabbits with purified saline extracts (section 2.14.). After a pre-immunisation blood sample had been taken, each rabbit was inoculated with 1 ml of antigen (estimated at 16 ug of carbohydrate, unless otherwise stated) in incomplete Freund's adjuvant (Difco) distributed between two intramuscular and two dorsal subcutaneous sites. A boost was given with the same dose 2 weeks later. Starting one week after that, blood samples (10ml) were taken at weekly intervals, assessed for antibody by IFA and purified for IgG

Flowchart of immunisation schedules for raising immunodiagnostic antisera

	Method of McKinney	Method of Kohler	Method of Tang and Toma	Method of Conlan and Ashwortl
Inoculum	Formolised whole cells (40 I.U.)	Boiled whole cells (40 I.U.)	Formolised whole cells (40 I.U.)	Purified serogroup antigen 16/32 ug carbohydrate
1st immunisation	2ml of Ag in CFA	1ml of cells in CFA	1ml (no adjuvant) cells	16/32 ug of Ag in IFA
2nd immunisation	21 days/ 2ml Ag in IFA	3 days/ 1ml cells in IFA	30 days/ 1ml cells	14 days/ Ag in IFA
3rd immunisation	28 days/ 2ml cells	7 days/ 1ml cells in IFA	37 days/ 1ml cells	84 days/ Ag in IFA
4th immunisation	35 days/ 2ml cells	10 days/1ml cells in IFA	(8 monthly	-
5th immunisation	-	14 days/1ml cells in IFA	immunisations each of	-
6th immunisation	-	21 days/1ml cells in IFA	1ml of cells)	-
7th immunisation	-	28 days/1ml cells in IFA	-	-
1st test bleed	Day 28	Day 42	Day 51	Day 21
2nd test bleed	Day 35	Day 49 (Exsanguinated)	Day 81	Day 28
3rd test bleed	Day 42 (Exsanguinated)	-	(then monthly	Day 35
4th test bleed	-	-	for 6 months)	Day 42
5th test bleed	-	-	-	Day 49
6th test bleed	-	-	-	Day 56
7th test bleed	-	-	-	Day 63
8th test bleed	-	-	-	Day 70
9th test bleed	-	-	-	Day 77
10th test bleed	-	-	-	Day 98 (Exsanguinated)
11th test bleed	-	-	-	

Figures given are post 1st immunisation.

KEY

CFA : Complete Freund adjuvant

IFA : Incomplete Freund adjuvant

Ag : antigen

to test its suitability as a capture antibody.

After the 12th week from the start of the protocol a further intravenous boost of antigen (1ml) was given, followed two weeks later by exsanguination under anaesthesia.

2.6 IgG preparation

2.6.1 Ammonium sulphate precipitation of immunoglobulins

This procedure was carried out for all test bleeds taken up to number 148, after which Protein-A Fast Flow (Pharmacia LKB Ltd.) was employed for IgG purification directly from whole sera. Test bleeds were placed immediately in a 37°C hot room for 30 min to allow clotting to take place and then left overnight at 4°C. After centrifugation at 3500 rpm for 15 min (1350g), the serum was carefully removed and 1ml of this used to determine the antibody titre by indirect immunofluorescence (IFA). The remainder was transferred to visking tubing (24/32 Scientific Instrument Centre Ltd.) and dialysed overnight, with stirring against 50% saturated ammonium sulphate (291 g per litre of distilled water at 0°C, BDH Ltd.) at 4°C. The contents of the sac was then centrifuged at 3500 rpm for 15 min on an MSE centaur 2 (1350g) and the supernate discarded. The precipitate was washed with 50% saturated ammonium sulphate to remove any lysed red blood cell products and centrifuged as before. The pellet was recovered and dissolved in 2 ml of 0.01M phosphate buffer (pH 7.5) and

dialysed with stirring for 24h or for 8h with several changes of buffer at 4°C. A sample from the sac contents was assayed for protein by the Biuret method and the volume noted to ensure the total protein concentration did not exceed the capacity of the DEAE 52 column. The sample was concentrated by transferring it to a length of visking tubing and placing it on a bed of polyethylene glycol 4000 (BDH Ltd.) at 4°C until the volume was less than 1 ml.

2.6.2 Purification of serum IgG on DEAE 52

A column (6 x 2cm) containing 8 g (wet weight) of DEAE 52 (Whatman Chemical Separation Ltd.) was equilibrated with 5 column volumes of 0.01 M phosphate buffer (pH 7.5) and the level of buffer allowed to drain to the top of the bed before application of the serum sample to the column. IgG eluted in the void volume with 0.01 M phosphate buffer (pH 7.5) as a single peak. Other serum proteins were eluted with 0.1 M and 0.3 M phosphate buffers (pH 7.5).

2.6.3 Purification of serum IgG on Protein-A Sepharose Fast Flow

The method of Miller and Stone (1978) was employed to isolate IgG rapidly from whole serum using insolubilised protein A.

A column of Protein-A Sepharose Fast Flow (7.9 x 0.9cm, Pharmacia LKB Biotech.) was equilibrated at 4°C with buffer A (See Appendix I) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF, BSA,

Sigma) and 0.02 % (w/v) sodium azide, (pH 8.0). Rabbit immune serum (2ml) prepared as previously described, was mixed with an equal volume of buffer A and applied directly to the column. The column was then washed with 5-10 volumes of buffer A (flow rate of 40 ml/ h) and bound IgG was eluted from the column using buffer B (See Appendix I) containing 0.1 mM PMSF and 0.02 % (w/v) sodium azide (flow rate of 40 ml/h). An elution profile of IgG was obtained by measuring A_{280} of each fraction. Proteins eluted with buffer B were then dialysed with stirring at 4°C against three changes (each of 1L) of buffer A without PMSF and sodium azide.

PMSF is required in buffers to inhibit proteolytic activity found in whole serum so that column deterioration is prevented.

2.6.4 Coupling polyclonal rabbit antibodies to CNBr-activated-Sepharose 4B

The method described by Pharmacia (1988) was followed. Briefly, 3g of freeze-dried cyanogen bromide-activated-Sepharose 4B, (Pharmacia Ltd., Separation Division) was swollen for 15 min in 1mM HCl and washed on a sintered glass filter (G3) with the same solution. Each gram of freeze-dried powder gives a gel volume of approximately 3.5ml. A total of 200ml per gram dry gel was added in a number of aliquots, the supernatant being sucked off between successive additions. The use of HCl preserves

the activity of the reactive groups which hydrolyze at high pH. The gel was then washed with 0.1 M $NaHCO_3$ coupling buffer (pH8.3), containing 0.5 M NaCl (5ml per gram dry gel) and immediately transferred to a solution of the rabbit antibody at a concentration of 5-10mg protein (antibody) per ml of gel. Four antibody solutions were used where IgG's were Protein-A purified. These were Nos. 43, 221, 228, and a control gifted by Fehrenbach (raised by the Kohler method of antibody production and used at 3mg/ml concentration). These solutions were prepared by dialysis against the coupling buffer with 3 changes of buffer over a period of 24 h at 4°C with stirring to dialyse out the Tris buffer and their protein concentrations determined by the method of Biuret. The CNBr-activated Sepharose-4B and antibody solution in coupling buffer was gently mixed for 2 h at room temperature (at a gel: buffer ratio of 1:2). After this time, any remaining active sites were blocked by transferring each gel/antibody mixture to a solution of 0.2 M glycine (pH 8.0) for 2 h at room temperature. Excess uncoupled protein was washed away on a sintered glass filter (G3) with coupling buffer followed by acetate buffer (0.1 M, pH 4.0) containing 0.5 M NaCl followed by coupling buffer. The antibody/Sepharose reagents were stored at 4°C until use.

2.7 Chemical analyses

2.7.1 Protein determination

Method of Biuret (peptide bond estimation in serum proteins).

This test was employed to estimate the amount of serum protein prepared by ammonium sulphate precipitation in rabbit blood samples to prevent overloading either DEAE 52 and Protein-A columns and to estimate the quantity of pure IgG for ELISA testing.

Either serum protein or IgG (0.1 ml) was mixed with 5ml of Biuret reagent (BDH Ltd.) and left for 30 min for the colour to develop. This was read at A_{540} on a Corning Colorimeter 253. BSA standards ranged from 0-100 mg/ml.

2.7.2 Method of Lowry

To 0.5 ml of sample, 0.5 ml of 1N NaOH was added and the mixture placed in a water bath at 100°C for 5 minutes. After the sample had cooled, 2.5 ml of reagent A (2 ml of 0.5% (w/v) $CuSO_4.5H_2O$ in 1% (w/v) sodium potassium tartarate added to 50 ml of 5% (w/v) Na_2CO_3) was added, the sample vortex mixed and allowed to stand for 10 min. Folin and Ciocalteu's phenol reagent (0.5ml) previously diluted 1:1 in distilled H_2O was added carefully and the contents vortex mixed. The colour was allowed to develop for 30 min and the A_{750} was measured on a Pye-Unicam PU-8620 spectrophotometer. Reagent blanks consisted of 0.5 ml distilled water substituted for the protein sample and a

standard curve was obtained with dilutions of bovine serum albumin (BSA, Sigma) containing 500, 400, 300, 200, 100 and 50 ug/ml. The protein concentration in the standard was assessed by measuring the A_{280} (1 mg/ml = 0.660).

2.7.3 Method of Bradford

To a 100 mm by 15 mm test tube , 0.1 ml of standard or sample was added. Coomassie Blue reagent (5ml, Bio-rad) made up to the manufacturers' instructions was added to each tube and the mixture of reagent and sample vortex mixed and left at room temperature for 10 min. The colour development was determined at A_{595} on a Cecil CE292 spectrophotometer and the total protein estimated from standards of 20, 15, 10 and 5 mg/ml of BSA (Sigma).

2.8 Carbohydrate determination

The carbohydrate content in a sample of purified LPS from L.pneumophila SG 1 (Knoxville-1) was determined by the method of (Dubois et al. 1956)

Briefly, 2 ml samples of sugar-containing solution were transferred to test tubes (14 x 150 mm) and 0.05 ml of 80% (w/w) phenol added followed by the rapid addition of 5 ml of concentrated sulphuric acid (sp.gr 1.84) to each tube. The tubes were allowed to stand for 10 min, vortex mixed, then placed for 20 min in a water bath at 30°C. The A_{480} was measured in a Cecil Instruments CE 292 UV spectrophotometer.

Blanks were prepared by substituting distilled water for the sugar solution and a standard curve was prepared

from dilutions of glucose (Mannheim Boehringer 1 mg/ml) at 25, 50, 75 and 100 ug\ml final concentration. All solutions were prepared in triplicate to minimise errors resulting from contamination with cellulose fibres.

2.9 Detection and semi-quantitation of endotoxin

The Limulus amoebocyte lysate test (LAL) for endotoxins, developed by Bang and Levin (1968), was utilised to determine the presence of lipopolysaccharide in CIE precipitates and as an aid in assessing the sensitivity of the ELISA by providing a quantitative value for the amount of LAL-reactive material in ELISA-positive urine.

The E-Toxate kit (Sigma) was employed and all glassware was treated with 1% E-Toxa-Clean (Sigma), rinsed with tap water, distilled water and lastly with pyrogen-free water (Baxter Healthcare Ltd.), autoclaved for 1 h and heated at 180°C for a further 3 h before use. The test was carried out according to Sigma Technical Bulletin No. 210 (4-86), using a positive control of lipopolysaccharide from E.coli 055 : B5 standardised against USP Reference Standard Endotoxin (RSE). Dilutions of this standard, ranging from 0.08 ng/ml to 0.005 ng/ml were used to estimate the sensitivity of the Limulus amoebocyte extract and results expressed as Endotoxic units/ml (EU).

2.10 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Gels were poured as 16 x 20 x 0.1 cm vertical slabs and were run using the discontinuous buffer system of

Laemmli (1970). The separating gels contained 10% (w/v) acrylamide, 0.3% (w/v) NN'-methylenebisacrylamide (Electran grade, BDH Ltd.), 0.375 M Tris-HCl, pH 8.3, 0.1% (w/v) SDS, 0.125% (v/v) TEMED, and 0.02% (w/v) ammonium persulphate. The stacking gels contained 4.5% (w/v) acrylamide, 0.125% (w/v) bisacrylamide, 0.125 M Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.15% (w/v) TEMED, and 0.04% (w/v) ammonium persulphate.

The running buffer consisted of 186 mM glycine, 25 mM Tris-HCl, pH 8.9, and 0.1% (w/v) SDS. Gels were run at 20-25 mA until the bromophenol blue tracking dye reached 1 cm from the bottom of the gel.

Molecular weights of unknown proteins were determined by construction of a standard curve plotting the mobility of protein standards (No. SDS-6H, Sigma) against the $\log_{10}$ of their molecular weights. These were :

Proteins	Mol. Wt.
Myosin	205,000
Beta-galactosidase	116,000
Phosphorylase B	97,400
Albumin (Bovine Plasma)	65,000
Albumin (Ovalbumin)	45,000
Carbonic anhydrase	29,000

2.10.1 Silver staining for proteins

All SDS-PAGE gels were silver stained either by

the method of Hitchcock and Brown (1983) or, for LPS, by the method of Tsai and Frasch (1982).

Before silver staining, the SDS-PAGE gel was fixed in a solution containing 10% (v/v) acetic acid and 30% (v/v) methanol with gentle agitation for 30 min. After rinsing in distilled water the gel was transferred to 100 ml of 10% (v/v) glutaraldehyde for 30 min and rinsed several times with distilled water. The gel was then agitated gently in a large volume of distilled water overnight. After removal of the glutaraldehyde by the rinsing procedure, the gel was then shaken in a solution of 5 ug/ml dithioerythritol (Cleland's reagent, Sigma) for 30 min. Without rinsing, the gel was transferred to 100 ml of 0.1% (w/v) silver nitrate in H_2O and gently agitated for 30 min before the silver nitrate solution was poured off and the gel rinsed first with H_2O, then with 100 ml 3% (w/v) Na_2CO_3 containing 50 ul formaldehyde while being gently agitated by hand. The gel is rinsed again in 3% Na_2CO_3 with formaldehyde (50 ul/100 ml) and then allowed to develop in the same solution until the bands appeared. The reaction was stopped by adding 5 ml of 2.3 M citric acid and left for at least 10 min to prevent over development before being photographed.

2.10.2 Silver staining for LPS

The following procedure described by Tsai and Frasch (1983) was used for specific LPS staining of SDS-PAGE gels. Overnight fixation in 200 ml of 25 % (v/v) isopropanol

in 7% (v/v) acetic acid was followed by 5 min oxidation in 150 ml distilled water with 1.05 g of periodic acid (GPR from BDH Ltd.) and 4 ml of 25 % (v/v) isopropanol in 7 % (v/v) acetic acid (made up just before use). Eight washes, each time in 200 ml distilled water for 30 min, was followed by 10 min silver staining in a solution consisting of 0.1 N NaOH (28 ml), concentrated (29.4%) ammonium hydroxide (1 ml), 20% (w/v) silver nitrate (5 ml) and distilled water (115 ml) (made up just before use and stirred constantly on a magnetic stirrer). After staining, gels were given four 10-min washes, each in 200 ml distilled water, was followed by a 10-20 min period of development at 25°C in 250 ml of developing solution (citric acid (12.5 mg), 37% formaldehyde (0.125 ml) and distilled water to 250 ml. This was made up just before use and gently heated to the optimal temperature of 25^0C before addition to the gel (proteins are also stained at lower temperatures). One hour in a stop bath (200 ml of distilled water and 10 ml of 7% (v/v) acetic acid) was followed by a final wash in 200 ml of distilled water before the gel was photographed.

Use of ammonium hydroxide is essential for the preferential staining of LPS and to prevent the oxidation of the ammonium hydroxide reagent it was transferred into small bottles thus excluding air until use.

2.11 Serological assays

2.11.1 Determination of antibody titre in rabbit antisera

The level of antibody was determined by the indirect immunofluorescent antibody test (IFAT) modified from Wilkinson et al., 1979. This test is based on the immunospecific reaction of rabbit antiserum with a heat-killed suspension of legionellae, in which the coating of organisms by the rabbit's antibody is revealed by staining with fluorescin-conjugated anti-rabbit globulin. Multispot PTFE-coated microscope slides were used (Hendley-Essex). A drop of heated legionellae organisms (5ul), sufficiently diluted to show discrete bacteria under a x40 objective lens and x6 eyepiece lens combination (Fallon and Abraham, 1982) was added to each well.

The slides were dried under tungsten lighting and fixed in acetone for 10 min at room temperature. Doubling dilutions of test antisera were made in PBS starting at 1/16 to 1/32000 and 10 ul of each dilution was spotted onto an individual well on the slide which was then incubated at 37°C in a humid chamber for 30 min. The slides were then washed in PBS for 10 min and dried by blotting between sheets of Whatman No. 1 filter paper.

The anti-rabbit IgM\IgG FITC conjugate (Nordic Immunology, Norway) at a working dilution of 1/40 was then spotted onto the slides, 5 ul to each well and incubated at 37°C in a humid chamber for 30 min and washed as before.

After drying the preparations were mounted in polyvinyl alcohol mounting medium (Serva), and covered with a glass cover slip.

Fluorescent microscopical examination was carried out using a Leitz Dialux EB fluorescence microscope fitted with an ultra-high pressure mercury lamp and an H2 filter block, consisting of a BP390-490 exciting filter, RKP-510 dichroic mirror and LP 515 suppression filter. The result was scored according to the level of fluorescence, the end point (titre) being the reciprocal of the dilution of the antiserum giving a level of 1+, where the degrees of fluorescence are +++, ++, +, +/-, and -.

2.11.2 Slide agglutination test

The method of Thacker et al (1985) was followed to test rapidly the ability of antibody in test bleeds to react with whole cells of L.pneumophila SG 1 (Knoxville-1). Briefly ten fold dilutions up to 1/1000 were made from the antisera of interest and these spotted onto a clean glass microscope slide (75 x 26 mm). A drop (25ul) of bacterial suspension (made up in 10 % (v/v) formol saline and incubated at 37°C overnight) was spotted alongside each antiserum dilution. The adjacent drops were each mixed with a clean glass rod and the glass slide gently rocked and any agglutination within 30 s was noted.

2.11.3 Ouchterlony double diffusion test

Gel diffusion medium was made by adding 15g Ionagar

No.2 (Oxoid), 16 g NaCl to 1l of distilled water and placed in a steamer for 45 min. Molten gel (12ml) diffusion medium was carefully pipetted onto a level glass plate (8 x 8cm) until it was evenly coated and then allowed to set. After refrigeration for 1 h, wells (4mm in diameter) were cut in the agar with a cork borer (No.1) to the desired pattern. The wells positioned 10 mm apart were each loaded with neat antisera (0.02ml) and run against sonicated bacteria or concentrated urine (0.02ml) prepared as in section 2.13.1 except that these were not proteinase-K treated. These were prepared by scraping one BCYE plate of lawn culture of Legionella into 0.85% (w/v) saline and sonicating at full power on a Soniprep 150 MSE at 8-10 microns for 5x 1 min bursts, with cooling for 30 s between each sonication. After loading, the plates were incubated in a moist chamber at 37°C for 24 h before results were noted.

2.11.4 Two-dimensional (crossed) immunoelectrophoresis (CIE)

Electrophoresis was performed in 1% (w/v) agarose (Type A-Sigma, with a setting temperature of about 35°C) 1.5 mm deep, on glass plates (5 x 5 cm). Barbital buffer (Appendix V), pH 8.6, ionic strength 0.1, specific conductivity = 7 mS (23°C) was used for agarose preparation and with 1% (v/v) Triton X-100 (Sigma) added when used as a running buffer during electrophoresis.

First dimension electrophoresis was performed in electrophoresis tanks (V. Holm-Nielson Aps, Copenhagen),

using 10 ul of Tris-saline EDTA (T.S.E.) extract, sonicated whole cell preparations (Collins et al., 1983) of L.pneumophila SG 1 (Knoxville-1) or concentrated positive (by ELISA) antigen-containing urine. A current of 40 mA (80 V) was applied for 45 min or until ovalbumin (100 ug/ul) labelled with bromophenol blue migrated a distance of 30 mm in the first dimension. Temperature was maintained at 10-12°C.

Second-dimension electrophoresis was performed at 12°C, applying a current of 30 mA for 20-24 h. The antisera used were a) dialysed against 50 % saturated ammonium sulphate to salt out immunoglobulins b) salted out as in a) followed by DEAE 52 chromatography to isolate IgG or c) added directly to Protein-A Fast Flow (Pharmacia LKB Ltd) to isolate IgG. Antigens were reacted with each serum at 0.1, 0.2 and 0.4 ml diluted in 3 ml of 1 % (w/v) agarose.

The agarose gels were removed and washed for 24 h in saline and then transferred to distilled water for further washing with several changes over 24 h. Where staining was required, the washed agarose gels were pressed with care to prevent air bubbles by four alternate wet and dry sheets of chromatography paper (Whatman), layers of tissue and a 2 kg weight for 15 min. The layers of wet tissue and chromatography paper were removed carefully and the film of agarose gel adhering to the glass plate stained in 0.1% (w/v) amido black in methanol: acetic acid : dH_2O

(3:1:6) for 1 min. After removal, the agarose film was destained for 48 h in 5 % (v/v) acetic acid with several changes and pressed again if necessary. Final drying of the agarose film was at 37°C overnight.

Where antigen-antibody precipitates were assayed for lipopolysaccharide, the sonicate was prepared from L.pneumophila SG 1 (Knoxville-1) grown on medium made with pyrogen-free water (Baxter Healthcare Ltd.). All glassware and electrophoresis tanks were pre-treated with 1 % E-Toxa-Clean (Sigma) and rinsed with pyrogen-free water. Also, barbital buffer (Appendix V) was prepared with pyrogen-free water and the wicks and glassware were treated as described in section 2.9.

2.12 Enzyme-linked immunosorbent assay (ELISA)

The original method (personal communication, McKinney, R.) was followed. To each well of a 96 well microtitre tray (Immulon III / I from Dynatech microelisa systems.) 0.1 ml of capture antibody (20 ug/ml) was added at either 1/50 or 1/100 dilutions in 0.01 M phosphate buffered saline with 0.01% (w/v) merthiolate (pH 7.4) and incubated in a 37°C water bath for 2 h. BSA (0.2 ml of 2% (w/v)) in 0.01 M PBS was then added and the plates incubated again at 37°C in a water bath for 2 h. The plates were then washed twice with 0.01 M PBS (pH 7.4) and 0.1 ml of neat urine added.

Two wells of control antigen, known positive and negative urines against capture antibody (McKinney) at a

dilution of 50 were included as controls for all test plates set up to examine either purified IgG from rabbit test bleeds or urinary or purified antigen.

The plates were washed with 0.01 M PBS (PH 7.4) before 0.1 ml of conjugate (consisting of capture antibody conjugated to horse radish peroxidase) at a working dilution of 1/800 in 0.01 M PBS containing 2% (w/v) BSA was added to each well and incubated for 1h at 37°C. The plates were then washed with 0.01 M PBS (pH 7.4) and 0.1 ml of substrate added to each test well. The substrate was made up fresh from 8.5 ml of distilled water, 0.5 ml of 4% (w/v) orthophenylenediamine (prepared in advance and stored in 0.5 ml amounts at -20°C), 1 ml of substrate buffer (containing 170 mM citric acid, 650 mM Na_2HPO_4, and 0.1% (w/v) merthiolate), and 50 ul of 3% (v/v) H_2O_2. Following addition, plates were incubated at 37°C in a water bath for 30 min before 4.5 M H_2SO_4 (0.05 ml per well) was added to stop the reaction.

Each plate was read in a Titertek Multiscan Plus ELISA plate reader with a 492 nm filter and Vers. 1.4 software installed. Blank values were recorded for wells in column A where pre-immune sera or 0.1ml of 2% (w/v) BSA in 0.01 M PBS (pH 7.4) were substituted for capture antibody.

Positive control antigen was extracted from L.pneumophila SG 1 (Knoxville-1) by the method described later in section 2.16.3.

Peroxidase was shown to be a good choice of enzyme for conjugation by Nakane and Pierce (1966) and Nakane and Kawaoi (1974). It has high activity, is cheaper than alkaline phosphatase, and yields a visible (brown) reaction product. Conjugation of high titre antisera, determined by IFA, to horseradish peroxidase was achieved by the one step glutaraldehyde method described by Voller et al., (1976). IgG raised against L.pneumophila SG 1 (2mg in 1ml of PBS pH 7.2) was added to 5 mg of horse radish peroxidase (HRP-4, Bioenzyme Labs. Ltd.) and mixed at room temperature. The mixture was then dialysed against PBS for 18 h at 4°C with three changes of buffer. Glutaraldehyde (25% v/v) was added to give a final concentration of 0.2% (v/v) and the mixture was incubated at room temperature for 2-3 h before dialysis against PBS overnight at 4°C with several changes of buffer. The conjugate-containing dialysis sac was transferred to 0.05 M Tris-HCl, pH 8.0 and dialysed overnight with several changes of buffer. The sac contents were then diluted to 4ml with 0.05 M Tris-HCl, pH 8.0 buffer containing 1% (w/v) BSA and 0.02% (w/v) NaN_3 to give the stock conjugate which was stored at 4°C in the dark until use. The working strength of the conjugate was determined by chess-board titration in the ELISA system using working capture antibody and both control antigen and antigen-containing urine. Usually, the conjugates had a working dilution of > or = 500.

2.13 Immunoblotting

Electrophoretic transfer of LPS from polyacrylamide gels to Hybond-C nitrocellulose (Amersham International plc.) or to Immobilon-P polyvinylidene difluoride (Millipore), both of pore size 0.45um, was accomplished in a Bio-Rad transfer tank (transfer buffer made up of 7.5 g Tris-HCl, 36.0 g glycine, 500 ml of methanol made up to 2.5 l with distilled water). Nitrocellulose paper of the same size as the gel was presoaked in transfer buffer, positioned next to the gel and sandwiched between 3 MM chromatography paper (Whatman) and Scotchbrite pads and retained in a plastic holder. The polyvinylidene difluoride membrane was treated similarly except that it was presoaked in methanol for 1-3 s, immersed in distilled water for 2 min and finally equilibrated in transfer buffer for 2 min before use. A current of 0.02 A was applied overnight across the buffer in the transfer tank, which was water-cooled. After transfer of the components from the gel, the membranes were immersed in TTS (20 mM Tris-HCl with 1% (v/v) Tween 20 (Sigma) in 0.85% (v/v) saline, pH 7.2/5% (w/v) BSA (Sigma)) and gently shaken at room temperature for 2-3 h. The membranes were washed in TTS/ 0.1% (w/v) BSA three times with shaking for 15 min for each wash and then incubated with antiserum diluted to 100 or 200 in TTS/ 1% (w/v) BSA for 2-3 h at room temperature. The membranes were washed three times in TTS/ 0.1% (w/v) BSA as before and incubated with donkey anti-rabbit IgG

conjugated to horse radish peroxidase (SAPU) diluted to 100 or as directed in TTS/ 1% (w/v) BSA for 2 h at room temperature on a shaker. The blots were then rinsed in TBS (20 mM Tris-HCl in 0.85% (w/v) saline pH 7.2) and the bands developed in 8 mM 4-chloro-1-napthol in 0.02% H_2O_2 (v/v) with shaking for 30 min at room temperature. The reaction was stopped when the blots were washed in distilled water.

2.14 Concentration of antigen-containing human urine from patients with a L.pneumophila SG 1 infection

Human urine to be analysed for the presence of LPS from Legionella pneumophila SG 1 by SDS-PAGE was concentrated using a Minicon macrosolute concentrator type B15 (Amicon). Briefly, for each human urine, 20 ml was divided between 4 chambers (5 ml to each chamber) and the unit placed at 4°C until the volume of urine was reduced to 200 ul (50 ul for each chamber). Each concentrated urine sample (50ul) was mixed with 100 ul of lysing buffer and incubated with 10 ul of Proteinase-K (2.5 mg/ml) at 60°C for 1 h. Then each urine (10ul) sample was loaded into a lane of an SDS-PAGE gel.

2.14.2 A modification of procedure (i) was adopted to concentrate human urine to detect the presence of any protein moiety and LPS in ELISA-positive human urines.
As before, for each urine, 20 ml in total was added to each chamber (5 ml at a time) and the accumulated concentrated volume of 100 ul was removed to a sterile Eppendorf tube.

The chamber of the macrosolute concentrator was then washed with 1 ml of 3 % (w/v) SDS to dissolve any bound LPS and protein.

The combined washings and concentrate for each sample were freeze-dried overnight at -70°C and transferred to storage at -20°C. Each sample of urine was dissolved in 100 ul of distilled water and 25 ul of each urine was mixed with an equal volume of double strength solubilising buffer before loading onto a gel (10 ul/track).

Samples of urines which were not concentrated before analysis were proteinase-K digested treated in the following way. Urine (25ul) were mixed with 25 ul of solubilising buffer and 10 ul of Proteinase-K in lysing buffer (2.5 mg/ml) and incubated at 60°C for 1 h. Digestion was terminated by placing the sample in a boiling water bath for 5 min.

2.14.3 Concentration by polyvinylalcohol

50 ml of each urine for study was also concentrated in dialysis tubing against polyvinyl alcohol (M.W. 125,000) at 4°C until the volume was < or = to 1 ml (24-36 h).

2.14.4 Affinity chromatography

Columns (7 x 55mm) of antibodies raised to L.pneumophila SG 1 coupled to CNBr-activated-Sepharose 4B as described in section 2.6.4 were poured. These were exposed to urine, either antigen-containing (G.L.) or non-antigen-containing urine from a healthy human (R.F.)

at flow rates of 16 ml/h. After exposure to these human urine specimens, 100ul gel volume was taken from each column, mixed with 30ul of 2x solubilising buffer, vortex-mixed, centrifuged to deposit the gel in the buffer and placed in a water bath heated to 100°C for 5 min. 30ul samples were then loaded on to 10% SDS-PAGE gels at a current of 30mA for 3-4h before immunoblotting against IgG (antiserum Nos. 43, 59 and control McKinney each at dilutions of 50, 100 and 200 as described in section 2.13 except that Immobilon-P (Millipore) was used instead of Hybond-C (Amersham) as the transfer membrane.

2.15 Preparation of Cellular Antigens

2.15.1 L.pneumophila - Lipopolysaccharide

It was noted by Conlan and Ashworth (1986) that according to the method of Westphal and Jann (1965) extraction of L.pneumophila SG 1 yielded LPS contaminated with cellular protein in the phenol phase. Therefore Conlan and Ashworth (1986) devised an alternative extraction method which was adopted in this study.

Thirty BCYE plates inoculated with L.pneumophila SG 1 (Knoxville-1) were scraped into 0.9 % (w/v) saline and the suspension was adjusted so that 1 ml of saline contained 100 mg wet weight of cells. This was stirred constantly on a heated magnetic stirrer in a water bath at 100°C for 1 hour. The cell suspension was centrifuged at 17,000 g (W50-1 Rotor in a Beckman Model L5-50 ultracentrifuge) at 4°C and the

supernate dialysed against PBS (see Appendix III). Extracts were treated sequentially, each for 2 h at 37°C, with ribonuclease-1, then deoxyribonuclease-1 was added (in the presence of 0.1 M $MgCl_2$) and finally pronase (enzymes from BDH Ltd.) at final concentrations of 0.01 mg/ml for each enzyme. At the end of this treatment (6 h in total) residual enzyme activity (except ribonuclease-1) was destroyed by heating to 100°C for 5 min, before centrifugation at 1,250 g for 2 min to remove insoluble denatured proteins. Extracts were then dialysed for 24h at 4_oC against 0.01 M Tris-HCl, pH 8.2 and the enzyme-treated saline extracts further purified by gel filtration using Sepharose 4B medium (Pharmacia).

The volume of the saline extract was reduced by rotary evaporation at 70°C on a Buchi Rotavapor-R until a volume of 5 ml was attained. This was then applied to a column (90 cm x 2.6 cm internal diameter) of Sepharose 4B (Pharmacia LKB), equilibrated with 0.01 M Tris-HCl (pH 8.2) at a flow rate of 10 ml/hour. Eluted proteins were detected at A_{280} (LKB Bromma 2158 Uvicord). The fractions collected (LKB Ultrorac or Redifrac) for each peak were pooled and analysed for LPS by ELISA (McKinney antisera as capture antibody), for protein by the method of Lowry (1965) and also examined by SDS-PAGE.

2.15.2 L.pneumophila, Proteinase-K preparations

L.pneumophila SG 1 LPS was prepared by

Proteinase-K digestion of whole-cell lysates by the method of Hitchcock and Brown (1983). Bacteria were grown on BCYE agar, harvested after 48 to 72 h of incubation at 37°C, and suspended in distilled water to an A_{660} = 1. A 1.5 ml volume of this suspension was centrifuged at 14,000 rpm for 2 min in an eppendorf microfuge. The supernate was decanted, and the pellets were solubilised in 50 ul of lysing buffer (2% SDS (w/v), 4% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 1 M Tris (pH6.8), and 0.05% (w/v) bromophenol blue by incubation in a water bath at 100°C for 10 min. A fresh solution of Proteinase K (Sigma) was prepared for each experiment at a concentration of 2.5 mg/ml in lysing buffer. A sample (10 ul) of this was added to each boiled lysate and the lysates were then incubated at 60°C for 1h. The proteinase-K digested lysates were centrifuged for 3 min to remove any remaining cellular debris, and the supernates retained for analysis by SDS-PAGE.

2.15.3 Tris saline EDTA (T.S.E.) outer membrane extraction

The growth of L.pneumophila SG 1 (Knoxville-1) from 4 BCYE plates after 96 h incubation was harvested in PBS and bacteria were sedimented by centrifugation at 1250g for 20 min. The supernate was decanted and 2 ml of TSE buffer, pH 7.4 (0.01 M Tris-HCl, 0.15 M NaCl, 0.002 M EDTA) added to the cell pellet and the cells gently resuspended. Glass beads (1ml, Sigma No. G3753, Type 1, 75-150 um.) were then added and the mixture allowed to stand on ice for 45 min

before being vortexed for 2 min. The cells were sedimented by centrifugation as before and the supernate removed, filter-sterilised (0.22 um), aliquoted into 0.5 ml volumes and stored at - 20°C.

2.15.4 Lp1-Ag preparation

LP1-Ag for L.pneumophila SG 1 was prepared by ultrasonication following the method of Collins et al. (1983).

L.pneumophila SG 1 (Knoxville-1) cells were harvested from BCYE after 96 h growth and suspended in a minimal amount of sterile distilled water and transferred to an eppendorf tube. After centrifugation at 10,000 g for 10 min at 4°C, the supernate was removed and the cell pellet was weighed. A portion of the supernatant was then added back to the cell pellet at a ratio of 2 ml/g wet weight of cells and the cells resuspended.

Bacterial disintegration was accomplished by sonication with three periods of 45 s each at 20,000 Khz/s using an MSE Soniprep 150 with a stainless steel probe (3mm tip). The suspension was cooled with ice during sonication and for 1 min periods between each sonic treatment. Cell fragments were removed from the sonicate by centrifugation at 48,200 g for 1 h at 4°C using an SS-34 rotor in a Sorvall refrigerated Superspeed centrifuge. The supernate was filter-sterilised (0.2 um, Millipore) and stored in 100 ul aliquots at - 20°C.

2.16 Commercial kit evaluation

Two *in-vitro* diagnostic test kits for qualitatively detecting the presence of a L.pneumophila SG 1-related antigen in human urine have been manufactured and marketed within the last two years. The "Dupont" kit is only licensed for sale in the U.S.A. and was a generous gift for evaluation purposes only.

2.16.1 "Merigen" latex agglutination test

This kit, a latex agglutination assay, consists of a suspension of latex particles sensitised with monoclonal antibody which is claimed to react specifically with "S-1 antigen" (Merigen terminology in accompanying literature for L.pneumophila SG 1 antigen) when present in urine. Thus, the presence of this antigen is signified by no or a weak agglutination (i.e. a "positive" result).

The materials provided in this kit for the testing of specimens include 1) latex detection reagent which is an anti-L.pneumophila S-1 monoclonal antibody coated onto latex particles in a buffer containing sodium azide as a preservative 2) a positive antigen control reagent which is a non-viable suspension of S-1 antigen in a buffer with sodium azide 3) a negative antigen control reagent which is a pooled human negative urine with sodium azide 4) plastic pipette stirrers and 5) a test slide.

All specimens were allowed to reach room temperature before testing and urine samples were centrifuged at 1000 g

for 10 min. One free-falling drop of latex suspension, after gentle inversion to ensure even mixing, was put onto each marked oval on the test slide. Next to each drop of latex reagent 50 ul of either the specimen to be tested using the pipette-stir tubes or a free-falling drop of the positive or negative controls was added to the oval next to but not touching the latex reagent. Each specimen or control was mixed with the paddle end of its "pipette-stir" and the slide was rotated at 100 +/- 20 rpm in a moist chamber for 15 min. After this time the slide was rocked manually two or three times to resuspend the latex particles in each of the ovals and the results were immediately recorded. A positive test was recorded if either a weak agglutination (1+) or no agglutination was observed. A negative result was recorded if agglutination was observed greater than or equal to 2+ (1+ and 2+ agglutinations were defined photographically in the literature accompanying the Merigen kit). Results were only valid if the positive and negative controls gave no agglutination and agglutination respectively. This test was evaluated purely for its efficacy in distinguishing between known positive antigen-containing and negative urines previously evaluated by the ELISA in this laboratory. Also some of the urines used in this kit evaluation have been assessed by other workers in addition to our own testing with the common results agreed between laboratories.

2.16.2 "Dupont" Legionella urinary antigen [^{125}I] RIA kit

This kit is a radioimmunoassay based system intended for *in-vitro* diagnostic use. It consists of 1) 50 reaction tubes coated with polyclonal rabbit anti-L.pneumophila SG1 IgG ready for use 2) concentrated (x25) saline rinse with 0.8% (w/v) sodium azide as a preservative, 3) positive control human urine with 0.8% (w/v) boric acid as a preservative, 4) negative control human urine with 0.8% (w/v) boric acid and 5) [^{125}I] labelled antibody in 0.1M Tris (pH 8.0), 0.9% (w/v) NaCl and 5% (w/v) BSA as a detector antibody. The IgG-[^{125}I] had an activity of less than 370 KBq (10 uCi) on the calibration date. All the reagents were stored with the appropriate precautions at 2-8°C until used before the expiry date. All specimens and controls were allocated four reaction tubes precoated with polyclonal IgG (normally two reaction tubes would be allocated to each specimen and four to each of the two controls [tested in duplicate] but a quality control note with the kit indicated a 1.4% incidence of non-coated reaction tubes) and these labelled accordingly. Positive and negative control urines supplied with the kit were added (100ul) to their respectively labelled reaction tubes and the same volume of the test specimens to their reaction tubes without leaving droplets down the wall of the polystyrene tubes. Taking care not to disturb the tubes they were incubated at 37°C +/- 1°C for 60 min in a water bath. After this time the

concentrated saline rinse, diluted 25 fold with fresh distilled water, was used to wash out the urine samples from each tube by aspiration. Each tube was washed three times with approximately 1 ml of saline rinse. One-hundred ul of the [^{125}I] detector antibody was pipetted directly to the bottom of each tube. Tubes were incubated as before. After incubation the unbound radioactive tracer was aspirated into a separate vacuum flask for safe disposal. Then the outside wall of each test tube was dried of any extraneous rinse whilst maintaining the tubes in an upright position. Disintegrations per min (dpm) over a 60 s period were measured on a United Technologies Packard crystal multi detector gamma system scintillation counter whose counting efficiency of ^{125}I was > or = 75%. Duplicate background counts were also made over the same time period with every 10 tubes measured for radioactivity. According to the manufacturers of the kit, the net (i.e. corrected for background) negative control urine mean dpm should be less than 250 dpm and the positive control urine net dpm should be greater than three times that of the negative control. In addition all four tubes must score as either positive or negative for the test result to be valid and if not, the test for that specimen must be repeated.

2.17 SPECIES IDENTIFICATION

2.17.1 Direct extraction of cellular ubiquinones for analysis by RPTLC

Direct extraction of ubiquinones (isoprenoid quinones) by a modified procedure of Karr et al. (1982) was employed for the extraction of ubiquinones from fresh whole cells.

Each species and strain to be studied was inoculated onto two BCYE agar plates and incubated at 37°C for 48-72 h. Growth was then scraped off with a bent Pasteur pipette (0.4 g wet weight) into 3 ml of methanol-hexane (3:2) in a glass universal container and the mixture vortex mixed for 3 min. To this suspension, 8 ml of n-Hexane was added, the mixture was vortex mixed vigorously for 3 min and then centrifuged at 120 g for 10 min on an MSE GF-8 centrifuge.

The hexane layer was removed, and the aqueous phase was re-extracted with 8 ml of n-Hexane. The hexane extracts were pooled, evaporated to dryness *in-vacuo* before the residue was redissolved in 0.1 ml ethyl acetate and analysed by RPTLC.

2.17.2 Extraction of ubiquinones by saponification

A modified procedure of Abe et al. (1978) was used. Fresh whole cells (0.4 g wet weight) were scraped from two BCYE agar plates and suspended in 3 ml of 1% (wt/v) pyrogallol in methanol in a 10 ml Quickfit flask. To this, 0.2 ml of 50% aqueous KOH was added and the mixture refluxed

for 10 min at 100°C followed by immediate cooling under running tap water. Distilled water (1ml) and n-Hexane (5ml) were added and the mixture shaken vigorously for 5 min and then centrifuged at 120g for 10 min before the hexane layer was removed and the aqueous phase re-extracted with an additional 5 ml of hexane. The pooled n-hexane extracts were evaporated to dryness *in-vacuo* and the resulting residue redissolved in ethyl acetate before separation by RPTLC.

2.17.3 Preparative TLC purification of cellular ubiquinones for mass spectrometry

Ubiquinones were prepared as before except that the growth from eight plates of BCYE was required to provide a sufficient amount of crude extract for ultimate analysis by mass spectrometry. Each hexane extract was applied as a band 1 cm from the bottom of an aluminium-backed TLC plate (10 x10 cm) coated with silica gel 60 $WF_{254}s$ (Merck from BDH Ltd.) along with a Q-10 standard (Sigma). Plates were eluted in equilibrated tanks with n-hexane : diethyl ether (85:15) as solvent for about 15 min or until the solvent front had advanced 3/4 the height of the TLC plate. The plate was examined under ultra violet light (wavelength 254 nm) and the position of the ubiquinone band marked with pencil. This area of silica gel was scraped off into a sintered glass funnel connected to a side-arm vacuum flask and the ubiquinones eluted with 20 ml of chloroform (AnalaR, BDH Ltd.) under vacuum and transferred to a conical-bottomed

glass container.

The chloroform was evaporated *in-vacuo* and the dry sample flushed with 90% N_2/10% C_2O, covered with aluminium foil and stored at -20°C until analysed by mass spectrometry.

2.17.4 Reversed-phase thin layer chromatography (RPTLC)

Each sample (20-50ul) was spotted onto a reversed-phase thin layer chromatography plate (Whatman octadecylsilane bonded reversed-phase $KC_{18}F$ plates, 20 x20cm, 200um layer) together with a control extract from L.pneumophila SG 1 (Knoxville-1) and two ubiquinone standards, Q-6 and Q-10 (Sigma). The plate was developed in a mobile phase of acetone: water (19:1) in a Whatman TLC tank lined with Whatman No. 1 chromatography paper. After elution, the plates were sprayed from a distance of 20-25 cm with 10% (v/v) phosphomolybdic acid in ethanol (Sigma) and placed in a hot air oven at 160°C for 30 to 50 s until the separated components could be seen after which it was removed, allowed to cool and photographed. A tentative identification of ubiquinones was made by comparing Rf values with those of commercial standards and of ubiquinones extracted from L.pneumophila SG 1. Confirmation of the presence of ubiquinones and their identity in these extracts was by fast atom bombardment mass spectrometry.

2.17.5 Preparation of whole-cell fatty acid methyl esters (FAME) by acid methanalysis

Four plates of BCYE were inoculated with the Legionella species or strain of interest and incubated at 37°C for 96 h in CO_2 jars after which the growth was carefully recovered using a bent Pasteur pipette and transferred to a test tube (14 x 150 mm) containing 8 ml methanol. To this, 8 ml of toluene was added followed by 0.4 ml of concentrated sulphuric acid. The test tube was sealed with a teflon cap, vortex mixed for 1 min and heated overnight in a water bath at 54°C. After cooling, the contents were transferred to a glass universal container and 8 ml of n-hexane added before vortex mixing and centrifugation (120 g for 10 min) to achieve phase separation. The top hydrophobic immiscible layer was removed and transferred to a clean glass universal before washing in excess 0.3 M NaOH. Again the top layer containing the fatty acid methyl esters was removed to a clean universal and the FAME's were concentrated *in-vacuo* to about 2 ml before transfer to 4ml amber-coloured sample vials (Phase Separations Ltd.) and evaporated to dryness. The dry sample was flushed with 90% N_2/10% CO_2 and sealed with an inert teflon-lined screw cap (Phase Separations Ltd.) before storage at -20°C.

2.17.6 Whole-cell FAME analysis of the **Legionellaceae** by gas-liquid chromatography (GLC)

FAME extracts were solubilised in 0.04 ml toluene

("Special for Chromatography" BDH Ltd.) and 0.1-0.5 ul of each sample was injected into the GLC (Chrompack Packard Ltd. Model No. 437A) for analysis. The chromatographic conditions were as follows :

Injector temperature : 270°C

Oven temperature programme : 150°C/ 5 min, oven rise of 4°C/ min to 250°C, 250°C/ 5 min.

Detector temperature : 300°C

Injector : Splitter, ratio 60-100:1 (dependent on sample concentration)

Split flow : 47.91 ml/min (at 60 : 1 split ratio)

Carrier gas : Helium Grade A(BOC), flow rate = 0.79 ml/min

Make-up gas : 20 ml/ min (He)

Column : 50 M x 0.25 mm CP Sil 5 CB (Chrompack U.K. Ltd.)

Liquid phase : 100% dimethyl polysiloxane (non-polar)

Detector : Flame ionisation detector

Attenuation : 2^{10}

Range : 0 (Auto select)

On-line gas filters were used as follows :

Helium : Oxygen and moisture removing filters

Air : Charcoal filter

H_2 : Charcoal filter (All from Chrompack U.K. Ltd.)

A commercially available standard bacterial fatty acid mix containing 26 fatty acid methyl esters found in some members of the family Legionellaceae (Wait, R. 1988) was obtained (Supelco Inc.). Tentative identification of unknown

FAME's in samples prepared from whole cell extracts were made by comparison of retention times of unknown components with the Supelco standards. When possible this was cross-referenced with fatty acid methyl esters obtained from Sigma. Ultimate identification was by GLC-Mass spectrometry.

2.18 Mass spectrometry

2.18.1 Confirmation of the identity of whole-cell fatty acids of the Legionellaceae by gas chromatography-mass spectrometry (GC-MS)

GLC-Mass spectrometry enabled the identity of each of the extracted bacterial whole-cell fatty acids to be confirmed by the analysis of their fragmentation patterns and molecular ions produced after bombardment with high speed electrons.

Each sample was dissolved in 0.1 ml of dichloromethane and injected using the same technique as in previous GLC analyses and analysed on a HRGC\MS MFC500 Carlo Erba gas chromatograph interfaced with a Kratus MS80 RFA mass spectrometer.

The parameters used for gas-liquid chromatography were identical with those previously defined in section 2.16.6 except that a 25 M x 0.25 mm BP 1 capillary column (Supelco Inc.) whose chemical phase is compatible with CPSil 5CB was employed.

Other conditions were as follows :

Ionisation mode : Electron impact

Ionisation energy : 70 Ev

Scan rate : 0.3 s/ decade mass

Start mass : 600

Accelerating voltage : 4 kV

Temperature of the source : 230°C

2.18.2 Confirmation of the identity of ubiquinone species by negative-ion Fast Atom Bombardment (FAB) mass spectrometry

Identity of ubiquinones in cell extracts from four species of Legionella was provided by mass spectrometry. In addition, the mass spectra of two standards Q-6 and Q-10 (Sigma) were determined since this technique has not been applied previously to ubiquinones yet provides valuable data with good sensitivity (Wait and Hudson, manuscript in preparation).

The samples for analyses were dissolved in triethanolamine (Sigma), a liquid of low vapour pressure and bombarded with a beam of fast xenon atoms, causing ionisation and spattering directly into the gaseous phase where the ions are mass-analysed. A Kratos MS-80 RFA mass spectrometer, fitted with an Ion-Tech BNF11 fast atom gun with a Kratos FAB source was employed for all ubiquinone analyses. Conditions for analysis were: an accelerating voltage of -4KeV, starting mass of 1600, resolution of 1000 and a scan rate of 30 s/ decade of mass, with the raw data analysed by the DS 90 software package (supplied by Kratos).

RESULTS

3.1 Production of immunodiagnostic antisera

Sera produced by different immunisation schedules from twenty-six rabbits by following different immunisation schedules were compared to investigate the ability of rabbit anti-*Legionella* IgG from each schedule to act as avid capture antibody (Cab) for detection of urinary antigen in the ELISA assay. Conjugate for all ELISA testing was supplied by McKinney and for reasons of consistency used throughout this research. No loss of activity of this reagent was observed over a 36 month period of storage at 4°C.

3.1.1 Evaluation of rabbit IgG as capture antibody for ELISA

After only a small number of rabbits had been used to raise antibody for the ELISA test, evaluation of sera showed that only one test bleed from one rabbit produced avid IgG specific only for *L.pneumophila* SG 1 urinary antigen. This showed that production of such antibody appeared to be unpredictable as noted previously (Kohler *et al.*, 1981, Fehrenbach *et al.*, 1986, Samuel *et al.*, 1990).

In all these evaluatory tests, the accepted minimal differential A_{492}, determined spectrophotometrically, used to distinguish antigen-containing "positive" urine from negative urine controls in the ELISA and which defined a capture antibody as acceptable was that the former should exceed the latter A_{492} result by at least a factor of 2. In addition, the negative urine controls (from healthy donors

with no known history of Legionella infection) should not exceed an A_{492} value > 0.2 (normal values observed are >0.02 and <0.08). The A_{492} value with the T.S.E. extract from L.pneumophila SG 1 (positive control antigen) should exceed 1.751.

Rabbit-2/88, inoculated according to the McKinney method described in section 2.5.1 (see p45 for flowchart) produced effective IgG at the time of its second test bleed (day 35 from the first inoculation). No effective capture antibody was demonstrable on exsanguination one week later (table 2) so that a quicker method for the purification of IgG by NH_4SO_4 precipitation followed by fractionation on a DEAE 52 column to avoid the 5 day or more delay in serum evaluation was needed. If the evaluation of working IgG could be established immediately or soon after blood was taken, the rabbit could then be exsanguinated to obtain all the circulating avid capture antibody produced. A number of tests were appraised for their ability to distinguish between avid (obtained infrequently) and non-avid IgG. Some of these tests were carried out in retrospect following early success in producing avid IgG. This provided the required effective capture antibody for the ELISA available as a control.

3.1.2 IFA testing

All pre-immune and post-immunised test bleeds taken

Table 2 IFA titres and ELISA results of test bleeds from R2/88 immunised according to the method of McKinney.

Rabbit No.	Immunogen	* Day	IFA titre (K)	Bleed No.	Effective as Cab/ELISA
2/88 (NZW)	(Knoxville-1) whole cells in 0.1% formol saline. (40 I.U.)	28	32	41	No
		35	32	43	Yes
		42	32	51	No

Key

Cab - capture antibody

* - figures given are post 1st inoculation

from the 26 rabbits were titred by the IFAT. Low IFAT titre antisera (< 4000 or 4K) did not react with positive control T.S.E.-extracted antigen and positive control urines to differentiate them from negative control urines in the ELISA. Only antisera of higher IFA titres (> 8-128K) which reacted with the T.S.E.-extract were of particular interest as they were reactive in the ELISA. Antiserum which produced low IFA titres (< 4K) only produced a positive slide agglutination at low dilution (undiluted or 1/10, table 3).

Slide agglutination

This is a very simple test to carry out, but agglutination gave no indication of the effectiveness of the IgG as capture antibody in the ELISA system. e.g. a serum of IFA titre of 16K or more could be diluted 100 fold and would still agglutinate formalised whole cells of L.pneumophila SG 1 whereas a serum of lower IFA titre (<4K) would only agglutinate organisms at a 10 fold dilution or less (table 3). All high IFA titre antisera, including those not effective as capture antibody, agglutinated whole cells so that this was an unsuitable test for the determination of the capture antibody status of isolated IgG.

3.1.4 Purification of IgG by Protein-A

This method was employed in parallel with and then chosen in preference to, NH_4SO_4 precipitation and fractionation on a DEAE 52 column. It reduced considerably the time taken to purify IgG for ELISA testing from > 5 days

Table 3 IFAT titre and slide agglutination of formolised whole cells of L.pneumophila SG 1.

Antiserum Number and immunisation details [Effective as capture antibody in ELISA]	Titre by IFAT (K)	Agglutination of formolised whole cells in a slide test Dilution Factor		
		Neat	1/10	1/100
No. 21 (Kohler) R 1/86 [No]	32	Yes	Yes	Yes
No. 27 (Kohler) R 1/87 [No]	4	Yes	Yes	No
No. 30 (Tang and Toma) R 7/87 [No]	1	Yes(weak)	No	No
No. 40 (McKinney) R 1/88 [No]	32	Yes	Yes	Yes
No. 43 (McKinney) R 2/88 [Yes]	32	Yes	Yes	Yes
No. 59 (McKinney) R 6/88 [Yes])	32	Yes	Yes	Yes
No. 139 (Conlan & Ash.) R 6/89 [No]	32	Yes	Yes	Yes
No. 141 (Conlan & Ash.) R 6/89 [Yes]	8	Yes	Yes	No

to < 3 days as well as being more simple to perform because whole serum could be applied to the column. The time advantage was gained by the omission of the many dialysis steps associated with NH_4SO_4 precipitation.

It was observed (table 5) that when Protein-A and NH_4SO_4/DEAE 52 methods for the isolation of IgG were run in parallel, different ELISA results were observed. This stimulated the screening of all antisera with IFAT titres >8K which had previously been purified by the NH_4SO_4/ DEAE 52 method for IgG isolation. The results (table 4) showed that IgG from some test bleeds were effective in the role of specific capture antibody when purified by Protein-A. This was not the case when the same antiserum (except No.43, used as a control) was purified by the NH_4SO_4/ DEAE 52 technique.

3.1.5 ELISA testing

As one of the main aims of this research was to produce a sensitive and specific ELISA diagnostic system, all test bleeds taken from rabbits were evaluated by this method. Control positive and negative human urines were used so that different IgG preparations could be designated as effective or non-effective in differentiating between antigen-containing and non-antigen-containing control urines.

Initially, the IgG fraction from immune serum was tested in the ELISA until the first effective test bleed was identified. The time between blood sampling and testing by

Table 4 Immune sera fractionated by Protein-A for IgG and tested by ELISA.

Test bleeds purified by protein-A affinity chromatography for IgG with results of the IgG used as capture antibody at neat and at 10 or 25 fold dilutions (in PBS) in ELISA testing against positive control antigen (C/Ag T.S.E. extract, two wells), five positive "urines" from patients with L.D. (Mill., Mun., G.L., J.M. and Co.), two urines from patients with a non-*Legionella* related pneumonia (pn) and two specimens of urine from patients with a urinary tract infection (UTI).

Test Bleed No. (IFA)	C/Ag	C/Ag	Mill.	Mun.	G.L.	J.M.	Co.	pn	pn	UTI	UTI
43. 32K (control) (1/25 dil.)	++	++	+	+	+	+	+	-	-	-	-
25.nt.32K	++	++	-	-	-	+	-	-	-	-	-
1/10 "	++	++	-	-	-	+	-	-	-	-	-
28.nt.16K	++	++	-	-	-	+	-	-	-	-	-
1/10 "	++	++	-	-	-	+	-	-	-	-	-
35.nt.16K	++	++	-	-	-	++	-	-	-	-	-
1/10 "	++	++	-	-	-	+	-	-	-	-	-
40.nt.32K	++	++	-	-	-	+/-	-	-	-	-	-
1/10 "	++	++	-	-	-	+/-	-	-	-	-	-
41.nt.32K	++	++	-	-	-	+/-	-	-	-	-	-
1/10 "	++	++	-	-	-	+/-	-	-	-	-	-
42.nt.32K	++	++	-	-	-	+/-	-	-	-	-	-
1/10 "	++	++	-	-	-	+/-	-	-	-	-	-
43.nt.32K	++	++	+	+	+	++	+	-	-	-	-
1/10 "	++	++	+	+	+	++	+	-	-	-	-
51.nt.32K	++	++	+	+	+	++	+	-	-	-	-
1/25 "	++	++	/	/	/	+	+	-	-	-	-

Table 4 cont.

57.nt.32K	++	++	+/-	+/-	-	+	-	-	-	-	-	
1/25 "	++	++	+/-	+/-	-	+	+/-	-	-	-	-	
59.nt.32K	++	++	/	/	/	++	+	-	-	+/-	-	
1/50 "	++	++	/	/	/	++	+	-	-	-	+/-	
60.nt.64K	++	++	++	++	++	+	++	++	++	++	++	
1/25 "	++	++	+/-	+/-	+/-	+/-	+/-	+/-	-	-	+/-	
62.nt.32K	++	++	+	+	++	++	+	+	+	+	+	
1/25 "	++	++	+	-	+	+/-	+	-	+/-	-	+/-	
63.nt.64K	++	++	+/-	+/-	+	+	+	+	+	+/-	+	
1/25 "	++	++	-	+/-	+/-	+	+/-	+/-	-	-	+	
64.nt.32K	++	++	-	+/-	-	+/-	+/-	+/-	-	-	+	
1/25 "	++	++	+/-	+/-	+	+	+	+	+/-	-	+	
65.nt.28K	++	++	+	+	+	++	+	+	+/-	-	+	
1/25 "	++	++	+/-	+/-	+	++	+/-	+/-	-	-	+	
66.nt.64K	++	++	+	+	+	+	+	+	+	+	+	
1/25 "	++	++	+	+/-	+	+	+	+/-	+	-	+	
67.nt.32K	++	++	+	+	+	++	+	-	-	-	-	
1/10 "	++	++	++	++	++	++	+	+/-	+/-	+/-	+/-	
68.nt.32K	++	++	++	++	++	++	++	++	+	++	++	
1/25 "	++	++	++	++	++	++	++	+	+	+	+	
70.nt.64K	++	++	+	+	+	+	+	+	+	+	+	
1/25 "	++	++	+	+/-	+	+/-	+	+/-	+/-	+/-	+/-	
81.nt.32K	++	++	+	+	+	+	+	+	+	+/-	+	
1/25 "	++	++	+	+/-	+	+	+/-	+/-	+/-	+/-	+/-	

Table 4 cont.

139.nt.32K	++	++	++	++	++	++	++	-	-	-	-
1/25 "	++	++	+	+	+	+	+	-	-	+/-	-
141.1/25.8K	++	++	+	+/-	+	+	/	-	-	-	-
147.nt.32K	++	++	++	++	++	+	++	-	-	-	-
1/25 "	++	++	++	++	++	++	++	-	-	-	-
148.nt.32K	++	++	++	++	++	++	++	+/-	+/-	+/-	+/-
1/25 "	++	++	+	+	+	+	+	-	-	-	-

KEY

nt. - tested at a neat concentration

1/10- tested at a 1/10 dilution

1/25- tested at a 1/25 dilution

/ - not tested

++ - O.D.'s > 0.601

+ - O.D.'s 0.201- 0.600

+/- - O.D.'s 0.051-0.200

- - O.D.'s < 0.050

ELISA was important because antibody titres and capture efficacy were not always stable. For this reason, whole antisera from test bleed Nos.43 and 59 (from R2/88 and R6/88 respectively) were compared with their fractionated IgG counterparts. Results showed (table 5) that whole antiserum and the derived IgG fractions differed in that only IgG fractions were able to differentiate between positive and negative urines. It was concluded, therefore, that it was necessary to isolate IgG from test bleeds in order to determine effectiveness as capture antibody.

A study of fractionated IgG from the first 59 test bleeds taken from rabbits; R1/86, R1/87, R1/88, R2/88, R3/88, R4/88, R5/88 and R6/88 (i.e. from 3 of the 4 protocols [excluding the Conlan and Ashworth (1986) protocol examined in this thesis) produced results which allowed division of IgG from individual test bleeds produced, into three categories A, B and C. These are defined as follows:

Category A was defined as those IgG's which, when used as capture antibody in the ELISA, produced an $A_{492} > 1.751$ when reacted with control antigen (T.S.E. extract of L.pneumophila SG 1) and A_{492} values which overlapped when tested against both known positive and negative urine controls (i.e. no differentiation was observed).

Category B was defined as in A except that an $A_{492} < 1.750$ was observed with control antigen in the ELISA.

Table 5 Comparison between whole antisera (Nos. 43 and 59) and their IgG fractions in ELISA.

	IgG obtained by NH_4SO_4/ DEAE 52 chromatography		Whole serum as CAb	
Antigen	ELISA $O.D._{492}$ values with IgG from test bleed No.43	No.59	No. 43	No. 59
T.S.E. (positive control antigen)	2.77	2.70	2.34	2.43
Mill. (positive urine)	0.44	0.44	0.09	0.07
Mun. (positive urine)	0.86	0.65	0.53	0.34
89/00382 (negative urine)	0.04	0.03	0.86	0.40
89/000390 (negative urine)	0.03	0.04	0.12	0.06
Negative (non-*Legionella* pneumonia)	0.02	0.02	0.00	0.03
Negative (non-*Legionella* pneumonia)	0.048	0.02	0.03	0.03
89/00379 (UTI-*Proteus*)	0.039	0.02	0.30	0.18
89/00633 (UTI-*E.coli*)	0.012	0.30	0.10	0.19
89/00338 (UTI-*Klebsiella*)	0.039	0.05	0.13	0.07

KEY

T.S.E.-positive control antigen

UTI - urinary tract infection

Category C was defined as in A except that the IgG tested differentiated known positive urine from negative urine controls.

The ELISA spectrophotometer was blanked against wells with either pre-immune serum substituted for capture antibody or where wells were blocked by 2% (w/v) BSA solution in PBS [see section 2.12.1]). Typical A_{492} values with positive urines found to be > 0.70 with < 0.08 observed for negative urines with this last value increasing to 0.20 with some capture antibodies of category C.

The following four subsections describe results obtained when each of the four protocols were assessed.

3.1.6 Method of McKinney et al., (1979)

This was one of the first protocols published for the production of antiserum in rabbits (see flowchart, p45, for immunisation summary) for use in an ELISA system to detect urinary antigen from patients with a L.pneumophila SG 1 infection, and was found in these studies to be successful in producing effective capture antibody in 6/14 (43%) rabbits. It was therefore studied in detail as one aim of this project was to produce avid antiserum with consistency so that a continuous diagnostic service of this type could be offered to the N.H.S. in Scotland, at least.

Of the 13 rabbits that were set up by this method, only one (R7/88, a 1/2 Lop) failed to produce high titre antiserum (16-64K by IFAT were usual) from the first test

bleed of its inoculation schedule (table 6). R7/88 produced titres consistently of 4K by IFAT and although this improved to 16K upon extended immunisation beyond the standard 6 week protocol, it later fell again to 4K. IgG purified from blood samples taken from R7/88 was of no diagnostic value when evaluated under conditions described in section 2.12.

IgG produced in 6 NZW rabbits (R2/88, R6/88, R8/89, R9/89, R3/90 and R4/90) were effective as capture antibody in the ELISA. Of these, four (R8/89, R9/89, R3/90 and R4/90) produced avid IgG from blood taken on the 35th day after the priming immunisation but none was detectable from blood taken at exsanguination 7 days later (table 7). The other two of the six NZW rabbits (R2/88 and R6/88) produced avid capture antibody on two occasions within their respective protocols. Blood samples taken from these rabbits by IFA testing gave titres > 16K and were therefore purified by Protein-A during re-screening. This produced interesting results in that R2/88 was found to be producing avid IgG which was detectable 7 days after day 35 and R6/88 was also producing avid IgG detected 7 days before day 35 post primary immunisation. High titre (> 8K by IFAT) samples of blood taken from the other rabbits did not produce avid IgG on re-screening with Protein-A to check the results produced by the NH_4SO_4/DEAE 52 method of IgG purification.

To investigate the importance of formolisation of the organisms two other rabbits (R8/90 and R9/90) were

Table 6 Titres obtained from test bleeds taken from a rabbit, R7/88, immunised by the method of McKinney upon extended immunisation.

Rabbit No.	Immunogen	* Day	IFA (K)	Bleed No.
7/88 (half lop)	(Knoxville-1)	21	4	71
		25	4	73
		30	4	75
		33	8	77
		43	16	80
		48	16	85
		54	16	87
		60	16	89
		67	4-8	92
		88	8	93
		109	4	97
		112	4	98

KEY

* - figures given are post 1st immunisation

Table 7 The transient response of avid IgG production when rabbits were immunised by the method of McKinney.

IgG from antiserum taken after the priming inoculation and tested by ELISA for avidity.

Rabbit No.	4th week	5th week	6th week
8/89	-(143)	+(147)	-(151)
9/89	-(144)	+(148)	-(152)
3/90	-(192)	+(197)	-(202)
4/90	-(193)	+(198)	-(203)

KEY

+: IgG was effective in differentiating positive and negative control urines according to the conditions described in section 2.12.

-: IgG not effective as above.

( ) - Test bleed no.

inoculated according to this protocol (except that R9/90 was inoculated with boiled organisms instead of formalised organisms of the same strain) and were exsanguinated on day 35. However, both rabbits produced IgG not effective as capture antibody in the ELISA. The importance of formolisation of the organisms in relation to the production of capture antibody could not therefore be substantiated. Titres produced from both of these rabbits were high (>16K) by IFAT.

3.1.7 Method of Kohler et al., (1981)

Two rabbits (R1/86 and R1/87) were inoculated with L.pneumophila SG 1 (R1/86 with the Knoxville-1 strain before this project was started and R1/87 with the RH (Bellingham type) strain, according to this schedule (section 2.5.2). From the test bleeds of R1/86, only one contained avid IgG. This sample was taken 19 months after the priming immunisation (many months beyond the stated end point of the protocol). Even though this rabbit was immunised and its antiserum tested for a further 14 months, no effective capture antibody for the ELISA system was produced. Blood samples taken and tested by IFA from both R1/86 and R1/87 showed erratic titres ranging from 1 to 64K rising and falling over a period of time (tables 8 and 9 respectively), and this feature persisted even when immunisation was extended following the same regime.

This protocol under its more limited evaluation

Table 8 IFAT titres of R 1/86 whose immunisation after a rest period was by that of Kohler.

Rabbit No.	Immunogen	* Day	IFA titre (K)	Bleed No.
1/86 (NZW)	Autoclaved (Knoxville-1) whole cells (40I.U.)	-	65	7
		150	32	9
		270	8	10
		420	8	19
		467	32	21
		499	32	23
		513	32	25
		520	16	28
		527	16	35

KEY

* - figures given are post 1st immunisation

Table 9 IFAT titres of antisera during the immunisation course of R 1/87 according to the method of Kohler.

Rabbit No.	Immunogen	* Day	IFA titre (K)	Bleed No.
1/87 (NZW)	Boiled (Knoxville-1) whole cells. (40 I.U.)	42	16	15
		63	32	16
		147	16	17
		165	2	20
		197	2	22
		204	4	24
		214	4	27
		244	4	29
		274	1	37
		304	4	52
		364	64	60
		394	64	63
		398	64	66

KEY

* - figures given are post 1st immunisation

compared with that of the McKinney (1979) and Conlan and Ashworth (1986) schedules yielded no other samples of blood containing IgG "effective" in the ELISA even when antiserum was screened using Protein-A to purify IgG.

3.1.8 Method of Tang and Toma (1986)

This protocol, with a duration of 13 months (section 2.5.3), used formalised organisms as in the McKinney schedule (1979) but utilised a different strain of L.pneumophila SG 1 and was reported to produce antiserum of broad specificity detecting urinary antigen excreted from patients infected with different serogroups of L.pneumophila as well as L.micdadei and L.longbeachae (Tang and Toma, 1986, and Tang et al. 1989). In this respect it differed from the other three protocols investigated and raised some interesting questions (see discussion).

One rabbit (R7/87) was immunised according to this protocol. IgG production monitored by IFA testing revealed very low titres of 0.128K initially, with this titre rising steadily to 1K and jumping to 32K five months after the protocol was started with this status continuing at this level before falling to 2K at the end of the protocol. All antisera tested by ELISA before the rise in titre to 32K could be described as category B antisera, as would be expected, but this changed to category A when all blood samples taken had IgG titres of 32K. No positive urine controls showed higher A_{492} values compared with those of

negative urine controls by ELISA.

3.1.9 Method of Conlan and Ashworth (1986)

This protocol (see flowchart, p45, for immunisation summary) was investigated for two reasons; the first was that whole organisms used as inocula in the early stages of the project in the other schedules did not consistently stimulate the production of IgG effective in the ELISA. The second was that some support existed for the idea of urinary antigen having characteristics like lipopolysaccharide (LPS). This concept was an interesting development as Harrison and Taylor (1982) claimed that antibodies to a major heat-stable, pronase-resistant antigen provided a means of serogrouping strains of L.pneumophila SG 1. The nature of this serogroup antigen was uncertain, although its chemical composition and endotoxicity together with its behaviour in two-dimensional electrophoresis and SDS-PAGE suggest it may be LPS. ELISA results (table 11) using avid IgG (from test bleed Nos. 43 and 59) detected urinary antigen in the same specimens whether these were boiled or unboiled.

This and other experiments described in this thesis lend further support to the concept of urinary antigen being related to or derived from LPS, and that it is most probably a component of the carbohydrate "O" side chain.

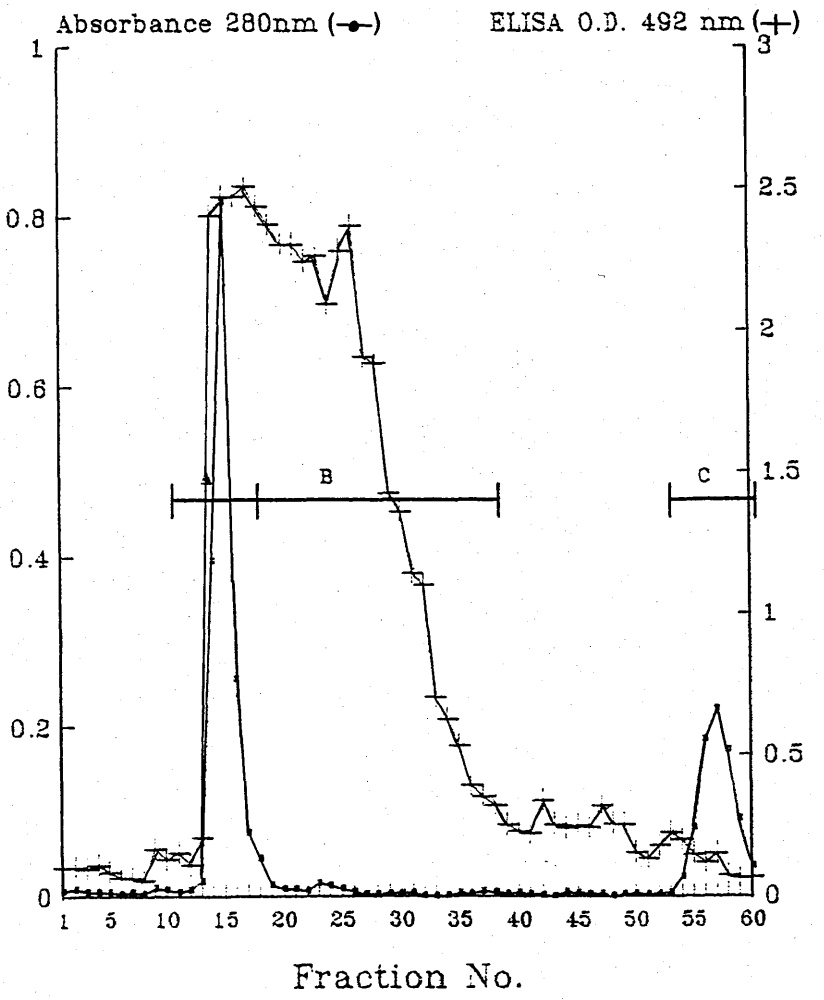
LPS has been extracted from L.pneumophila SG 1 cells by the more common methods for isolation of LPS. The

phenol/water extraction method (Westphal and Jann, 1965) yielded LPS in the phenol phase, the same phase into which protein partitions. The methods of Darvaeu and Hancock (1983) and Galanos et al. (1969) were used by (Sonesson et al. 1989) for further studies. These did not include antiserum production. Here, saline extracts produced by the method of Conlan and Ashworth (1986) were used to raise antisera for diagnostic purposes and further attempts were made to characterise the extracted material. Three preparations were produced from strain Knoxville-1 of L.pneumophila SG 1 (SE.I, SE.II and SE.III) because of earlier successes in raising avid capture antibody. SE.I was divided into three pools of antigen, A, B and C taken by pooling fractions A, B, and C (similar to figure 2) respectively after gel filtration. A and B were used for inoculating rabbits. Peak C was not investigated in this way.

NZW Rabbits (R2/89, R3/89 and R4/89) were inoculated with pooled antigen from peaks A and B according to the protocol described by Conlan and Ashworth (1986), but with R3/89 being given 32ug of carbohydrate, ($[CH_2O]n$) instead of 16ug at mid-protocol, as it was observed that R2/89 and R3/89 responded poorly to this antigen at 16ug concentration (IFAT titres ranged from only 256-2K during the course of the protocol). R3/89, after its dosage was raised to 32ug $[CH_2O]n$ produced titres up to 16K by IFAT. The schedule for R2/89 continued with 16ug $[CH_2O]n$

Figure 2 Gel filtration of crude saline extract (S.E. I) through a bed of Sepharose 4B.

The separation of serogroup antigen reactive in the ELISA, detected at A_{492} and protein detected at A_{280} are shown.



inoculations as a control and R4/89 was then started and inoculated with the same dose of antigen (16ug) as R2/89. Its IFA titre was 64-256 during the protocol. Another saline extract (SE.II) from the same strain of Legionella was prepared. This antigen was inoculated into three NZW rabbits (R5/89, R6/89 and R7/89). R5/89 was inoculated with 16ug $[CH_2O]n$ throughout its schedule and produced titres of only 512-1K as observed with SE.I at this concentration. Both R6/89 and R7/89 were inoculated with 32ug $[CH_2O]n$ and titres up to 16K were found on analysis of the test bleeds. However, only one test bleed (No.139) was specific and sensitive when purified IgG was used as capture antibody and tested by ELISA. The final saline extract (SE.III) was again separated into three pools (A, B and C as before) after gel filtration using Sepharose 4B (figure 2). Pool A and pool B were inoculated into different rabbits; R1/90 with pool A and R2/90 with pool B. Fraction nos.13-19 (Pool A, figure 2) were found to give the highest ELISA A_{492} values compared with those of other eluted peaks. Pool A also coincided with a peak of protein (determined spectrophotometrically at A_{280}) from the Sepharose 4B gel. Carbohydrate and protein estimates of pool A were 12ug/ml and 55ug/ml respectively.

Peak B (fraction nos. 20-35) also gave high A_{492} values but contained little or no detectable (A_{280}) protein. Chemically estimated values for carbohydrate and

protein for pooled fractions in peak B were 2ug/ml and 5ug/ml respectively. Pool A was inoculated into R1/90 where IFA titres of test bleeds up to 8K were noted at a $[CH_2O]n$ concentration of 16ug.Pool B was non-immunogenic in rabbit R2/90 where negative (< 16) titres by IFAT were found.

Another rabbit (R11/90) was inoculated with pooled antigen composed of fractions from peaks A and B where titres of 2K were produced with a $[CH_2O]$ dosage of 32 ug/ml per inoculum. This was, however, unsatisfactory for use in the ELISA.

Furthermore, one saline extract (a gift from Williams, CAMR, Porton Down, Salisbury), which was manufactured by Conlan and extracted from the Corby strain of L.pneumophila SG 1 (No.74181, originally isolated in this laboratory) was also inoculated into two rabbits (R5/90 and R10/90). Its composition estimated by Conlan was; protein 0.79 mg/ml and 0.69 mg/ml $[CH_2O]n$.

The extract from the Corby strain (No.74181) of L.pneumophila SG 1 inoculated into R5/90 and R10/90 produced in each rabbit high titre antisera determined by IFAT. Only the last two bleeds from R5/90, one taken before the final boost and blood taken at exsanguination in the 13th week of the protocol, produced effective capture antibody which distinguished between positive and negative control urines (A_{492} values from positive controls were > 2 times A_{492} obtained with negative urines).

Rabbit-10/90, produced IgG (purified in the same way as IgG from R5/90) that gave greater differential A_{492} values for positive and negative control urines making IgG from R10/90 more desirable for clinical *in-vitro* testing. These A_{492} differential values produced by IgG from both R5/90 and R10/90 fulfilled the criteria to be described as working antisera but the differential was observed to vary between 2 and 15 times (when A_{492} values for positive urines were 0.3 and negatives, 0.02).

In terms of the A_{492} differential described, other samples of blood (Nos. 43 and 59 in particular) which produced IgG effective as capture antibody and obtained via the McKinney (1979) schedule gave greater values with the same "positive" control urines and may therefore be more sensitive. The A_{492} values for positive urine controls usually exceeded a value of 1.0, a colour change easily observed by eye without the aid of an ELISA spectrophotometer.

3.2 Capture antibody evaluation and ELISA-related studies

Sera identified in the initial screening tests with known "positive" and "negative" urine samples in ELISA were investigated further. These sera were tested against a larger number (77) of clinical specimens. These were previously tested using gifted McKinney capture and detecting antibody so that their antigen status was known (table 10). These control urines consisted of 15 known

KEY

MSSU - midstream specimen of urine
CSU - catheter specimen of urine
S.galactiae - Lancefield group B heamolytic Streptococcus
Ps. - Pseudomonas
E.faecalis - Enterococcus faecalis
S. - Staphylococcus
E. - Escherichia
C. - Candida
? - +ve by ELISA (non-boiled specimen)
-ve on retesting after boiling for 10 min

L.D. - Legionnaires' disease (diagnosed on serological or cultural evidence)
pneu.- non-L.D. pneumonia (no serological or cultural evidence of a Legionella infection)

( ) - ELISA result

Table 10 Characteristics of test urines used to validate ELISA.

Clinical diagnoses are given where these were established at the time of each patient's illness.

No. of test well.		Lab. No.	Patient	Date of specimen/test	Diagnosis ELISA
1		84/30161	Mill.	02/08/84	L.D. (+)
2		86/45961	Mun.	22/10/86	L.D. (+)
3		-	Cross.	-	L.D. (+)
4		84/38706	McN.	17/09/84	L.D. (+)
5		83/40233	McSkim.	-	L.D. (+)
6		84/25731	Rals.	28/06/84	L.D. (+)
7		83/41223	Gord.	09/11/86	L.D. (+)
8		87/15518	Hamil.	-	L.D. (+)
9		84/37190	Conn.	29/04/84	L.D. (+)
10		84/46987	Gall.	16/05/84	L.D. (+)
11		82/28418	Duff.	27/08/82	L.D. (+)
12		87/46305	Mell.	30/11/87	L.D. (+)
13		84/54100	Flec.	24/04/84	L.D. (+)
14		87/35176	Co.	24/09/87	L.D. (+)
15		88/06204	McIn.	-	L.D. (+)
16		85/14398	McClau.	02/04/85	pneu. (-)
17	*	83/43927	Bowd.	07/12/83	pneu. (-) ?
18		86/46637	Ham.	27/10/86	pneu. (-)
19		84/78798	Hay.	-	pneu. (-)
20		86/23339	Bark.	23/05/86	pneu. (-)
21		85/06647	Kan.	15/02/85	pneu. (-)
22		-	McKen.	-	pneu. (-)
23		85/14641	Kell.	16/05/85	pneu. (-)
24		84/32677	E.St.Hil.	16/08/84	pneu. (-)
25		84/41371	Gra.	08/10/84	pneu. (-)
26		- /37838	Max.	-	pneu. (-)
27		86/ -	Cad.	19/05/86	pneu. (-)
28		85/14397	Andr.	02/04/85	pneu. (-)
29		87/ -	Doug.	23/05/87	pneu. (-)
30		84/77477	Salis.	16/05/84	pneu. (-)
31		85/06793	Aitch.	18/02/85	pneu. (-)
32		85/14906	Kel.	-	pneu. (-)
33		84/22344	Hend.	05/06/84	pneu. (-)
34		85/02147	Rog.	-	pneu. (-)
35		85/16348	-	16/04/85	pneu. (-)
36		85/11899	Brisb.	16/05/85	pneu. (-)
37		84/41942	Donnac.	10/10/84	pneu. (-)
38		86/23340	Bark.	-	pneu. (-)
39		85/14788	Kel.	04/04/85	pneu/ (-)

* possible L.D.,IFAT=128 /only one serum tested

Table 10 cont.

UTI test urines used to validate ELISA

No. of test well.	Lab. No.	Specimen Type	UTI-causative organism/s
40	88/45348	MSSU	S.saprophyticus
41	88/45975	MSSU	E.coli
42	88/48029	MSSU	E.coli
43	88/47462	MSSU	E.coli
44	88/46185	MSSU	E.coli
45	88/47206	MSSU	S.epidermidis
46	88/45871	MSSU	E.coli
47	88/46928	CSU	Ps.aeruginosa/C.albicans
48	88/46981	MSSU	E.faecalis
49	88/47149	MSSU	E.coli
50	88/45850	MSSU	E.coli
51	88/46545	MSSU	E.coli
52	88/48409	MSSU	E.coli
53	88/47511	MSSU	E.coli
54	88/47207	MSSU	E.coli
55	88/47186	MSSU	E.coli
56	88/46927	CSU	K.aerogenes
57	88/47340	MSSU	E.coli
58	88/46936	MSSU	E.coli
59	88/47271	CSU	Acinetobacter/Ps.aeruginosa
60	88/47266	MSSU	E.faecalis
61	88/47185	MSSU	E.coli
62	88/46984	MSSU	S.galactiae
63	88/47188	MSSU	E.coli
64	88/46934	MSSU	E.coli
65	88/46139	MSSU	E.coli
66	88/47646	MSSU	E.coli
67	88/47214	MSSU	E.coli
68	88/47313	CSU	Ps.aeruginosa/C.albicans
69	88/47440	MSSU	E.coli
70	88/45872	MSSU	Ps.aeruginosa
71	88/48375	MSSU	S.epidermidis
72	88/46930	CSU	Ps.aeruginosa/C.albicans/E.coli
73	88/46362	MSSU	E.coli
74	88/47464	MSSU	E.coli
75	88/47330	MSSU	E.coli
76	88/46967	MSSU	S.aureus
77	88/45909	MSSU	E.coli

positives, 24 known negative urines from patients diagnosed as having pneumonia but where infection by a legionella was not demonstrated by serology or by culture, and 38 known negative urines from patients with urinary tract infection (table 10).

No figures are available to quantitate the amount of Legionella urinary antigen in each of the 15 positive urines. Testing positive urine controls with McKinney reagents was used to give an indication of the optimal dilution at which antisera produced by the McKinney protocols (from R2/88, R6/88 etc.) and the Conlan and Ashworth protocols (R5/90 and R10/90) should be used.

IgG from both test bleed Nos. 43 and 59 gave optimal A_{492} values at dilutions of 1/50. These were the same as the dilution of the McKinney control capture antibody (personal communication, McKinney)

The antisera produced by the Conlan and Ashworth protocol had an optimal dilution of 10 fold (table 4) when used as capture antibody. This difference between 50 and 10 fold dilution was not connected with production of IgG or a measure of its avidity. It was more likely due to the method of IgG purification. The Conlan and Ashworth protocols were set up with blood samples taken after the Protein-A method for IgG purification had been chosen in preference to the NH_4SO_4/DEAE 52 method. The protein-A method produced a more dilute sample of IgG/ml of antiserum

purified (about 4-5 times) because only a 2ml portion of serum could be loaded onto the column at a time. This accounts for the differences seen. Also, no differences were observed with protein-A purified IgG from R2/88 (No. 43) when evaluated at a dilution of 10 and compared with the NH_4SO_4/DEAE 52-purified IgG at a dilution of 50.

All of the blood samples taken from the rabbits which differentiated positive from negative control urines in the screening stages by ELISA did so when tested against these 77 urines. Each of the 15 positive urine controls was detected (100% sensitivity) both by IgG from those blood samples (e.g. No. 43, No. 59 etc.) evaluated in addition to IgG from McKinney (positive control) and thus all were confirmed to contain urinary antigen. However, 3 of these urine samples gave lower A_{492} results than when they were originally tested (urine test Nos. 3, 7 and 8, table 11). None of the 62 negative control urines tested as positive (100% specificity) with any of the capture antibody (IgG) assayed at this level. These capture IgG antibodies are now in clinical use in an ELISA system and experience here has shown no "false positive" test results with boiled specimens of urine from patients where investigation by other methods has failed to produce evidence of Legionella infection. All positive urines identified by the ELISA originated from cases where clinical symptoms raised suspicion of Legionella infection and most of these have been supported by

KEY

+++ = A_{492} > 0.501
++ = A_{492} > 0.201 but < 0.500
+ = A_{492} > 0.101 but < 0.200
- = A_{492} < 0.050

Controls

All A_{492} values for +ve control antigen (T.S.E. extract) >2.600

Antisera evaluated

Nos. 43 and 59 were raised by the McKinney protocol from rabbits 2/88 and 6/88 respectively and were both collected 35 days after the primary immunisation.

No. 231 was raised by the Conlan and Ashworth protocol from rabbit 10/90 and was collected 31 days after the primary immunisation.

Table 11 Capture antibody evaluation by ELISA using urine controls as defined in table 10.

Test urine No.	Status of urine	Control (McKinney)	Test Bleeds/Cab No. 43		No. 59		No. 231	
		Dilutions (1/ ..) 50	100	50	100	50	25	10
1	+ve	+++	++	+++	++	++	+	++
2	"	+++	+++	+++	+++	+++	++	+++
3	"	++	++	++	++	++	+	++
4	"	++	++	+++	++	+++	+	+++
5	"	++	++	++	++	++	+	+
6	"	+++	+++	+++	+++	+++	++	++
7	"	++	+	++	++	++	+	++
8	"	+	+	+++	++	+++	-	+
9	"	+++	++	+++	++	++	+	++
10	"	+++	+++	+++	++	+++	++	+++
11	"	+++	+++	+++	+++	+++	+	++
12	"	+++	+++	+++	+++	+++	++	+++
13	"	+++	++	+++	++	+++	+	++
14	"	+++	+++	+++	++	+++	+	++
15	"	+++	++	+++	++	++	+	++
16	-ve (pn)	-	-	-	-	-	-	-
17	" "	-	-	-	-	-	-	-
18	" "	-	-	-	-	-	-	-
19	" "	-	-	-	-	-	-	-
20	" "	-	-	-	-	-	-	-
21	" "	-	-	-	-	-	-	-
22	" "	-	-	-	-	-	-	-
23	" "	-	-	-	-	-	-	-
24	" "	-	-	-	-	-	-	-
25	" "	-	-	-	-	-	-	-
26	" "	-	-	-	-	-	-	-
27	" "	-	-	-	-	-	-	-
28	" "	-	-	-	-	-	-	-
29	" "	-	-	-	-	-	-	-
30	" "	-	-	-	-	-	-	-
31	" "	-	-	-	-	-	-	-
32	" "	-	-	-	-	-	-	-
33	" "	-	-	-	-	-	-	-
34	" "	-	-	-	-	-	-	-
35	" "	-	-	-	-	-	-	-
36	" "	-	-	-	-	-	-	-
37	" "	-	-	-	-	-	-	-
38	" "	-	-	-	-	-	-	-
39	" "	-	-	-	-	-	-	-

Table 11 cont.

Test urine No.	Status of urine	Control (McKinney)	Test bleeds/Cab No. 43		No. 59		No. 231	
			Dilutions (1/ ..)					
		50	100	50	100	50	25	10
40	-ve (UTI)	-	-	-	-	-	-	-
41	" "	-	-	-	-	-	-	-
42	" "	-	-	-	-	-	-	-
43	" "	-	-	-	-	-	-	-
44	" "	-	-	-	-	-	-	-
45	" "	-	-	-	-	-	-	-
46	" "	-	-	-	-	-	-	-
47	" "	-	-	-	-	-	-	-
48	" "	-	-	-	-	-	-	-
49	" "	-	-	-	-	-	-	-
50	" "	-	-	-	-	-	-	-
51	" "	-	-	-	-	-	-	-
52	" "	-	-	-	-	-	-	-
53	" "	-	-	-	-	-	-	-
54	" "	-	-	-	-	-	-	-
55	" "	-	-	-	-	-	-	-
56	" "	-	-	-	-	-	-	-
57	" "	-	-	-	-	-	-	-
58	" "	-	-	-	-	-	-	-
59	" "	-	-	-	-	-	-	-
60	" "	-	-	-	-	-	-	-
61	" "	-	-	-	-	-	-	-
62	" "	-	-	-	-	-	-	-
63	" "	-	-	-	-	-	-	-
64	" "	-	-	-	-	-	-	-
65	" "	-	-	-	-	-	-	-
66	" "	-	-	-	-	-	-	-
67	" "	-	-	-	-	-	-	-
68	" "	-	-	-	-	-	-	-
69	" "	-	-	-	-	-	-	-
70	" "	-	-	-	-	-	-	-
71	" "	-	-	-	-	-	-	-
72	" "	-	-	-	-	-	-	-
73	" "	-	-	-	-	-	-	-
74	" "	-	-	-	-	-	-	-
75	" "	-	-	-	-	-	-	-
76	" "	-	-	-	-	-	-	-
77	" "	-	-	-	-	-	-	-

serological and/or cultural evidence implicating L.pneumophila SG 1 as the cause of illness.

3.2.1 Reactions of avid antisera with "soluble" antigens of L.pneumophila strains

Purified IgG from samples of blood taken (No.43 from R2/88 and No. 59 from R6/88) was tested as capture antibody using soluble antigens of several strains of L.pneumophila SG 1 instead of urine (Tang and Toma, 1986). These antigens were prepared as in section 2.14.4 and heated to 100°C for 15 min to denature heat-labile components leaving mainly LPS. The preparations were filter-sterilised (0.2um) which also removed particulate material.

Results by ELISA gave $\bar{x}$ A_{492} values > 1.400 on multiple testing for all seven strains used as antigen (table 12).

Investigations of boiled and non-boiled samples of both control urines and test urines (clinical specimens) revealed the incidence of false-positive results with unboiled samples. For this reason all clinical specimens are heated to 100°C for 15 min, centrifuged and the supernate tested. No false-positive results were identified after all clinical specimens were treated in this way and most positives have been substantiated with either cultural or serological evidence (or both) from sputum and blood respectively.

3.2.2 ELISA optimisation

The ELISA system established as a result of successfully raising effective capture antibody was further

Table 12 ELISA results with sonicates prepared from seven different strains of *L.pneumophila* SG 1.

Strain of *L.pneumophila* SG1	IgG's purified by DEAE 52 at a dilution of 1/50 as capture antibody: $O.D_{492}$ values No. 43	No.59	($\bar{x}$ of 3 values) No.231
Pontiac-1	1.426	1.328	1.102
Knoxville-1	1.534	1.388	1.182
Philadelphia	1.632	1.496	1.200
Camperdown	1.574	1.430	1.168
RH-Bellingham-type	1.416	1.204	0.980
Olda 1a	1.524	1.296	1.030
Olda 1c	1.560	1.313	1.057
Range	1.416 - 1.632	1.204 - 1.496	0.980 - 1.200
Mean ($\bar{x}$)	1.523	1.351	1.102
S.D.	0.078	0.096	0.084

investigated. It was observed from previous results that the majority of high titre antisera could not differentiate between positive and negative control urines and were grouped as category A. This category could be further subgrouped into groups I and II. Group I antisera gave high A_{492} values with both positive and negative control urines and group II gave low A_{492} values with both positive and negative controls urines.

The efficiency of blocking and also the plate type used as the solid phase in the ELISA (using No.43 as avid capture antibody) were both investigated. Two types of bovine serum albumin (BSA) were compared; product no. A-7096, (Sigma) and bovine albumin fraction V (Miles Scientific Ltd.) at 1, 2 and 4% (w/v in PBS) tested on 8 different plates (Immulon F III [flat-welled], Immulon Dynatech I [flat-welled], Virion F [flat-welled], Nunc, Denmark [flat-welled], Elkay 000-Mico-Tpu [flat-welled] and three Costar plates, No. C3590 [flexible, U-shaped well], No. C6595 [flexible, blue, U-shaped well], No. C2595 [flat-welled] from Northumbria Biologicals Ltd).

The two BSA preparations gave different results. Bovine albumin fraction V did not block any of the plates at each of the three concentrations tested (4, 2 and 1% (w/v) in PBS) where A_{492} values of 3.00 to 1.40 were obtained with the negative control urine. Similar A_{492} values were observed between each of the eight plates with both the

positive control antigen (T.S.E. extract), the positive (G.L.) and the negative urine. This was due to the conjugate binding to unblocked sites in the plastic well. When BSA A-7906 (Sigma) was evaluated at each of the three concentrations the blocking effect was obvious. Examination of the results where the BSA A-7096 was used at 4% (w/v) produced A_{492} differential values (between the positive urine control and the blank) which were no greater than those obtained when used at 2% (w/v). BSA used at 1% (w/v) produced only slightly raised A_{492} values with the negative urine control when compared with results where BSA was used at 2% (w/v). BSA at 2% (w/v) in PBS was the standard adopted for use in the ELISA. Where the results were compared for eight different plates all negative urine controls produced A_{492} values ranging from 0.05 to 0.24 depending on the plate evaluated. Only one plate was excluded from the direct comparison with the other seven as this plate (Costar No.6595) was blue in colour which interfered with the ELISA assay. Figures 3-10 show the A_{492} values obtained with a positive urine (G.L.) when tested at dilutions from neat to 32. The A_{492} value with a negative control urine on each plate type is also shown.

The other seven plates would have been acceptable for use in the ELISA test with only small differences in A_{492} values for the undiluted positive urine. However, differences were observed with the background A_{492} value for

ELISA results with No.43 (CAb) and control antigens on different ELISA plate types.

Figure 3

Elkay

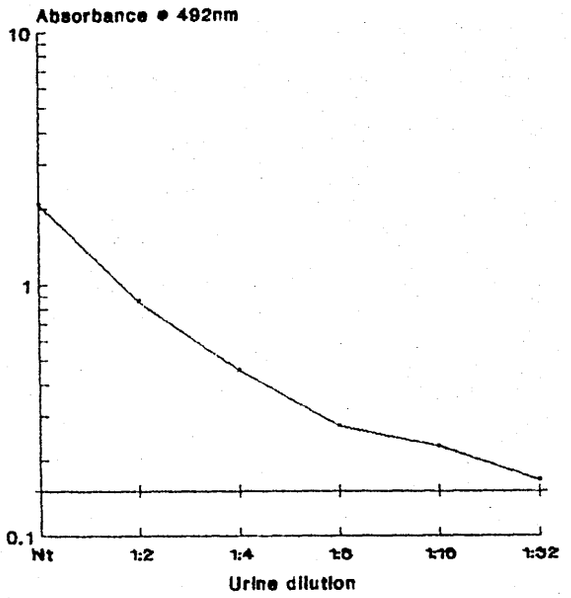


Figure 4

Costar No. C2595

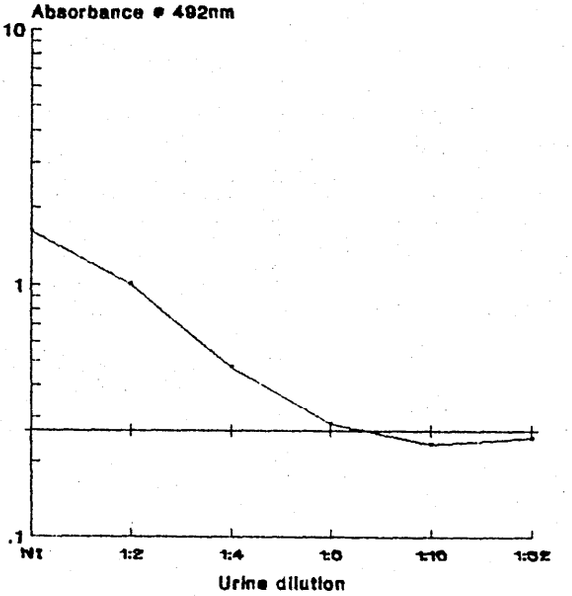


Figure 5

Costar No. C3590

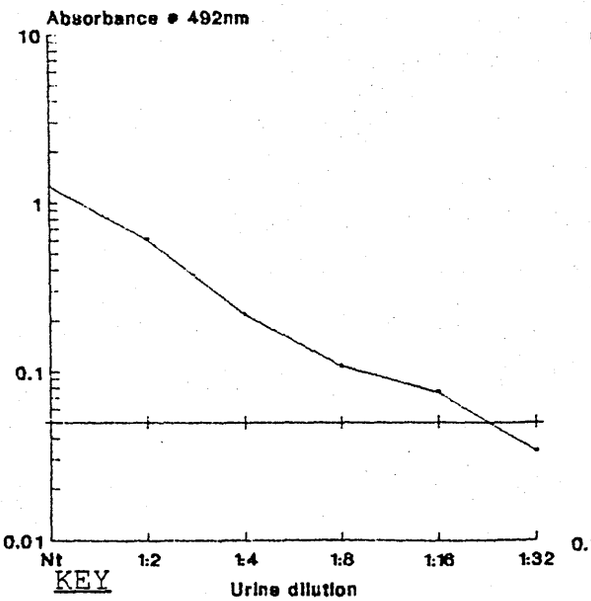
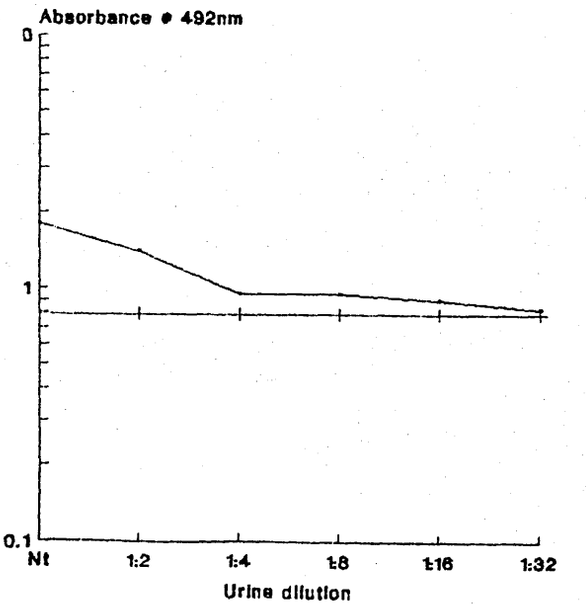


Figure 6

Costar No. C6595



KEY

Positive antigen-containing urine (G.L.) : [—•—]

Negative control urine : [—+—]

Figure 7

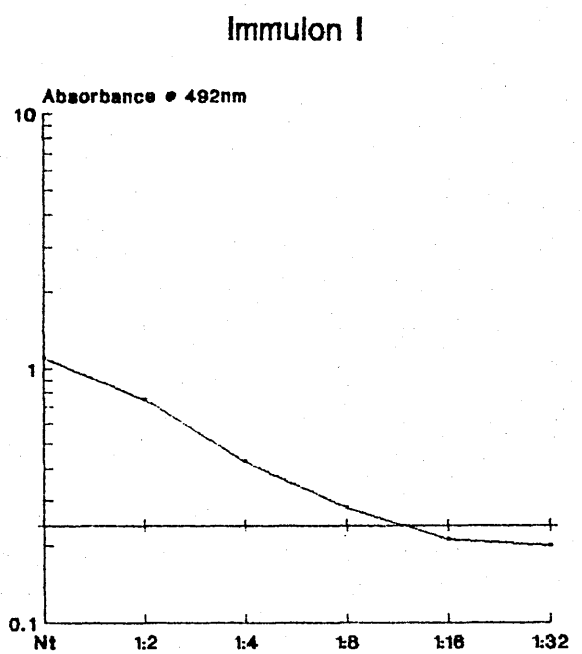


Figure 8

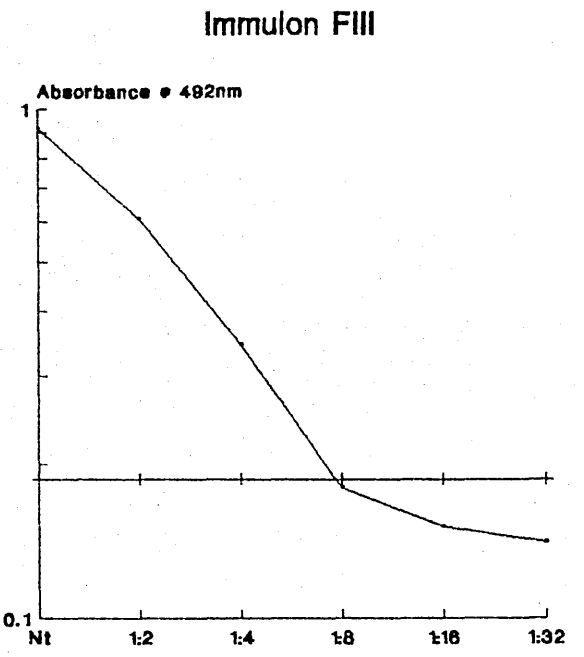


Figure 9

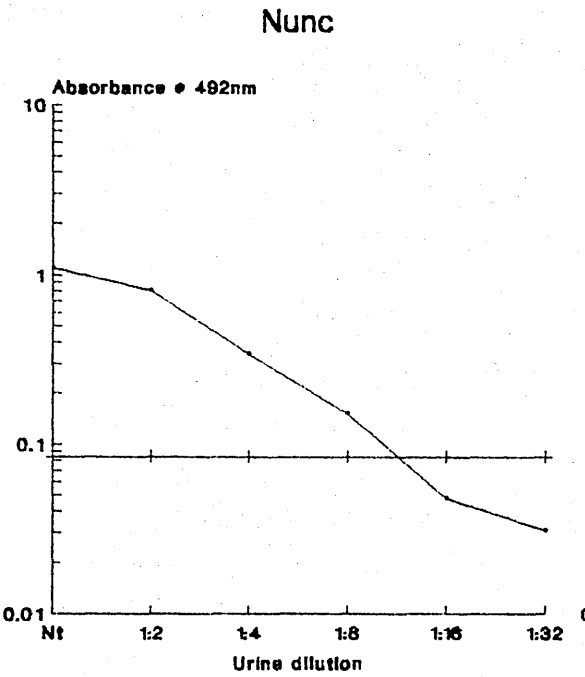
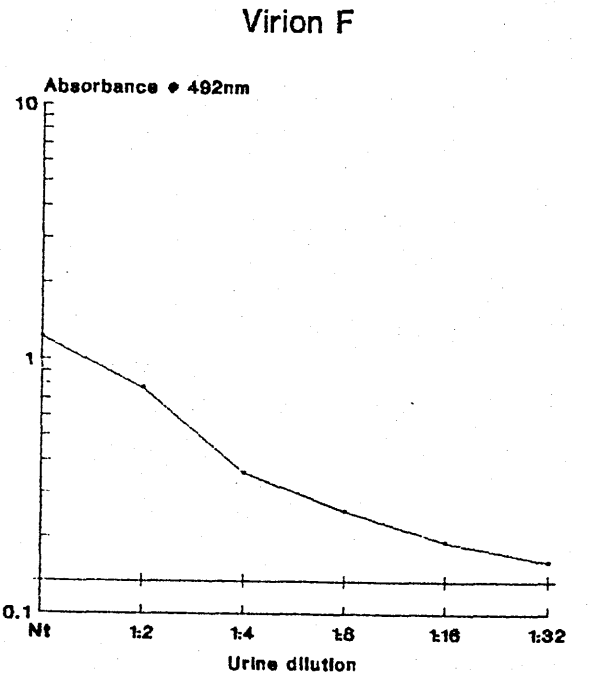


Figure 10



KEY

Positive antigen-containing urine (G.L.) : [—•—]

Negative control urine : [—+—]

the negative urine control where these ranged from <0.05 to 0.239. The latter A_{492} value was unacceptably high and produced with the Immulon I plate type. However, the differential A_{492} value between the undiluted positive urine and the negative urine controls was high (0.730). The greatest A_{492} differential between the undiluted positive and negative urine controls were obtained with three plates (Costar No. 3590 [1.196], Virion F[1.082] and Nunc [1.016]).

3.3 Evaluation of commercial test kits: a latex agglutination and a RIA for antigen detection in urine

Twenty-three human control urines whose status as either positive antigen-containing or non-antigen-containing urines were known (determined by ELISA, and supported by studies where other workers had tested some of these urines by Mab or polyclonal ELISA or by RIA in addition to clinical details, culture and IFA results where these have been available) were used to evaluate this kit. Their details are shown (tables 13 and 14).

pH values of all urines (stored at -20°C) examined were determined with a calibrated pH meter after the urines had reached room temperature. Their pH values shown in table 15 were within the physiological pH range (5-9) recognised by the manufacturer not to interfere with test results.

Results of these control urines, each tested three times and each boiled and centrifuged before the third test, are shown (table 15).

Table 13 Culture and serological details of patients whose urine samples were used to evaluate the "Merigen" latex agglutination test.

Lab. Nos.	+ ve by culture	IFA (to L.pneumophila SG1)
+ ve urine controls		
84/30296 (Mill.) *	NO	16-64
84/25731 (Ral.) *	YES	16-64
84/54100 (Fle.)	NO	16-256
86/44928 (Mun.)	NO	-
87/35176 (Cook) 25/9	NO	-
83/40233 (McSkim.)	-	-
83/57751 (McN.)	NO	> 512
84/37910 (Con.)	-	16-128
84/96957 (Fen.)	NO	512
87/46305 (Mell.)	NO	-
85/18487 (Wats.)	NO	-
87/32456 (Go.)	YES	128
82/28334 (Duff.)	NO	16-128
- ve urine controls		
84/41942 (Donac.)	NO	< 16
88/47149	-	-
84/78798 (Hay.)	NO	-
84/32677 (St. Hil. E.)	NO	< 16
88/45850	-	-
88/47188	-	-
88/46934	-	-
88/47340	-	-
88/45871	-	-

KEY

* - validated by McKinney in independent tests

( ) - patient

Table 14 Results of control urines validated.

Monoclonal Ab in ELISA and RIA tests were used to determine the presence of urinary antigen by other workers.

Urine	RIA titre (expressed as a binding ratio of the control)	Monoclonal (ELISA of urine)	Polyclonal (ELISA) Undiluted urine
Tested by McKinney			
84/25731 (Ral.)	-	1:128	0.87*(0.51)
84/30296 (Mill.)	-	1:64	0.27*(0.32)
Tested by Kohler **			
84/30296Mill.	6.01 (+ve)	-	-

KEY

* These are the mean results of two tests.
** Binding ratios between 1.50 and 2.00 have usually come from patients with Legionellosis.

Table 15 Results of 23 test urines used in the "Merigen" latex agglutination kit.

Specimen of urine tested (Lab. Number)	pH	Elisa result	Latex agglutination result on three occasions. 1st	2nd	3rd	Result
1.# 84\30296	6.0	+ve	2+	2+	2+	-ve
2.* 84\25731	6.1	+ve	0	0	0	+ve
3. 84\96957	6.3	+ve	1+	1+	0	+ve
4. 84\54100	5.9	+ve	0	0	0	+ve
5. 83\57751	5.6	+ve	1+	1+	2+	+ve\-ve
6. 82\28334	5.7	+ve	1+	2+	2+	+ve\-ve
7.* 87\32456	8.6	+ve	0	0	2+	+ve\-ve
8. 87\18487	5.7	+ve	3+	3+	3+	-ve
9. 87\46305	5.2	+ve	2+	3+	3+	-ve
10. 87\42752	5.5	+ve	4+	4+	4+	-ve
11. 84\37910	5.9	+ve	2+	2+	2+	-ve
12. 83\40233	5.4	+ve	2+	2+	2+	-ve
13. 87\35176	7.0	+ve	1+	1+	0	+ve
14. 84\41942	7.0	-ve	0	0	0	+ve
15. 84\32677	5.8	-ve	1+	0	0	+ve
16. 84\78798	6.3	-ve	2+	1+	0	-ve\+ve
17. 88\47149	6.1	-ve	3+	3+	2+	-ve
18. 88\48375	5.9	-ve	1+	0	0	+ve
19. 88\45850	6.8	-ve	0	0	0	+ve
20. 88\47188	5.6	-ve	3+	2+	0	-ve\+ve

Table 15 cont.

Specimen of urine tested (Lab. Number)	pH	Elisa result	Latex agglutination result on three occasions. 1st	2nd	3rd	Result
21. 88\46934	6.3	-ve	2+	1+	0	-ve\+ve
22. 88\47340	6.1	-ve	3+	2+	0	-ve\+ve
23. 88\45871	6.3	-ve	1+	1+	0	+ve

Key

* : Culture +ve for L.pneumophila SG 1

N.B. Specimen No.84\30296 was found to be +ve by R. Kohler using RIA where the RIA titre was 6.01, expressed as a binding ratio of the control. Patient suffered clinical L.D. with a low (16-64) 4 fold rise in IFA titre . Culture of the sputum was negative.

Of the 13 positive control urines tested (table 15), only 4 (31%, one of these from a culture positive patient) gave a clear positive result on all three occasions when tested by this latex agglutination kit. Six of the 13 positive control urines (46%) gave a negative result on all three occasions. One of these urines (84/37910) came from a patient with a significant rise in IFA titre from 16 to 128 and another urine (84/30296) was confirmed as positive by Kohler (using RIA), Toma (using Mab ELISA) and McKinney (using polyclonal ELISA). The three remaining positive control urines (23%), including one from a culture positive patient (Go.) gave mixed results on repeated testing.

Of the 10 negative control urines, 4 (40%) gave a positive result on each of the three occasions. Only one urine (10%) consistently gave a negative result. The remaining five (50%) each gave negative results before boiling, and positive results after boiling and centrifugation.

These results are clearly unacceptable and show that this test is not an alternative to a sensitive and specific ELISA system.

3.3.1 "Dupont" Legionella urinary antigen [^{125}I] radioimmunoassay kit (Lot# PA306)

This kit has already been evaluated by Aguero-Rosenfeld and Edelstein (1988) and was a gift from "Dupont" for further independent evaluation. There were two reasons

for further evaluation. The first was to assess the ability of the kit to distinguish between control positive and negative urines (all previously determined by the "in-house" ELISA and one by two other independent groups of co-workers using ELISA and RIA techniques (table 14). The second was to compare the sensitivity of our ELISA with that of the RIA in a simple dose/response investigation as well as to compare the specificity of the RIA with that of the ELISA.

Fifteen human control urines were used to test the RIA kit: 5 known +ve controls determined by ELISA with No. 4 (84/30296, table 14) verified by different groups of workers to contain detectable levels of antigen and the remainder from patients from whom L.pneumophila SG 1 was not isolated.

Four of the 5 positive control urines produced definite positive net dpm counts (table 16) with mean ($\bar{x}$) ratios greater than three times that of the kit-supplied negative control. Only one test urine No.5 (Go.) gave a $\bar{x}$ ratio of 2.8 but this increased to greater than three times when its net dpm + one standard deviation (S.D.) was considered.

The final 5 control urines negative by ELISA, were from patients' with UTI due to a variety of organisms (table 10). One catheter urine (88/46930) produced a mixed flora on culture. All gave a negative result by the RIA test.

Tests to establish the sensitivity of the RIA were performed with antigen-containing urine by diluting this

urine in two-fold steps to 128 (table 16). The end point at which the highest dilution gave a positive result was 16 (where the $\bar{x}$ + 1 S.D. was > three times the negative kit control).

The positive and negative controls with the kit produced net dpm (+/- 1 S.D.) counts of 495 +/- 61.7 and 72.8 +/- 20.7 respectively. Background counting (2 tubes) was performed with every set of 8 tubes where actual test dpm measurements were taken, and the $\bar{x}$ ratio was calculated from the test value divided by the net dpm of the negative test kit control.

Concluded from these results is that the RIA kit is as sensitive and specific for the detection of Legionella antigen in urine as the "in-house" ELISA.

3.4 Effective and non-effective Cab investigated and characterisation of urinary antigen

These studies were performed to establish the nature of the specific urinary antigen that was captured and detected by avid antibody used in ELISA testing with the hope that a more successful method to produce diagnostically useful capture antibody could be found and the urinary antigen further characterised.

3.4.1 Ouchterlony tests

Agar diffusion tests reacting antiserum taken from rabbits inoculated according to different schedules, with various antigens are described.

Table 16 Results of 15 urines tested by the "Dupont ^{125}I RIA kit for the detection of urinary antigen.

Each urine was tested four times and the mean dpm and S.D.'s calculated. Results of a positive urine (G.L.) diluted two-fold and tested for the presence of antigen to compare the sensitivity of the capture antibody produced for the ELISA with that of RIA are also shown.

	Assay No.									
Tube No.	1	2	3	4	5	6	7	8	$\bar{x}$	S.D.
Background	Range=34-40 (Two readings taken every 10 tests)									
+ve ctrl.	429	503	429	566	439	512	530	592	495	61.7
-ve ctrl.	80	83	102	69	69	90	50	39	72.8	20.7

(Net dpm)

Sample	Assay No. 1	2	3	4	$\bar{x}$	S.D.	Mean ratio (RIA result)
ELISA+ve urines (culture +ve)							
1 (Smi.)	4014	3689	3985	3970	3914	151	53.8 (+ve)
2 (G.L.)	1710	1678	1550	1870	1702	131.6	23.4 (+ve)
3 (J.Mcm.)	277	291	363	282	303.3	40.3	4.2 (+ve)
*4 (Mill.)	994	1058	523	616	797.8	267.6	5.0 (+ve)
#5 (Go.)	223	216	194	188	205.3	16.9	2.8 (+\-)
ELISA-ve urines (+ve pn's, non-Legion)							
1 (Hay.)	90	98	80	84	88.0	7.8	1.2 (-ve)
2 (E.St.Hil.)	64	69	69	81	70.8	7.2	1.0 (-ve)
3 (Gra.)	72	62	63	83	70.0	9.8	1.0 (-ve)
4 (Salis.)	69	83	69	85	76.5	8.7	1.0 (-ve)
5 (Brisb.)	81	79	96	122	94.5	19.9	1.3 (-ve)

Table 16 cont.

Sample	Assay No. 1	2	3	4	$\bar{x}$	S.D.	Mean ratio (RIA result)
-ve urines (UTI's)							
1 (88\45348)	80	94	60	67	75.5	15.0	1.0 (-ve)
2 (88\46927)	89	61	86	58	73.5	16.3	1.0 (-ve)
3 (88\47266)	65	75	87	71	74.5	9.3	1.0 (-ve)
4 (88\46139)	63	70	61	128	80.5	31.9	1.1 (-ve)
5 (88\46930)	87	71	56	46	65.0	17.9	0.9 (-ve)

Test Sensitivity Assay (net dpm)

(urine)	1	2	3	4	$\bar{x}$	S.D.	Mean Ratio (RIA result)
(G.L.)							
1\4 dil	598	622	554	565	584.8	31.1	8.0 (+ve)
1\8 "	251	291	311	347	300	40.0	4.1 (+ve)
1\16 "	218	180	211	158	191.8	27.9	2.6 (+\-)
1\32 "	181	95	163	129	142	38.0	1.9 (-ve)
1\64 "	121	81	113	99	103.5	17.5	1.4 (-ve)
1\128 "	113	128	135	85	117.5	18.2	1.6 (-ve)

-ve urines (+ve UTI's)	Specimen type\ Causative organism(s)
1 (88\45348)	MSSU-S. saprophyticus
2 (88\46927)	CSU -K.aerogenes
3 (88\47266)	MSSU-E.faecalis
4 (88\46139)	MSSU-E.coli
5 (88\46930)	CSU -Ps.aerug.\C.alb.\E.coli

KEY
* - culture -ve, but specimen confirmed by 3 groups of co-workers to contain antigen using ELISA and RIA.
# - culture +ve, ELISA (in-house) +ve, -ve by Merigen latex agglutination.

Two antigens, a sonicate (see section 2.14.4 for preparation) and known positive and negative antigen-containing urines at neat and concentrated x100 (using a macrosolute concentrator, Minicon) were reacted with capture antibody from rabbits inoculated according to the McKinney schedule.

Figure 11 shows pure IgG (purified by DEAE 52) from the sequential test bleeds taken from R1/88 (not effective as CAb in the ELISA), whose IFA titres fell during the protocol (an unusual feature). It was observed from the agar diffusion gel that when the IFA titre had fallen to 2K, two reaction lines were observed when this test bleed (No. 48) was reacted with sonicate prepared from the Knoxville-1 strain of L.pneumophila.

Figure 12 shows the agar diffusion test of the sequential test bleeds (whole serum) taken from R2/88 with test bleed No.43 (the only bleed from R2/88 able to capture urinary antigen in the ELISA test. Reactions of identity with each test bleed (Nos. 41, 43 and 51) against the sonicate of Knoxville-1 were observed when these tests were performed together with the antigen in the central well.

A similar agar diffusion gel was set up using the whole serum from sequential test bleeds from R6/88 where these were tested against the L.pneumophila sonicate. The same pattern was observed in this experiment with all three test bleeds producing one visible reaction line. Test

Figure 11 Agar diffusion gel (Ouchterlony) showing precipitin lines between IgG from test bleeds taken from R1/88.

Precipitin lines between test bleed Nos. 40, 42 and 48 (IgG purified by DEAE 52) and L.pneumophila SG 1 sonicate.

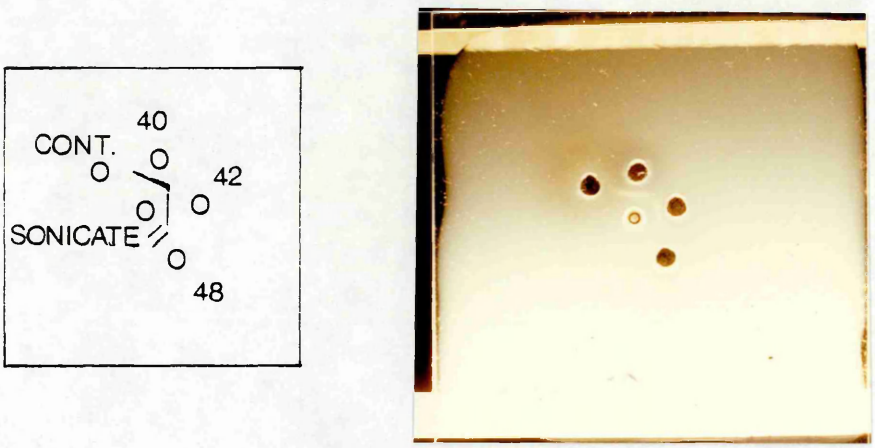
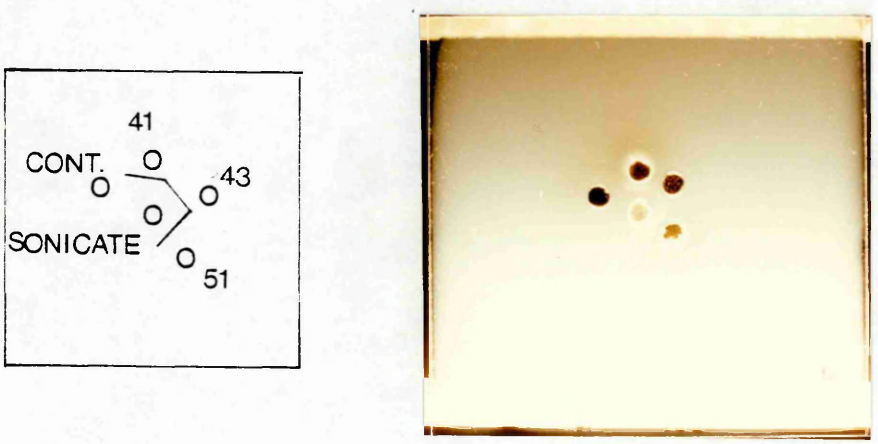


Figure 12 Agar diffusion gel (Ouchterlony) showing precipitin lines between IgG from test bleeds taken from R2/88.

Precipitin lines between test bleed Nos. 41, 43 and 59 (IgG purified by DEAE 52) and L.pneumophila SG 1 sonicate.



bleed No. 59 whose IgG was effective as capture antibody in the ELISA produced a diffuse reaction line of identity with the test bleeds taken one week either side of test bleed No. 59.

Whole serum samples containing IgG effective as capture antibody together with the purified IgG were each tested against the sonicate to establish whether they were reacting with the same antigen in the sonicate.

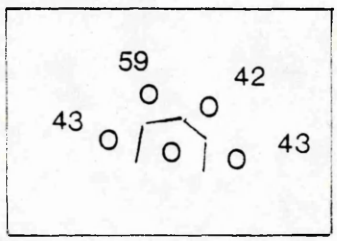
Figures 13 and 14 show the unfractionated and the purified IgG samples from test bleed Nos. 43 and 59 with No. 42 as a negative control (ineffective in the ELISA). Firstly, the IgG fractions from test bleed Nos. 43 and 59 produced much clearer results than those using whole serum. Only a single line was observed between the antigen and IgG-containing wells compared with a diffuse line of precipitation observed between whole serum and the sonicate. Secondly, this appeared to be a reaction of identity. In figure 14 there were two lines between serum IgG from test bleed Nos. 43 and 59 and the boiled sonicate. The weaker precipitin line was not observed before boiling between these two reagents nor was it seen on repeating the test. The reasons for this are not clear.

All test bleeds which, when fractionated for IgG and found to be effective in the ELISA as capture antibody together with non avid IgG controls, were tested against both negative and positive urine (concentrated 100x). No

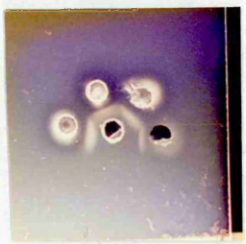
Figure 13 Agar diffusion gel (Ouchterlony) showing precipitin lines between whole serum from test bleed Nos. 42, 43 and 59.

Precipitin lines between test bleeds Nos. 41, 43 and 59 and L.pneumophila SG 1, Knoxville-1 sonicate, non-boiled (1) and boiled (2).

1.



2.



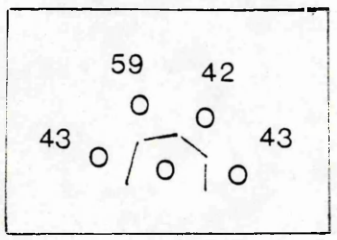
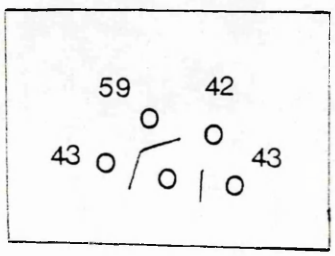


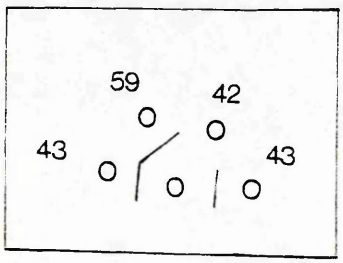
Figure 14 Agar diffusion gel (Ouchterlony) showing precipitin lines between IgG from test bleed Nos. 42, 43 and 59.

Precipitin lines between test bleeds Nos. 41, 43 and 59 and L.pneumophila SG 1, Knoxville-1 sonicate, non-boiled (1) and boiled (2).

1.



2.



precipitin lines were observed between any of the antisera and the concentrated urines.

identity.

3.4.2 Crossed two-dimensional immunoelectrophoresis (CIE)

This high resolution technique was performed to investigate further the nature of the antigens with which the antiserum samples reacted. This method has a resolving power which can differentiate between 85 different antigens (Collins et al., 1983) in L.pneumophila SG 1. Of these, 11 antigens were heat-stable. The photographs published by these workers provided a reference CIE pattern with antigen migration distances clearly defined.

Test bleeds taken from rabbits inoculated according to the schedules of McKinney (section 2.5.1) and Conlan and Ashworth (section 2.5.4) were reacted with the sonicate of L.pneumophila SG 1 (Knoxville-1) prepared as in section 2.14.4.

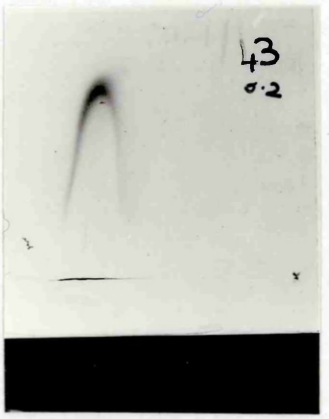
The two-dimensional electrophoretic patterns of IgG fractions from these test bleed Nos. 43 and 59 (taken from rabbits 2/88 and 6/88 respectively) are shown in figures 15 and 16. Only one diffuse peak was observed in each figure indicating that under these experimental conditions, IgG was raised in response to only one antigen of the formalised suspension of L.pneumophila SG 1. The migration distances (using the ovalbumin and bromophenol blue as a marker) of these two peaks were identical indicating that the antigen

Figure 15 CIE of L.pneumophila SG 1 sonicate reacting with test bleed No. 43 (R2/88).

Only a single precipitin line is observed between the antigen and the IgG. Albumin/bromophenol blue marker from the 1st dimension shown by - x.

+

(2nd)



-

+

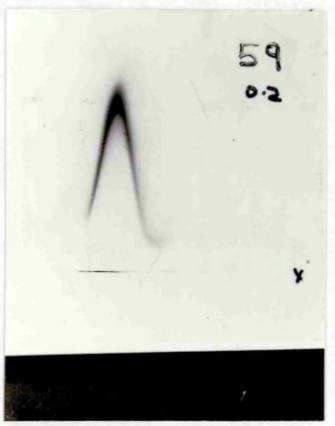
Direction of current (1st) →

Figure 16 CIE of L.pneumophila SG 1 sonicate reacting with test bleed No. 59 (R6/88).

Only a single precipitin line is observed between the antigen and the IgG. Albumin/bromophenol blue marker from the 1st dimension shown by - x.

+

(2nd)



-

+

Direction of current (1st) →

in the sonicate with which these two different pools of IgG antibodies (purified by DEAE 52 chromatography) reacted was the same. Further, using the figures published (Collins et al., 1983a and b) the antigen from the sonicate precipitated in the agarose gel was heat-stable lipopolysaccharide (group-specific antigen).

To determine why the antibody status had changed from being an effective CAb in the ELISA in differentiating positive and negative antigen-containing urines (test bleeds Nos. 43 and 59) to one which failed to meet this criteria, test bleed No. 67 (taken only seven days after No. 59) was reacted with the same sonicate (figure 17) of L.pneumophila SG 1. Figure 17 shows 8 antigen/antibody peaks indicating that IgG's were being produced to other component antigens of the formalised whole cell (i.e the immunogen). It was noted however, that this bleed was ineffective in the ELISA but when the same serum sample was purified for IgG by Protein-A chromatography only one major antigen/antibody peak was observed on the two-dimensional gel (figure 18) and this IgG fraction was able to distinguish between positive and negative urines in the ELISA. Faint antigen/antibody lines (, figure 18) were also seen above the most densely-stained peak whose peak apex had identically shaped arcs as the faint peaks perhaps indicating reactions with other O-specific chains of the LPS where these are of a different molecular weight (the heterogeneous nature of

Figure 17 CIE of L.pneumophila SG 1 sonicate reacting with test bleed No. 67 (R6/88).

Eight precipitin lines were observed between the antigen and IgG (purified by DEAE 52). Albumin/bromophenol blue marker from the 1st dimension shown by - x.

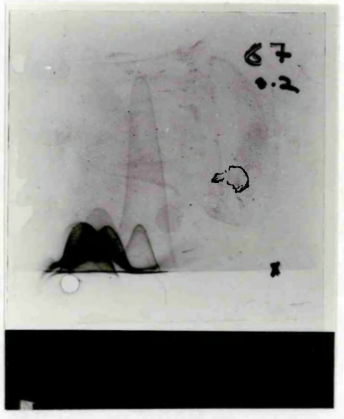
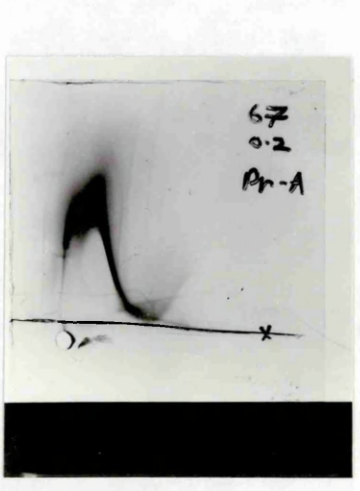


Figure 18 CIE of L.pneumophila SG 1 sonicate reacting with test bleed No. 67 (R6/88).

Multiple precipitin lines are observed between the antigen and the IgG (purified by Protein-A). Albumin/bromophenol blue marker from the 1st dimension shown by - x.



which is a feature of LPS).

This is seen more clearly in figure 19 where a control test bleed (No. 68) taken from R5/88 and ineffective as capture antibody to detect antigen-containing urines was reacted with the sonicate.

Finally, figure 20 shows antiserum (no. 141, IFA=8K) taken from R6/89, inoculated according to the protocol of Conlan and Ashworth (1986) and reacted with the L.pneumophila sonicate. Only when IgG from this sample of blood was purified by Protein-A chromatography was it effective as capture antibody in the ELISA. It shows a major antigen/antibody peak whose antigen component has the same electrophoretic mobility as LPS seen in other figures (15 to 19).

Positive antigen-containing urine concentrated x100 from two different patients was substituted for the sonicate of L.pneumophila SG 1. On repeated testing, no precipitin lines were observed.

Also, when the saline extract of L.pneumophila SG 1 (Knoxville-1) was used as the antigen to be electrophoresed in the first dimension and then reacted with the IgG in the second dimension no reaction lines were observed after staining.

3.4.3 Limulus Amoebocyte Lysate (LAL) Assay

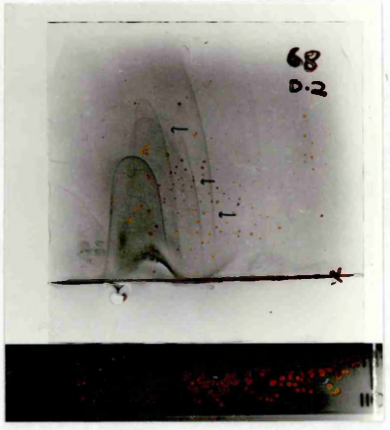
Table 17 shows the results when various antigens were reacted with Limulus polyphemus crab amoebocyte extract for

Figure 19 CIE of L.pneumophila SG 1 sonicate reacting with test bleed No. 68 (R5/88).

Multiple precipitin lines are observed between the antigen and the IgG (purified by Protein-A). Albumin/bromophenol blue marker from the 1st dimension shown by - x.

+

(2nd)



-

+

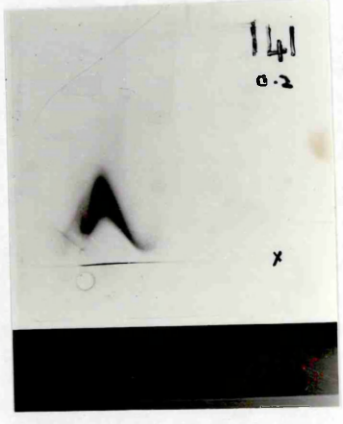
Direction of current (1st) →

Figure 20 CIE of L.pneumophila SG 1 sonicate reacting with test bleed No. 144 (R6/89).

Only a single precipitin line is observed between the antigen and the IgG. Albumin/bromophenol blue marker from the 1st dimension shown by - x.

+

(2nd)



-

+

Direction of current (1st) →

Table 17 Results obtained by testing precipitin lines from CIE gels as well as antigen-containing urine in the *Limulus* amoebocyte lysate assay.

Tube No.	Contents of reaction tube	Result
A	0.1ml Sonicate of *L.pneumophila* SG 1 (Knox-1)	+ve
A1	Ouchterlony precipitin line from McKinney\ sonicate reaction	+ve
A2	CIE precipitin line (Mobility value of LPS) No.43\ sonicate	+ve
A3	CIE (LPS) No.59\ sonicate	+ve
A4	CIE (LPS) No.67\ sonicate	+ve
A5	CIE (LPS) No.141\ sonicate	+ve
A6	CIE gel only (ctrl.)	-ve
A7	0.1ml Urine from healthy human (R.F.)	-ve
A8	0.1ml +ve by ELISA urine (G.L.) undiluted	+ve
A9	" 1/4 dil.	+ve
A10	" 1/8 dil.	+ve
A11	" 1/16 dil.	+ve
A12	" 1/32 dil.	+ve
A13	" 1/64 dil.	-ve
B1	0.1ml sonicate + 0.1ml LPS (*E.coli*) @ 0.04ng/ml	+ve
B2	CIE gel + 0.1ml LPS (*E.coli*)	+ve
B3	0.1ml G.L. +ve by ELISA urine (undiluted) + 0.1ml LPS (*E.coli*)	+ve
B4	0.1ml R.F. -ve by ELISA urine (nt.) + 0.1ml LPS (*E.coli*)	+ve
B5	No. 43/ sonicate precipitin line from CIE + 0.1ml LPS (*E.coli*)	+ve

Table 17 cont.

C	Negative control (0.1ml endotoxin-free water)	-ve
	Endotoxin standard (calibrated vs. USP Reference Standard Endotoxin for the semi-quantitation of LAL assay. 2ug of E.coli LPS serotype no. 055:B5 (Activity = 6000 EU per ug) diluted in endotoxin-free water (Sigma).	
D	0.1ml LPS @ 0.08ng/ml	+ve
E	0.1ml LPS @ 0.04ng/ml	+ve
F	0.1ml LPS @ 0.02ng/ml	+ve
G	0.1ml LPS @ 0.01ng /ml	+ve
H	0.1ml LPS @ 0.005ng/ml	-ve

N.B. 0.1 ml of a working solution of concentrate from lysed amoebocytes of the horseshoe crab, Limulus polyphemus (E-Toxate) is added to every test.

KEY

+ve - Hard gel formed after 1 hr at 37°C

-ve - soft gel,turbidity or clear liquid after 1 hr at 37°C.

E.coli - Escherichia coli

(G.L.)/(R.F.) - patients initials

the detection of endotoxin.

IgG from four antisera (Nos. 43, 59, 67 and 141) was reacted with a fresh sonicate of L.pneumophila SG 1, Knoxville-1 harvested in endotoxin-free water (Baxter Health Care Ltd.). This was carried out in agarose and electrophoresed in buffer both made with endotoxin-free water. Where the precipitin lines had formed (observed without staining), these were cut out with a sterile scalpel and transferred to small test tubes (E-Toxa-Clean-treated, section 2.9), reacted with horseshoe crab amoebocyte extract and incubated at 37°C without disturbance for 1h. Controls included the CIE gel with no precipitin line and the sonicate itself as well as the E.coli LPS (serotype No.055:B5).

All the reaction lines suspected to be precipitated LPS antigen were positive in the in the LAL assay. The gels alone and the negative control where endotoxin-free water was added to the Limulus reagent were negative in the test.

Also to obtain a guide to the sensitivity of the ELISA system in its detection for urinary antigen, a boiled specimen of antigen-containing urine (G.L.) was used as a standard and diluted two-fold to 1/64. The E.coli LPS standard was diluted from a concentration of 0.08 ng/ml to 0.005 ng/ml.

The highest dilution at which a positive result was obtained with the standard LPS was 0.01 ng/ml and the

highest dilution of antigen-containing urine was 1:32.

If it is assumed that the sensitivity of the LAL assay is the same for both the antigen (LPS) in the urine and the E.coli standard LPS then the sensitivity of the ELISA might be estimated at detecting as little as approximately 0.02 ng/ml or 20 pg/ml antigen.

The negative controls were negative in the LAL assay including the urine from a healthy human and all the results shown in table 17 were reproduced three times.

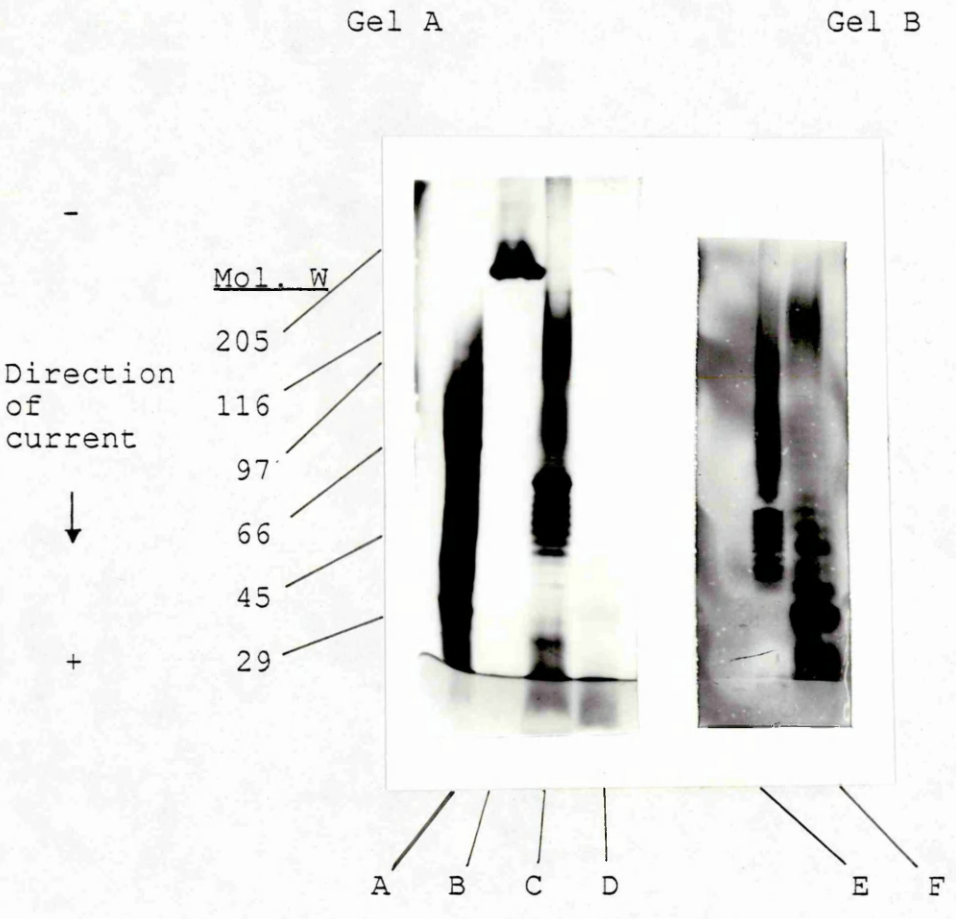
3.4.4 Probing immunoblots of "positive" urine and proteinase-K digests with capture antibody

To investigate the components present in ELISA-positive urine collected from a patient with Legionnaires' disease a sample was concentrated as described in section 2.13.1 and electrophoresed alongside Proteinase-K digested whole cells of L.pneumophila SG 1 (Knoxville-1) together with the molecular weight standards, SDS-6H (Sigma).

Positive (by ELISA) urine (Co.), seen in figure 21 gel B, produced a ladder-like profile similar to that expected from the analysis of a bacterial lipopolysaccharide , with areas of close banding similar to that of the L.pneumophila proteinase-K lysate. Gel A was silver stained for protein and gel B was stained for LPS. Also in gel B, in the area where < 29kD components were resolved at the base of lane F (loaded with the positive urine) a smear of undefined staining was observed. This was also observed

Figure 21 SDS-PAGE of proteinase-K digest of L.pneumophila SG 1 and "positive" urine.

Gel A, containing 10% SDS silver stained for protein and showing the electrophoretic profiles of proteinase-K whole -cell lysate in lane C, proteinase-K treated antigen -containing urine (Co.) concentrated x100 in lane D, molecular weight standards (SDS-6H, Sigma) in lane A and a proteinase-K standard in lane B. Gel B, containing 10% SDS was silver stained for LPS. Lane E contained proteinase-K whole cell lysate. Lane F contained the same sample as lane D.



in gel A in the same area but at the base of lane C (loaded with proteinase-K whole cell lysate).

A loopful of positive urine (Co.) was cultured on blood agar and incubated at 37°C for 48 h to exclude the possibility of other endotoxin-producing bacteria being present. No growth was detected.

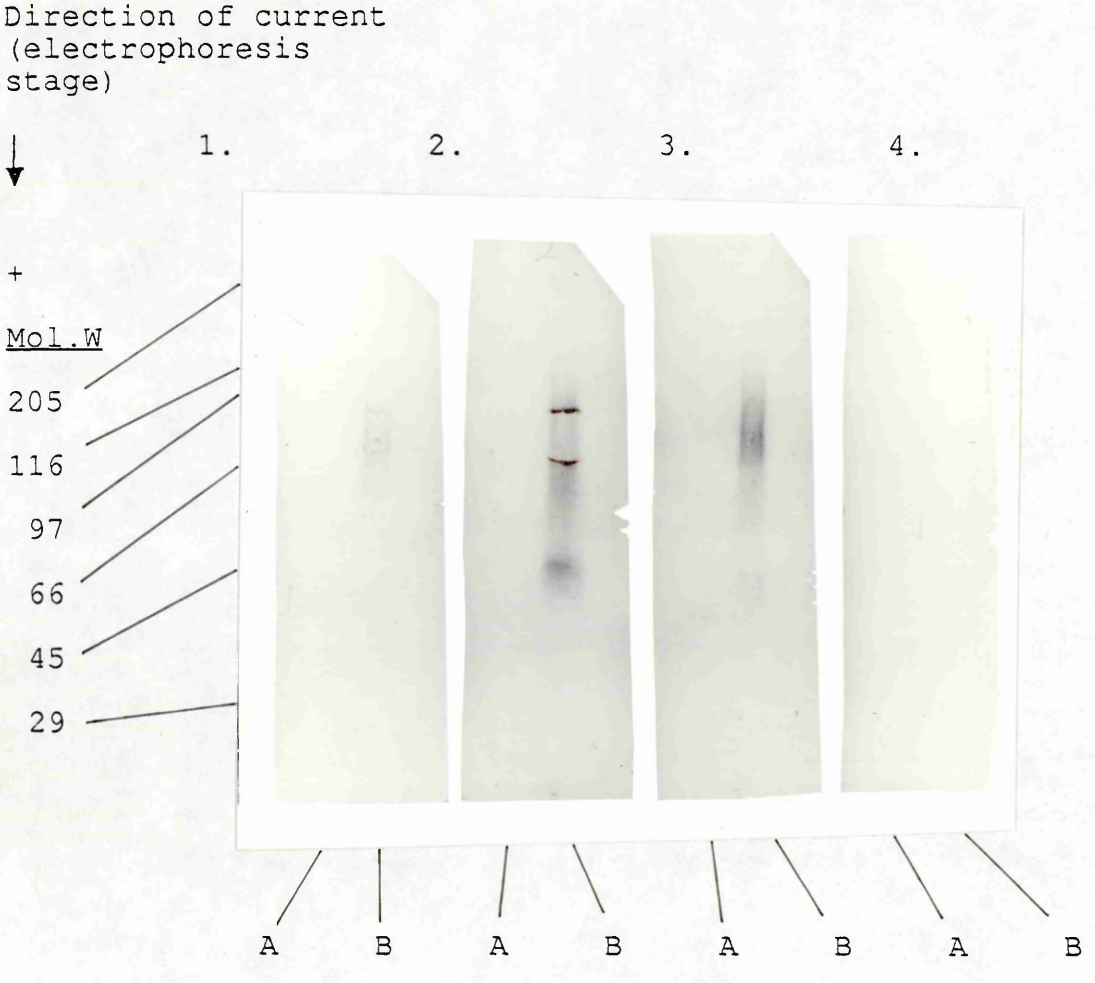
To determine with which antigens the antibodies raised in the animal protocols reacted, the concentrated (100x) ELISA-positive urine (Co.) as well as a whole cell proteinase-K lysate of L.pneumophila SG 1, as before, were prepared for analysis by SDS-Page. Lanes of the gel were loaded alternately with concentrated antigen-containing urine and whole cell proteinase-K lysate. These were transferred electrophoretically and immunoblotted individually against 4 different antisera effective in the ELISA test. These were No. 43, antiserum donated by Williams (raised by the Conlan and Ashworth method), McKinney antiserum and antiserum donated by Fehrenbach (raised by the method described by Kohler).

The results of the developed immunoblot where each of these antisera were reacted with the transferred material are shown in figure 22. None of the antisera stained blots from the positive urine lanes (Co.). However, faint staining was observed in three cases with the electrophoresed whole-cell proteinase-K lysate. Areas of development with No. 43, McKinney and the Williams antisera showed LPS-like

Figure 22 Immunoblot of 4 different CAbs with proteinase-K and "positive" urine.

Antibody pools (IgG) No.43 [1], Williams [2], McKinney [3] and Fehrenbach [4]) were reacted to proteinase-K digested whole-cell lysate of L.pneumophila SG 1, Knoxville-1 [B] and concentrated (x100) antigen-containing urine [A].

-



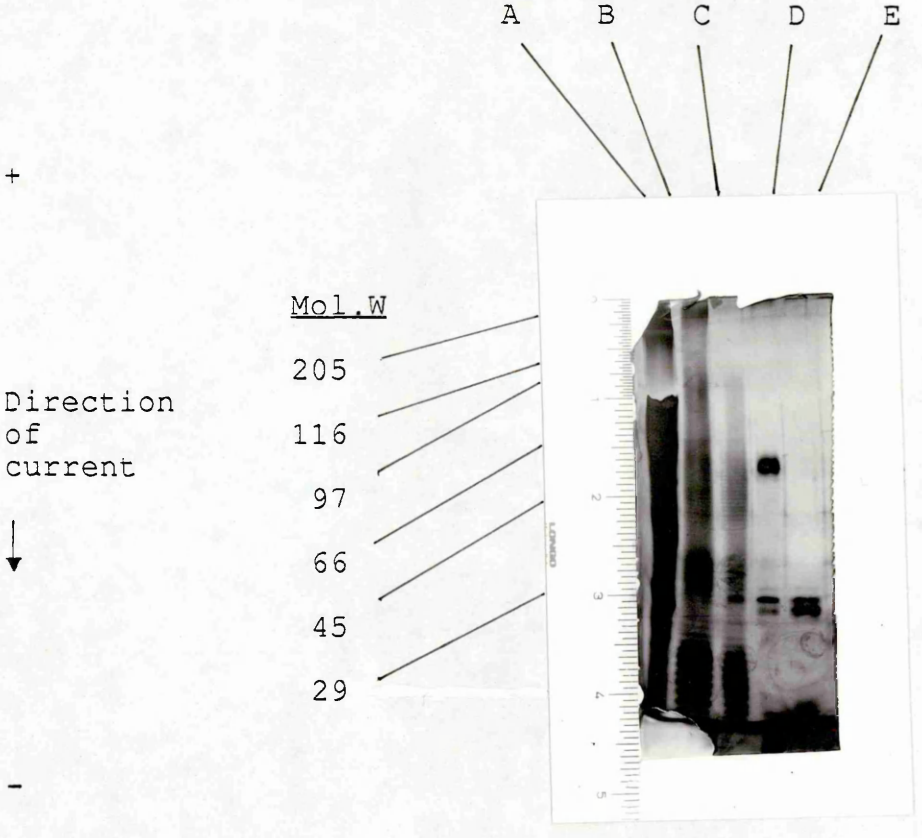
patterns staining more heavily in areas corresponding to close banding. Also, an additional two clear, dominant bands were observed with No. 43 and the Williams antiserum, which were less clear with the McKinney antiserum. The estimated molecular weights of the two immunodominant bands were 80 and 60 kD.

Figure 23 shows the SDS-Page gel where one positive and one negative urine (both proteinase-K treated) were compared to examine for any additional banding in the areas described which may indicate this to be the molecular weight of the urinary antigen detected by the ELISA. One band noted with the positive urine (Mill.) and absent from the negative urine was a band estimated to be 60kD. When the contents of this gel were transferred to nitrocellulose membrane and immunoblotted against both the McKinney and No. 43 antisera no binding of these antibodies was shown by staining. The urines were concentrated by the the Minicon method but no LPS-like profile was observed.

This experiment was repeated and extended by the examination of a further 12 specimens of urine of which 6 were positive for urinary antigen and 6 were negative (by ELISA). Of the 6 negatives, 3 were from healthy people and 3 were confirmed UTI's due to Escherichia coli, Klebsiella aerogenes and Proteus species respectively. All 12 urines were treated with proteinase-K at 56°C for 1h. The whole-cell proteinase-K lysate of L.pneumophila was

Figure 23 10% SDS-Page gel containing saline extract, "positive" and "negative" urines and silver stained for LPS.

Lane A contains molecular weight standards (SDS-6H), proteinase-K digested whole-cell lysate of L.pneumophila SG 1 in lane B, Saline extract I (non proteinase-K treated) in lane C, a positive urine (Mill.) in lane D and a negative urine (by ELISA), 89/00391 in lane E (both proteinase-K treated), both concentrated (x100).



overloaded to show the 60 and 80kD bands observed by the previous immunoblot (figure 24).

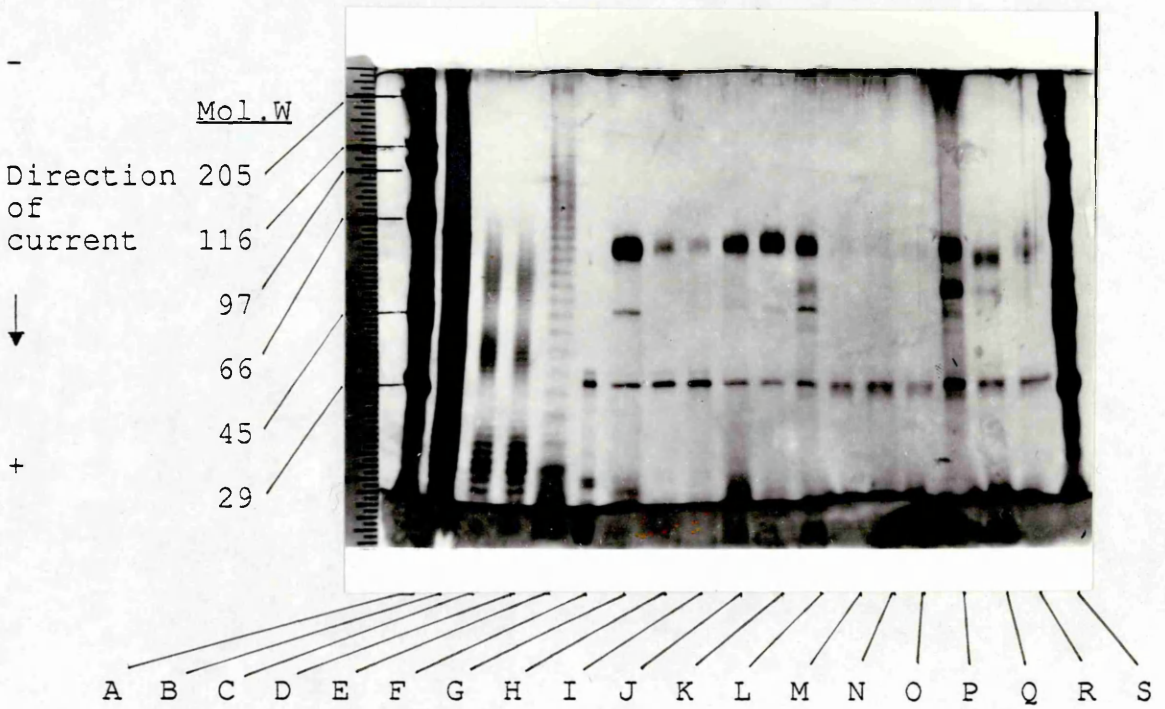
The saline extracts of L.pneumophila SG 1, before and after proteinase-K digestion were examined with no notable differences being observed between them. The predominant bands were similar to those seen in proteinase-K digests of whole cells. The control for the LPS-specific stain was a lane containing LPS (1ug) from S.minnesota (Sigma). Also, proteinase-K alone was electrophoresed so that common bands observed between the enzyme and the urines treated by this enzyme could be eliminated. Two or three bands seen in proteinase-K-treated urines were derived from the enzyme. Only enzyme-derived bands were evident in enzyme-treated negative urines.

Proteus and E.coli infected urines shared 2 common bands but an additional one was seen in the Proteus infected sample. Only one band was noted with UTI urine infected with K.aerogenes. All these urines were concentrated by the Minicon method (section 2.14.2) using 3% (w/v) SDS in the rinsing solution to extract the concentrate from the Minicon chamber. Still no LPS pattern was revealed by silver staining. When the urines were electrophoresed onto nitrocellulose and reacted with No. 43 and McKinney antibody at dilutions of both 1/100 and 1/50 and developed, again no staining was observed.

When samples of the same 12 urines, which were not

Figure 24 10% SDS-Page gel of 12 concentrated "positive" and "negative" urines silver stained for LPS.

The gel was loaded with molecular weight standards (SDS-6H) in lanes A and S; proteinase-K digested whole-cell lysate of L.pneumophila SG 1, Knoxville-1 in lane B, saline extract III (non-proteinase-K digested) in lane C, saline extract III (proteinase-K digested) in lane D, Salmonella minnesota LPS (1ug) in lane E, proteinase-K control in lane F, 6 positive (by ELISA) urines, Mill., Mun., McN., Co., Wat., and 84/86987 in lanes G to L respectively, 3 urines from healthy patients; 89/00382, 89/00390, 89/00391 in lanes M to O respectively and 3 UTI urines; 89/00279 (Proteus), 89/0063 (Eschericia coli and 89/00338 (Klebsiella aerogenes) in lanes P to R respectively where all urines were proteinase-K treated.



exposed to proteinase-K, were analysed by SDS-Page, bands (figure 24) identical in molecular weight to those found (to be unique) in the L.D. antigen-positive urines (figure 23) were seen. Therefore these characteristic bands may reflect incomplete digestion of urinary components common to these specimens. They could not be further differentiated by immunoblotting.

3.4.5 Affinity chromatography used to isolate urinary antigen and analysis by SDS-Page and immunoblotting

It was the purpose of this experiment to capture the antigen found in urine with the hope that it may be further charactrerised.

Cyanogen bromide (CNBr)-activated-Sepharose 4B was coupled with polyclonal rabbit antibodies raised in earlier experiments to capture the antigen in antigen-containing urine (tested by ELISA). IgG fractions from test bleeds No. 43 (category C antisera), 221 (category A antisera), 228 (category A antisera); all raised by the McKinney protocol and a positive control antisera, P1 (category C antisera donated by Fehrenbach and raised according to the method of Kohler, see section 3.1.5 (p93) for definitions.

Each of these antibody pools were reacted with enough CNBr-activated-Sepharose 4B to produce two columns. Four columns, each with bound antibodies from one of the pools, were loaded with positive antigen-containing urine (G.L.) and a parallel four were loaded with urine which was boiled

and centrifuged (from a healthy person). Samples of the eluate were taken after collection of 1ml, 10ml and 20ml of column effluent. These samples were then tested in duplicate by ELISA for unbound antigen. Also, before the antibodies were coupled to the CNBr-activated-Sepharose 4B, they were tested in the ELISA system against two different positive antigen-containing urines (Mill. and G.L.) as well as a negative urine (R.F.).

The ELISA results are shown in table 18 and the microtitre tray in figure 25. The controls consisted of both the positive (G.L.) and negative (R.F.) test urines where these were not exposed to any of the columns. Positive urine produced a mean $O.D._{492}$ of 1.189 and the negative urine, a value of 0.046 where these were tested in duplicate.

Where positive urines were passed down each of the four columns, binding was indicated by each antibody/Sepharose combination giving reduced $O.D._{492}$ results when the fractions at 1ml and 10ml were tested.

These $O.D._{492}$ values increased as more of the positive urine was passed down the column indicating saturation of the binding sites. The $O.D._{492}$ values and therefore the amount of antigen bound did vary between the antibody/Sepharose complexes. As this value for the P1/Sepharose started at a relatively high value of 0.777 (mean, $\bar{x}$) and rose to 1.189 (x) after 20ml had passed through the column, it suggested that relatively little

Table 18 ELISA assays of urine from Sepharose-4B/antibody columns.

	$O.D._{492}$ values of eluted urine (+ve) from columns			
Fraction No. (ml)	P1/+ve urine	43/+ve urine	221/+ve urine	228/+ve urine
1	0.780	0.155	0.687	0.091
1	0.774	0.106	0.497	0.072
10	1.089	0.051	1.054	0.261
10	1.085	0.049	0.916	0.276
20	1.190	0.087	1.120	0.434
20	1.188(f)	0.033	1.117(f)	0.414(f)

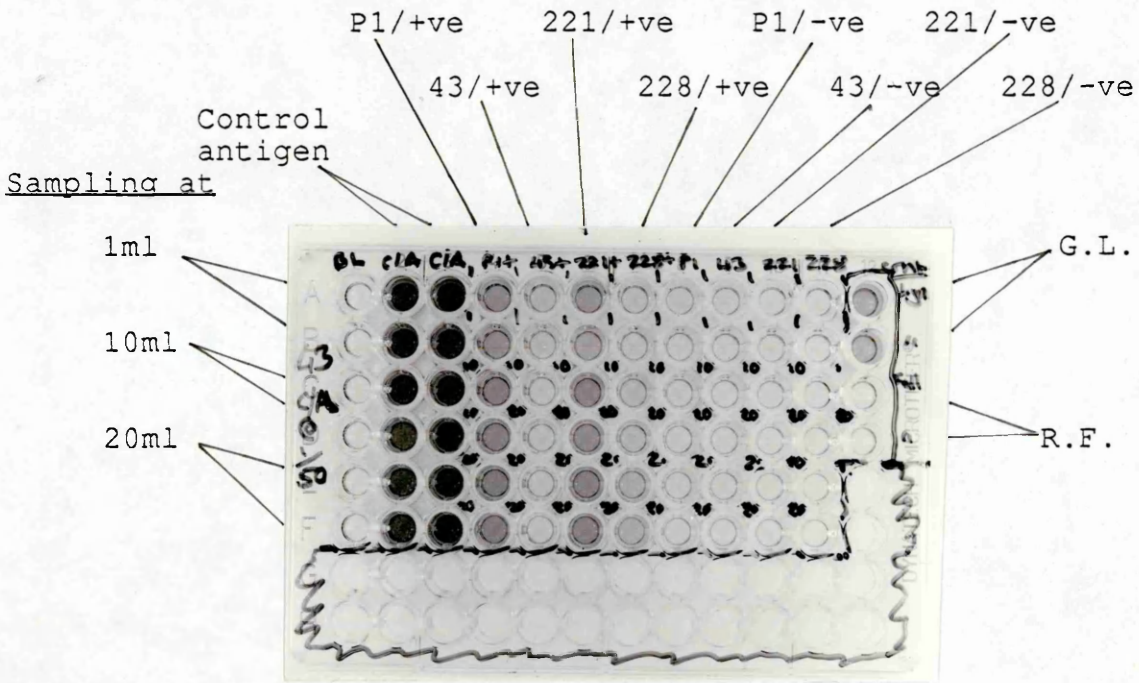
	$O.D._{492}$ values from eluted urine (-ve) form Ab/Sepharose 4B column			
Fraction No. (ml)	P1/-ve urine	43/-ve urine	221/-ve urine	228/-ve urine
1	0.074	0.067	0.035	0.050
1	0.055	0.041	0.039	0.047
10	0.030	0.026	0.024	0.039
10	0.019	0.029	0.017	0.008
20	0.034	0.041	0.017	0.008
20	0.052	0.035	0.013	0.003

Controls	$O.D._{492}$	Range	Mean ($\bar{x}$)	S.D.	n
Control antigen	-	2.131 -2.376	2.264	0.068	12
Positive urine (G.L.)	-	1.137 -1.241	1.189	0.073	2
Negative urine (R.F.)	-	0.034 -0.058	0.046	0.016	2

KEY (f) -free antigen eluted

Figure 25 ELISA testing of effluent collected from 8 affinity columns after 1, 10 and 20ml.

Samples of both positive (G.L.) and negative urine (R.F.) where these had not been exposed to the columns were also included in the ELISA as controls. Capture antibody was No.43 at a dilution of 1/50.



antigen was bound. This was probably due not to the lack of avidity of the antibody for the urinary antigen but because the antibody concentration in the coupling solution was very weak (3mg/ml protein). This is substantiated by the high $O.D._{492}$ values obtained using the P1 antibody in the ELISA at a concentration of 20ug/ml-the same concentration at which Nos. 43 and 59 are used in the ELISA for clinical purposes. The Sepharose/No. 43 complex captured relatively more antigen from the urine since the $O.D._{492}$ values were very low with the 1ml and 10ml fractions (these values approach those obtained with the negative urine controls). When No. 221 was tested for its ability to capture antigen in the ELISA, it produced low $O.D._{492}$ values with positive control urines which reflected the binding of antigen. The antibody coupled to Sepharose, also showed poor binding of urinary antigen as high $O.D._{492}$ values were detected in all eluate fractions by ELISA. When IgG from test bleed No. 228 was tested in the ELISA, high $O.D._{492}$ values were produced with both positive and negative urines showing poor specificity. When complexed with Sepharose, this IgG bound antigen from the positive urine as suggested by the ELISA results (table 18). When the equivalent columns were treated with urine devoid of antigen, $O.D._{492}$ values ranged from 0.019 to 0.067 which are background levels in this assay. These results are summarised in table 18.

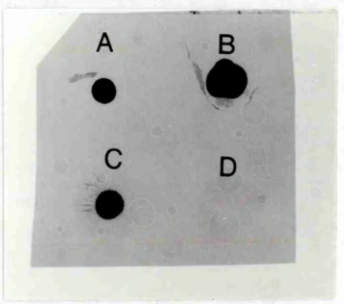
A sample of each antibody/Sepharose coupled mixture was

analysed directly after chromatography of urines by electrophoresis on 10% SDS-Page for bound urinary antigen and immunoblotted onto Immobilon-P or nitrocellulose membranes before being developed in antibody solutions of Nos. 43, 59, and McKinney each at dilutions of 1/50, 1/100 and 1/200. No staining developed using any of these antibodies at each of the dilutions, even with the Immobilon-P polyvinylidene difluoride membrane which is claimed to have increased protein binding properties, although level of LPS binding is unknown (personal communication, Millipore). This claim was substantiated by a test blot (figure 26) where 4 drops of different antigen solutions were spotted onto both Hybond-C (Amersham) and Immobilon-P (Millipore) membranes. These antigen solutions were T.S.E. (used as the positive control in the ELISA test), two saline extracts, one produced from L.pneumophila SG 1 (Knoxville-1), the other from the Corby strain of L.pneumophila SG 1 (donated by Williams) and concentrated (x100) urine, positive by ELISA. Only the first three antigen solutions reacted with both McKinney and No.43 antisera/donkey anti-rabbit IgG-HRP in this test blot (blot 1). No reaction was observed with the concentrated urine when either nitrocellulose or polyvinylidene fluoride (Immobilon-P, Millipore) membranes were used. Also in a similar experiment when the non-ionic detergent Tween 20 was included in the blocking buffer, the immunoblot reaction

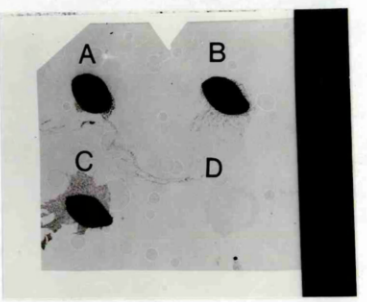
Figure 26 Test immunoblots where test antigens were reacted with different detecting antibody systems.

T.S.E.(A), saline extracts from Knoxville-1 (B) and from Corby-1 (C) strains of L.pneumophila SG 1 and concentrated (x100) urine (D) (Co.) were spotted onto two different membranes; nitrocellulose (1, Hybond-C) and polyvinylidene difluoride (2, Millipore). Blots 1a and 2a were treated with McKinney conjugate (1/200) before development and blots 1b and 2b were treated with No. 43 IgG (1/50) and then donkey anti-rabbit IgG-HRP (1/100) before development.

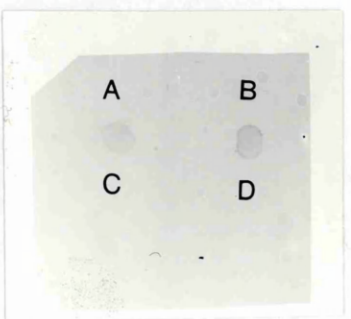
1a.



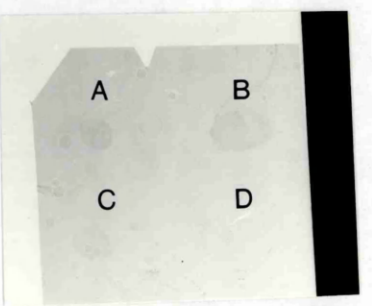
2a.



1b.



2b.



was very weak indicating some dissociation of the antigen from the membrane (results not shown). When the SDS-PAGE gel (figure 27) was silver stained for LPS after the transfer stage of immunoblotting, two bands were observed (50 and 80 kD) from samples of antibody/Sepharose complex taken from columns treated with positive urine. This pattern was also observed when SDS-PAGE was developed in silver stain with the omission of the immunoblotting stage.

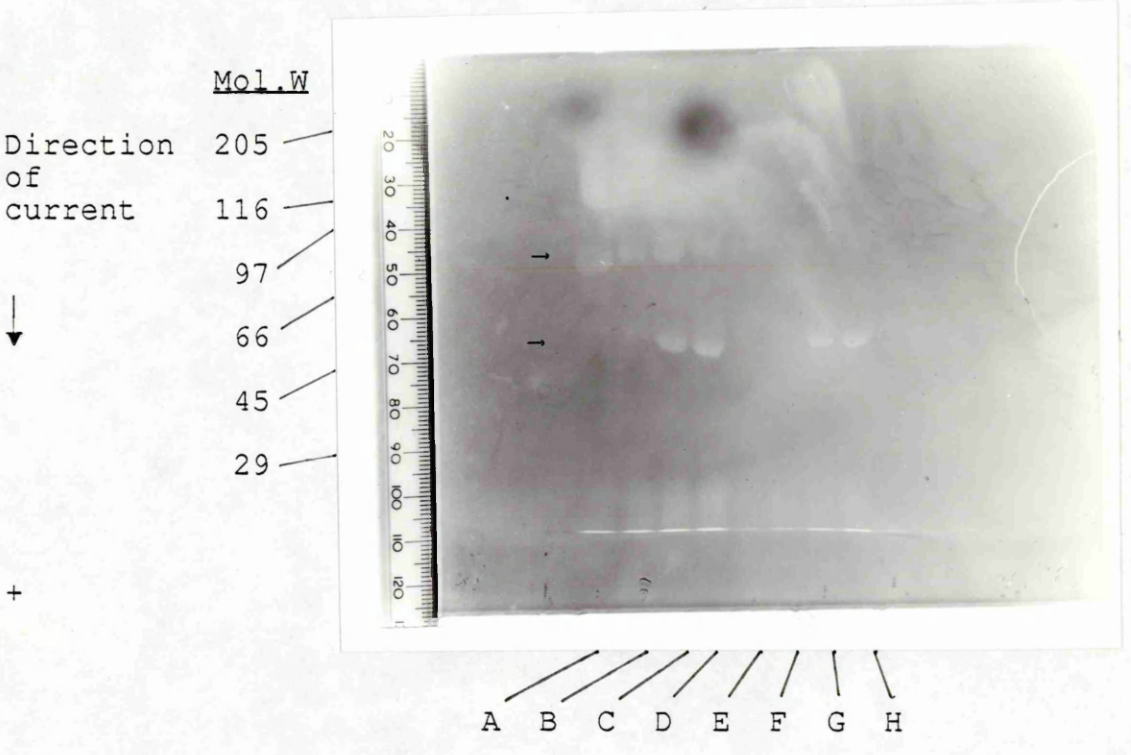
IgG is made up of Fc and Fab fragments and may explain why these two bands were observed when samples of antibody/ Sepharose/positive urine complex were analysed by SDS-PAGE. The combined effect of 2-mercaptoethanol, SDS and boiling may affect the structure of whole rabbit IgG to produce a fragment represented as one of these two bands. The heavier of the two bands is unique to Sepharose loaded with positive urine and it may be that this is related to the antigen found in urine. Highlighted bands were also noted at the buffer interface of the SDS-PAGE gel at approximately 20kD but these were common to both positive and negative urine-treated columns.

When samples were prepared in buffer without mercaptoethanol no bands were observed in the gel.

A further experiment to determine whether either of the two bands (60 and 80kD) was related to rabbit IgG was done. The above experiment was repeated but with an additional lane containing control rabbit antibody (No. 43). The gel

Figure 27 10% SDS-PAGE gel of components bound by affinity columns loaded with "positive" and "negative" urine.

The gel silver stained for LPS was loaded as follows: Lanes A to D were loaded with P1/, 43/, 221/, and 228/Sepharose 4B treated with positive urine (G.L.) and lanes E to H with P1/, 43/, 221/, and 228/Sepharose 4B complex treated with non-antigen-containing urine (R.F.).



was immunoblotted and developed with Donkey anti-rabbit IgG (SAPU) at a dilution of 1/100. The anti-rabbit IgG reacted only with two bands (50 and 55kD) in the lane where No. 43 control rabbit antibody had been loaded (figure 28). No reaction with any components of the other samples was observed.

3.5 Urinary antigen excretion profile of a patient with classical Legionnaires' disease

This case history is included as an example of the successful use of the ELISA where it produced evidence of infection with L.pneumophila SG 1 before antibodies manufactured by the patient's immune system were detectable and before any organisms were cultured by the investigating laboratory.

This patient (J M^{c}M), a male of 47 years who had a history of smoking, developed asthma in 1984 and who used an inhaler, returned prematurely from holiday in Gran Canaria with a one week history of febrile illness, cough rigors every hour, aches and pains and generalised lethargy. He was admitted to Ruchill Hospital where he was noted to be confused and febrile with diarrhoea and weakness together with consolidation of the right lung, demonstrated by x-ray. Specimens of sputum, blood and urine were taken for culture, IFA testing for antibodies and ELISA testing for antigen respectively.

Figure 28 Immunoblot stained with Donkey anti-rabbit-HRP antibody.

Figure A shows a 10% SDS-PAGE gel where Lane A was loaded with molecular weight markers SDS-6H (Sigma). Figure B: Lane B of the stained blot contained No.43 rabbit IgG. Lane C was loaded with No. 43/Sepharose/+ve urine complex, Lane D with P1/Sepharose/+ve urine complex, Lane E with No. 43/ Sepharose/-ve urine complex and Lane F with P1/Sepharose/-ve urine complex.

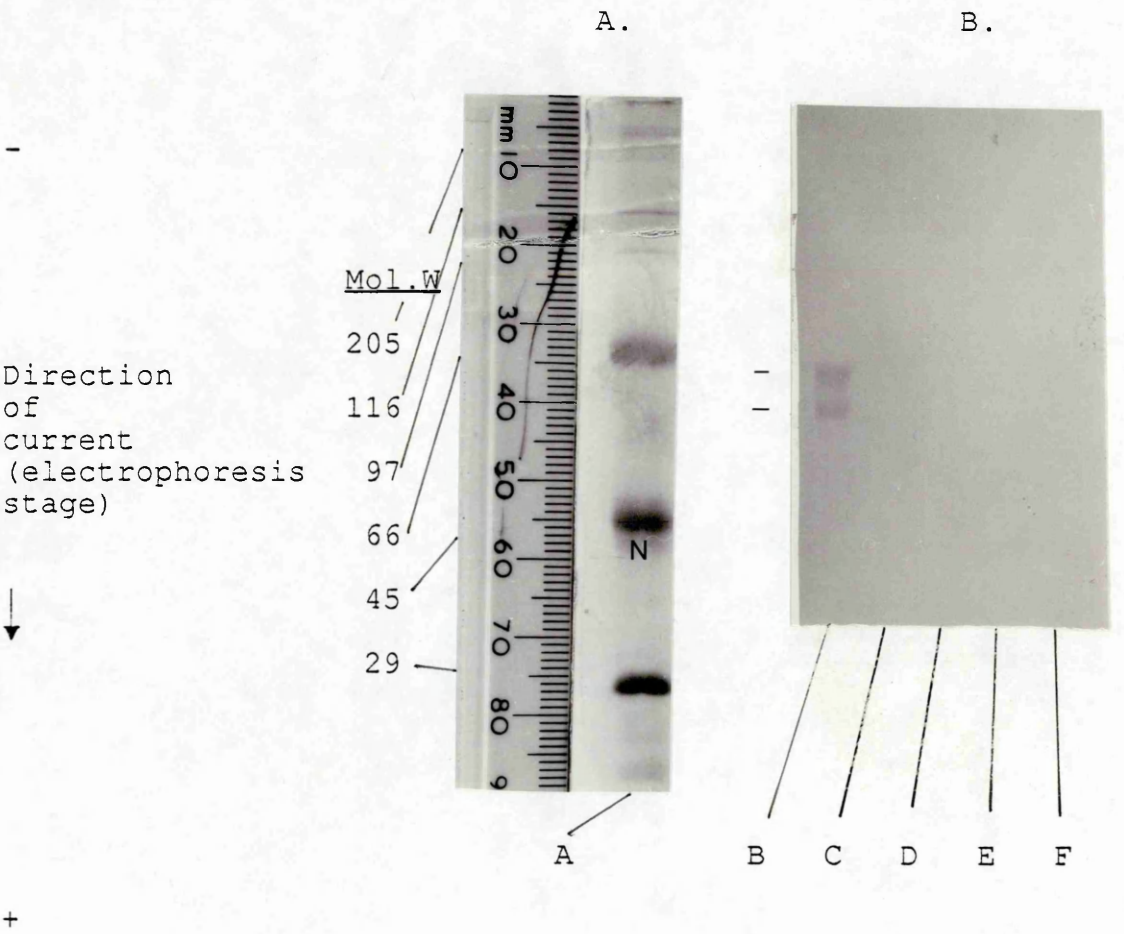


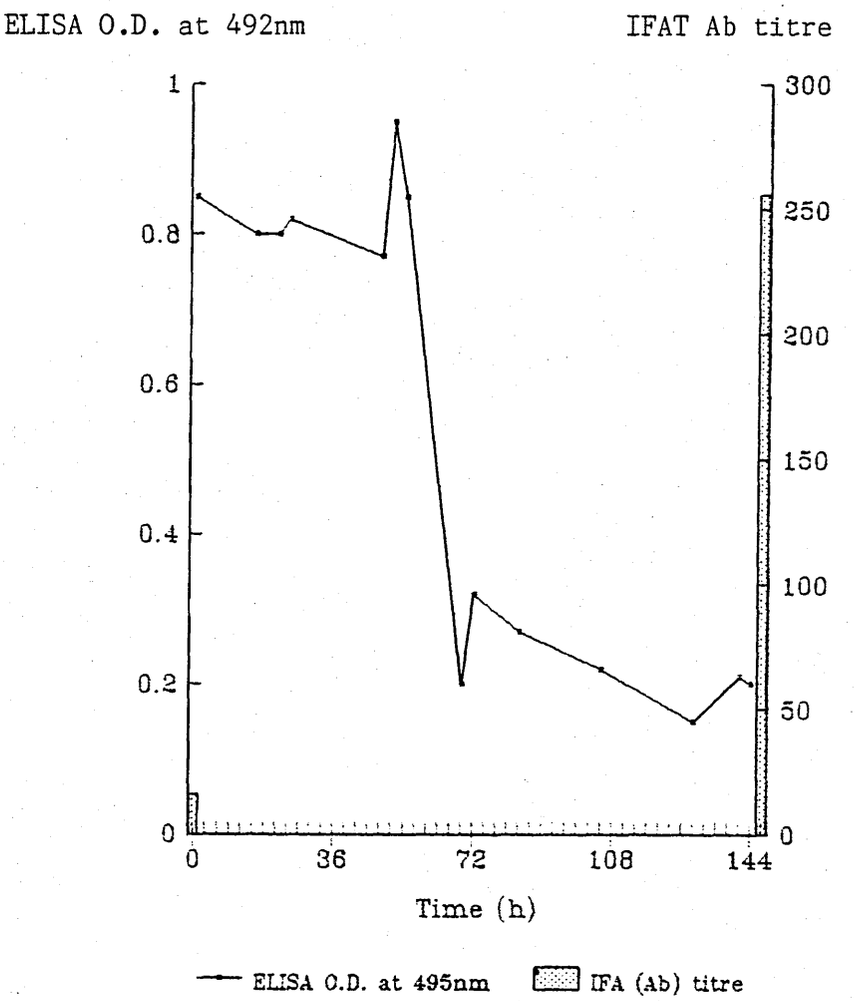
Figure 29 shows, in graphical form, the results of tests for urinary antigen from specimens voided every few hours. IFAT results of the patient's serum taken 8 days later on testing for antibodies to Legionella resulted in a positive four fold rise in titre to 256.

Initially the patient's urine tested positive with a high $O.D._{492}$ i.e. > 0.8. No organisms had grown at this early stage nor was antibody to L.pneumophila detectable at a dilution of < 16 fold (i.e. negative).

Erythromycin (500mg) was administered to the patient. Intravenous erythromycin was continued and oral rifampicin therapy introduced. The patient's condition improved three days later, although urinary antigen was still detected. On this third day after admission and with antibiotic therapy given the level of urinary antigen dropped significantly to an $O.D_{492}$ of 0.2 although this rose slightly hours later. This $O.D._{492}$ of 0.2 is technically positive as it was > 2 times $O.D._{492}$ of a negative control urine.

On the fourth day after admission, the route of erythromycin therapy was changed from IV to oral. The patient improved now having low grade pyrexia and tiredness. Seven days after admission L.pneumophila SG 1, Pontiac-1 strain was isolated from sputum. On the following day (i.e. eight days after admission) the patient's antibody

Figure 29 Urinary antigen excretion profile of a patient with L.D. showing IFAT results obtained on the date of admission (Time 0h) and 8 days later.



titre by IFAT was found to be 256 to L.pneumophila SG 1. The patient was eventually discharged 10 days after admission on oral ciprofloxacin. No further specimens of urine were tested after the patient was discharged.

SPECIES IDENTIFICATION

Fatty acid extracts from the Legionellaceae analysed by GLC

Fatty acids are important components of bacterial cell membranes and form the long hydrophobic tails of lipid, mainly phosopholipid molecules. They are released as free acids from the cell envelope upon hydrolysis and are generally 10-20 carbon atoms in length and have structures illustrated in figures 82-85 (p205-206).

In the unsaturated fatty acid the double bond(s) occurs near the middle of the backbone of the molecule. These structures may be represented in shorthand notation e.g. n16:1. The n = normal or straight-chained carbon backbone (other possibilities are a (anteiso) and i (iso) where these refer to the position of the subterminal CH_3 group), 16 = No. of C atoms and :1 = no. of double bonds within the molecule. It is the total complement of fatty acids which can be used to identify organisms at the genus and even at the species level.

FA's can be analysed using thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas-liquid chromatography (GLC). The latter is the most commonly used and the most convenient method to analyse the

complex mixture of FA's found in the Legionellaceae. Derivatives of the FA's are made (methyl esters) prior to chromatographic analysis as the carboxyl group is polar and would otherwise retard the progress of the molecule through the GLC column. The resultant peak "tailing" would obscure other peaks. Also, resolution is improved as a result of increasing the volatility and stability by methyl esterification of the FA.

Such derivatives have been used in the routine analysis of Legionella FA's.

3.6.1 Derivitisation of fatty acids

Methyl esters can be prepared by several methods, most of which are rapid and simple. All methyl esters of FA's in this thesis are produced by the acid methanalysis method.

Other methods, e.g. alkaline saponification procedure suffer from disadvantages where the release of amide linked acids may be incomplete (Jantzen et al, 1978) and unsaturated compounds may be artefactually produced from O-substituted 3(OH) acids (Reittschel et al, 1972), although hydroxylated acids are present in only small quantities in the legionellae.

Acid methanalysis has only one disadvantage in that cyclopropane acids may be degraded (Lambert and Moss, 1983). As cyclopropane 17 is a component of Legionella, and was found to vary in quantity between species, it was important to preserve it in the derivitisation procedure.

Most published methods recommend transesterification at 80°C but to prevent destruction of cyclopropane FA's the temperature was reduced to 54°C where quantitative analysis produced detectable and consistent quantities of cyc 17. The FA's of the Legionellaceae have been studied extensively here where variation and reproducibility has been investigated under a range of different conditions.

3.6.2 Technical points

Gas-liquid chromatography using capillary columns is considered the analytical method of choice. The vitreous silica phase is immobilised by cross-linking thus reducing column "bleed" (loss of liquid phase from the column) and increasing column life.

The use of a capillary column excludes the use of N_2 as a carrier gas as its optimum efficiency is attained only at low linear velocities (the linear velocity in cm s^{-1} ($\bar{u}$) is given by $\bar{u} = L / t_m$ where L is the column length in cm and t_m is the retention time of methane in seconds) leading to long analysis times. Both He and H_2 produce maximum chromatographic efficiency at higher linear velocities (20-30 cm s^{-1} for He, and slightly higher for H_2) and when increased beyond this range of optimum velocity their separating abilities are only slightly reduced. Analysis times with longer columns are therefore only slightly increased. Although He is more expensive than H_2 it is safer to use.

A split injection system where only a fraction of the total sample is introduced onto the column was employed. This is because a much higher flow rate can be maintained through the injector than through the column so that the sample is flash vaporised by the high temperature of the injector (270°C) and swept onto the column as a narrow band. This is then separated by the column phase as the sample migrates down the length of the column where the FA's are finally detected at the end of the column by the hydrogen flame ionisation detector (FID).

FID-type detectors respond linearly over several orders of magnitude of sample concentration and also respond to nearly all organic compounds with similar sensitivity. These are therefore the detectors of choice for FA detection and quantitation.

3.6.3 Calculation of results

In these studies all the FA profiles were obtained by GLC analyses (whose temperature conditions were optimised by experimentation) operated under identical conditions.

As part of the study, other factors were varied (e.g. time of incubation of the organisms, growth media, different batches of the same medium type) to examine their effect on the FA profile.

Data produced via the Chromatopac CR6A (Shimadzu) integrator was in the form of a chromatogram accompanied by figures relating to the following parameters: the retention

time (R.T.) of each peak (or a fluctuation detected by the FID) the area under each peak during the 35 min program and the total area under the curve.

For every chromatogram produced, 0.5% of the total area was calculated. Each peak >0.5% of the total area was identified (by comparison of its R.T. with known peaks of an L.pneumophila standard and other commercial bacterial FAME standards [Supelco, Sigma]) where possible and its individual peak area noted. The area of these individual peaks were summed (corrected total area, this removes the areas for the solvent peak, and any contamination peaks and other peaks <0.5% of the total area under the curve). Then each peak (>0.5%) was individually divided by the corrected total area and the resultant figure was = Mole % nonhydroxy fatty acid composition, for each fatty acid >0.5%.

All legionellae (unless stated) were analysed in this way at least three times and each fatty acid (>0.5%) for each profile entered into calculations to determine its mean ($\bar{x}$) and its standard deviation (S.D.). from the formula:

$$\text{S.D. } (n-1) = \sqrt{\frac{\text{Sum of } (x_i - \bar{x})^2}{n-1}}$$

Experiments were performed to determine the optimum

conditions for reproducibility of FA profile under which the legionellae should be grown before FA extraction, methylation and analysis. Once these conditions were established they were adopted as the "standard conditions" for growth of the organisms for analysis. Strict adherence (unless stated) to these conditions, described below, was necessary for the consistency of results so that comparisons could be drawn up between any environmental or patient isolate and the library of all described Legionella species including proposed and possible new species, described to date.

3.6.4 Analysis of fatty acid after growth of legionellae on BCYE after 24 and 96h

Two legionellae, L.pneumophila and L.cincinnatiensis were grown on the same batch of BCYE for incubation times of 24 and 96h at 37°C and the FA's analysed by GLC.

Table 19 shows the mole % nonhydroxy fatty acid composition of these two organisms grown after 24 and 96h.

It was observed that FA's may vary both qualitatively and quantitatively with the length of incubation. L.pneumophila produced some n19:0 and n20:0 but no a17:0 after 24h incubation whereas at 96h incubation no n19:0 or n20:0 but some a17:0 was evident. This latter FA profile is similar to that previously published (Wait,in Harrison and Taylor, 1988) for this organism.

After 96h incubation, L.cincinnatiensis produced n19:0

Table 19 Mole % nonhydroxy fatty acid profiles of legionellae after 24 and 96h growth on BCYE agar.

Legionella species	Mole % nonhydroxy fatty acid composition by gas-liquid chromatography *																
	i14:0	i15:0	a15:0	n15:1	n15:0	i16:1	i16:0	n16:1	n16:0	a17:1	i17:0	a17:0	cyc 17	n17:0	n18:0	n19	n20
L. pneumophila SG 1 (Knoxville-1) strain @ 24 h	3.1	-	12.3	-	-	1.4	32.1	19.2	10.3	-	-	-	10.3	-	4.1	2.9	4.4
L. pneumophila SG 1 (Knoxville-1) strain @ 96 h	5.6	-	14	-	-	3.5	40.3	10.9	5.7	-	-	9.9	6.4	1.1	2.6	-	-
L. cincinnatiensis 70-OH-H strain @ 24 h	5.8	-	14.9	2.2	1.9	-	20.9	23.5	15.4	-	-	7.1	3.7	1.4	3.1	-	-
L. cincinnatiensis 70-OH-H strain @ 96 h	9.8	-	19.6	2.2	1.4	-	26.1	17.9	7	-	-	7.5	2.7	1.3	2.5	0.9	1.1

* Figures are the mean for two different growth experiments for each time.

and n20:0 in addition to those FA's observed after 24h growth.

The growth of most legionellae on BCYE was limited in amount after 24h incubation. Cultures were therefore harvested at 96h incubation where there was no further growth detected, even with slow growing species.

3.6.5 Analyses after growth on different media

FA profiles produced by a number of legionellae grown on two different media were investigated.

FA profiles of cells grown on Oxoid BCYE, which was found to be particularly useful in the primary isolation of legionellae (unpublished observations), was compared with FA profiles of legionellae grown on BCYE.

Table 20 shows the FA content of 7 Legionella strains isolated in Australia (Wilkinson et al., 1990) including one proposed new species, L.adelaidensis, and 6 possible new species (A/36, B/86, C/449, D/500, D/532 and E/594).

Only minor qualitative differences between the same strains grown on the two media were observed perhaps supporting the concept that the organisms are in the stationary phase after 96h incubation. However, there were major quantitative differences evident when different strains are compared e.g. i16:0 in A/36, a15:0 in E/594).

Other strains (e.g. 86/35784, an environmental isolate, table 23) showed no qualitative and only small quantitative differences of up to 5% between FA profiles when grown on

Table 20 Effect of growth medium on FA profiles of legionellae.

* Legionella species (Australian strains)	Mole % nonhydroxy fatty acid composition by gas-liquid chromatography															No. of analyses
	i14:0	i15:0	a15:0	n15:1	n15:0	i16:0	n16:1	n16:0	a17:1	i17:0	a17:0	cyc 17	n17:0	n18:0	n19:0	n20:0	
A/36 (Oxoid-grown)	-	-	15.2(1.3)	-	-	21.1(0.7)	11(1.3)	6.7(0.7)	-	1.7(0)	36.4(1.6)	-	1(0.2)	1.8(0.4)	-	5.1(2.6)	3
B/86 "	0.5(0)	-	1.7(0.1)	2.1(0.1)	1.2(0.1)	15.4(0.1)	15(0.7)	14.6(0.5)	-	-	4.7(0.1)	19.3(0.2)	6.4(0.1)	11.4(0.3)	3.9(0.3)	3.2(0.3)	3
C/449 "	-	-	7.5(0.2)	1.7(0.2)	1.0(0)	15.3(0.4)	24.5(0.1)	8.1(0.2)	-	0.9(0.1)	25.4(0.2)	-	3.8(0)	6.4 (0.2)	2.3(0)	3.2(0.2)	3
D/500 "	6.8(1)	-	5.4(0.7)	2.6(0.3)	3.6(0.4)	16.5(5.9)	23.4(0.1)	25(1.6)	-	1.6(0.2)	-	-	2.0(0.1)	5.6(0.7)	2.0(0.1)	6.1(0.5)	3
D/532 "	7.4(0.3)	-	5.4(0.3)	3.4(0.1)	3.9(0.1)	10.5(0.4)	25.6(0.2)	24(0.5)	-	-	1.2(0.1)	-	1.8(0.1)	5.7(0.5)	2.1(0.1)	9.1(1.4)	3
E/594 "	1.1(0.2)	1.4(0.3)	40.7(0.7)	-	-	9.5(1)	6.2(0.3)	4.5(0.5)	-	1.5(0.1)	24.9(0.6)	3.9(0.2)	-	-	-	6.3(1.2)	3
F/636 " (L.adelaidensis)	3.1(0.2)	-	5.6(0.5)	3.6(0.1)	6.5(0.3)	7.3(0.7)	24.2(0.7)	28.6(0.6)	-	-	3.5(0.4)	-	2.3(0.3)	4(0.3)	1.5(0.3)	9.8(1.1)	3
A/36 (BCYE-grown)	-	-	9.1 (1.1)	-	-	10.2(1)	19.6(1)	14.1(1.2)	-	2.1(1)	29.2(2.8)	-	1.3(0.2)	6.2(0.7)	3.1(1.6)	5.2(0.9)	3
B/86 "	0.8(0.1)	-	2.6(0)	2.5(0.1)	1.2(0.1)	20.5(0.2)	14.9(0.2)	12.9(0.2)	-	-	5.8(0.1)	16.7(0.1)	6.3(0.1)	9.6(0.2)	3.6(0.2)	2.6(0.2)	3
C/449 "	-	-	7.1(0.5)	1.1(0.2)	1.4(0.2)	4.1(0.8)	29.9(0.9)	23.4(0.5)	-	1.3(0.1)	22.3(1.3)	-	3.1(0.5)	6.3(0.5)	1.1(0.1)	1.1(0.2)	3
D/500 "	7.6(0.3)	-	13.4(1.5)	3.4(0.2)	3.1(0.1)	16.6(0.1)	20(0.4)	16.8(0.6)	-	-	5.4(0.6)	-	1.9(0.1)	4.3(0.2)	2.3(0.1)	5.2(0.2)	3
D/532 "	2.4(0.2)	-	7.6(0.3)	1.7(0.1)	2.2(0.2)	6.7(0.3)	25.6(2.1)	30.9(2.7)	-	-	2.7(0.3)	-	2.0(0.1)	8.4(0.4)	2.1(0.2)	7.6(0.5)	3
E/594 "	1.8(0.2)	1.3(0.3)	23.8(4.9)	-	-	4.4(1.2)	8.7(3.6)	19(3.6)	-	1.1(0.3)	13.2(4.0)	2.8(2.0)	1.0(0.3)	13.3(4.8)	5.9(1.4)	3.8(2.9)	3
F/636 (No growth) (L.adelaidensis)																	0

* Proposed and possible new species (Wilkinson et al. (1990).

L.adelaidensis - proposed new species "

Figures are presented as mean values with the standard deviation shown in brackets.

Oxoid or "in-house" BCYE media.

This is within the limits observed for some FA's produced by a single species grown on the same media. As the production of in-house BCYE could be carefully controlled (i.e. Oxoid came as a preconstituted dehydrated powder) and was less expensive to produce, it was the medium of choice on which the legionellae were grown for analysis. It was only during the course of the production of the library of FA profiles that three Legionella species (L.adelaidensis, L.geestiae and 86/35784 after passaging) failed to grow on "in-house" BCYE. Their profiles after 96h growth on Oxoid BCYE at 37°C are therefore included (tables 20, 21 and 23 respectively).

3.6.6 In-house BCYE batch-induced differences of fatty acids

Different batches of BCYE (using identical ingredients) were used to culture various serogroups of L.pneumophila (SG2, SG3, SG6 [Chicago-2 strain] and SG10) to examine any differences in FA profiles due only to the BCYE batch. All the strains tested (table 22) showed only minor differences when grown on different medium batches which were no greater than differences observed between the same strains grown on the same batch of medium on different occasions. In addition the differences were less than the variations observed between different isolates of the same species.

3.6.7 Fatty acid library of the Legionellaceae

Once the standard conditions for the preparation of

Table 21 Mole % nonhydroxy fatty acid composition of Legionella species grown under standardised conditions.

Legionella species	Mole % nonhydroxy fatty acid composition of the Legionellae by gas-liquid chromatography															No. of analyses
	i14:0	i15:0	a15:0	n15:1	n15:0	i16:1	i16:0	n16:1	n16:0	a17:1	i17:0	a17:0	cyc 17	n17:0	n18:0	
L. anisa	4(0.4)	-	25.2(2.9)	1.6(0.4)	1.7(0.3)	-	22.4(1.2)	9.7(1.9)	9.3(0.6)	-	-	9.1(0.2)	12.5(4.6)	2.7(0.3)	2.6(0.2)	3
L. birminghamensis	2.3(0.2)	1.8(0.5)	30(2)	3.5(1.2)	3.4(1.7)	-	24.7(0.5)	9.4(2.6)	5.8(0.7)	-	-	-	16.8(3)	1.5(0.4)	0.7(0.1)	4
L. bozemanii SG 1	4.1(0.3)	-	33.9(5.6)	2.3(0.5)	2.4(0.8)	-	20.7(2.2)	8(1.4)	5.3(0.7)	-	-	14.1(1.3)	5.4(0.1)	4.2(0.8)	0.7(0.1)	3
L. bozemanii SG 2	3.7(1.2)	-	29.8(3.3)	1.4(0.9)	4.1(2.6)	-	15.6(3.3)	6.7(0.9)	8.8(1.8)	-	-	8.8(2.5)	14.6(3.2)	5.1(1)	1.9(0.8)	5
L. brunensis	1.2(0.2)	2.6(0.7)	41.2(1)	-	1.7(1)	-	11.3(0.9)	7.9(1.2)	3.9(0.5)	-	-	25.5(0.5)	1.2(0.2)	2.2(0.6)	-	3
L. cherrii	6.2(0.7)	-	29.1(3.5)	-	-	-	30.3(1.5)	9.5(2.8)	5.2(2.5)	-	-	11.8(1.1)	4.5(1)	1.8(0.2)	1.4(0.3)	3
L. cincinnatiensis	10.5(0.4)	-	21.4(1.8)	3.2(0.5)	1.2(0.1)	-	26.8(3.6)	19.1(2.1)	6.1(0.5)	-	-	7.8(0.1)	2.2(0.7)	1.8(0.7)	2.6(0.1)	3
L. dumoffii	2.5(0.1)	-	36.4(0.8)	3.2(0.9)	-	-	18.4(0.3)	8.6(0.6)	3(0.3)	-	-	18.9(1.6)	3.8(1.4)	5.2(1.3)	-	3
L. erythra	0.6(0.1)	-	12.1(0.8)	2.2(0.2)	2.4(0.1)	-	6.4(0.2)	35.9(0.4)	23(0.5)	-	-	11.5(0.2)	-	2.6(0.3)	3.8(0.4)	3
L. feeleii SG 1	3.4(0.5)	-	21.3(2.4)	2.8(0.8)	4.1(0.8)	2(0.3)	18.6(3.8)	21.6(1.9)	17.3(5.1)	-	-	5.9(1.1)	-	0.9(0.1)	1.2(0.2)	3
L. feeleii SG 2	3.3(0.7)	-	20.8(0.9)	2.6(0.4)	1.9(0.4)	1.9(0.6)	16.6(5.1)	27.6(2.8)	15.1(4.7)	-	-	7.6(0.8)	-	1.1(0.1)	1.7(0.4)	3
L. geestiae *	1.1(0.1)	44.6(3.1)	16.4(6.3)	-	-	-	2.6(1.3)	13.8(3.9)	6(2.8)	-	6.1(3.7)	6.9(1.6)	-	2.1(1.6)	2(1.8)	5
L. gormanii	6.1(0.1)	-	22.5(1.2)	2.9(0.4)	4.3(0.5)	-	22.3(1.6)	6.9(0.2)	8.7(0.6)	-	-	8.1(0.1)	13.3(0.5)	3.8(0.5)	1(0)	3
L. gratiana	10.4(0.2)	-	11.2(0.2)	2.7(0.1)	1.2(0.1)	0.5(0.1)	33.6(0.2)	20.7(0.2)	6.9(0.2)	-	-	2.5(0.1)	9.3(0.6)	-	1.2(0.1)	3

* Grown on Oxoid BCYE

Figures are presented as mean values with the standard deviation shown in brackets.

Table 21 cont.

Legionella species	Mole % nonhydroxy fatty acid composition of the Legionellae by gas-liquid chromatography															No. of analyses
	i14:0	i15:0	a15:0	n15:1	n15:0	i16:1	i16:0	n16:1	n16:0	a17:1	i17:0	a17:0	cyc 17	n17:0	n18:0	
L. hackeliae SG 1	3.4(0.1)	3.2(0.1)	40.4(5.3)	2.5(0.1)	1.6(0.1)	-	18.2(0.2)	9.4(0.2)	5.9(2.3)	-	-	13.9(1.5)	5.4(0.5)	-	1.2(0.2)	3
L. hackeliae SG 2	1.9(0.1)	3.4(0.1)	37.8(3.4)	1.5(0.1)	1.3(0.1)	-	12.6(0.8)	13.2(0.4)	8.3(0.5)	-	-	15.6(0.5)	5.5(1.4)	-	1.5(0.4)	3
L. israelensis	3.2(0.1)	-	32.8(0.8)	-	-	2.5(0.5)	21.5(1.4)	7.5(1.1)	6.3(0.4)	-	-	14.9(0.6)	5.8(2.5)	-	-	3
L. jamestowniensis	2.1(0.3)	-	32.4(0.3)	-	-	3.3(0.3)	23.2(0.3)	4.4(0.5)	4.3(1.4)	2.9(0.2)	-	21.3(0.8)	3(1.1)	-	-	3
L. jordanis	1.9(0.1)	-	52.9(2.1)	-	-	-	17.9(1.3)	3(0.3)	1.3(0.3)	1.1(0.1)	-	18.8(0.6)	3(1.1)	-	-	3
L. londiniensis	1.9(0.5)	-	33.7(0.6)	3.5(0.1)	2.8(1.4)	-	14.6(0.6)	15.7(1.1)	10(1.1)	-	-	10.8(1.6)	3.2(1.7)	0.9(0.5)	3.3(0.6)	4
L. longbeachae SG 1	6.2(0.3)	-	17(1.3)	1.6(0.1)	6(0.2)	-	28.9(0.7)	18.8(1.9)	6(0.5)	-	-	9.6(1.7)	8.3(4.8)	1.8(1.2)	-	3
L. longbeachae SG 2	7.2(0.8)	-	18.5(1.2)	1.2(0.3)	-	-	31(2.7)	20.3(0.7)	5.4(1)	-	-	9.2(0.1)	10.3(0.3)	2(1.7)	-	3
L. maceachernii	-	-	30.1(0.4)	3.6(0.4)	2.3(0.4)	2.2(0.6)	8.4(1.2)	16.2(1.1)	7.5(0.2)	5.2(0.4)	-	21.7(0.4)	-	-	2.9(0.2)	3
L. micdadei	-	-	32.3(3.5)	-	-	1.3(0.2)	11.4(0.5)	5.8(0.8)	11.5(4.4)	5(0.5)	-	28.1(0.9)	5(1.1)	-	-	4
L. moravica	7.2(0.1)	-	14.7(0.3)	3.1(0.7)	-	-	30.3(0.7)	24.9(2)	6.2(0.6)	-	-	6.6(1.3)	-	-	6.9(0.4)	3
L. nautarum	5.7(1.5)	-	16.1(0.6)	2.3(0.2)	-	3.6(0.2)	32.6(3.2)	12.5(3.1)	9.2(4)	-	-	7.9(2.6)	7(2.7)	1.6(0.1)	3.9(1.2)	3
L. oakridgensis	-	-	3.3(1.5)	2.3(0.8)	2.3(0.1)	2(0.3)	34.7(9.4)	13.5(1.4)	13.4(1.3)	-	-	2.8(0.4)	20.6(5.4)	3.1(1.2)	4.3(1.7)	3

Table 21 cont.

Legionella species	Mole % nonhydroxy fatty acid composition of the Legionellae by gas-liquid chromatography															No. of analyses
	i14:0	i15:0	a15:0	n15:1	n15:0	i16:1	i16:0	n16:1	n16:0	a17:1	i17:0	a17:0	cyc 17	n17:0	n18:0	
L. parisiensis	3.4(0.2)	-	25.5(1)	-	2.4(0.1)	-	16.5(1.1)	6.3(1)	13.2(1.5)	-	-	9.6(0.4)	16.6(1.2)	4.6(0.6)	2.6(0.5)	3
L. pneumophila	6.3(1)	-	17.5(4.6)	-	-	3.9(1.1)	40.3(5.9)	6.1(3.8)	5.8(2.9)	-	-	9.6(2.5)	6.6(2.4)	1.6(1)	3.5(0.7)	49
L. quateriensis	5.4(0.8)	-	11.4(0.6)	7.3(1.6)	2.7(0.6)	-	24.4(2.2)	26.7(1.8)	5.8(0.6)	-	-	5.5(0.2)	-	3.6(1.1)	8(0.2)	3
L. quinlivanii	1.4(0.5)	-	18.3(2.8)	3.2(0.7)	1.9(0.5)	-	17.6(2.2)	22.3(2.9)	11.7(3)	-	1.7(0.2)	15.6(1)	-	2.9(0.3)	2(0.6)	8
L. rubrilucens	2.6(0.2)	-	14(1)	2.4(0.1)	2(0.1)	-	23.2(4.4)	27.4(2.3)	14.4(2.5)	-	-	10.1(0.5)	-	1.8(0.1)	3.6(1.2)	3
L. sainthelensi	7.1(0.6)	-	24.3(1.9)	2.2(0.1)	-	-	20.4(0.5)	28.3(3.4)	7.9(0.5)	-	-	10.5(1.5)	-	-	-	3
L. santicrucis	7.4(1.4)	-	11.7(0.6)	2.1(0.1)	-	-	25.8(4.7)	24.9(1.8)	14.3(3.9)	-	0.7(0.1)	4.7(0.6)	2.5(1.4)	-	4.1(2.1)	3
L. spiritensis	2.6(0.4)	-	20.7(1.1)	-	-	2.7(2.2)	28.7(3.3)	17.2(2)	7.3(1)	2.2(0.1)	-	9.8(8.4)	-	-	1.7(0.3)	3
L. steigerwaltii	3.6(0.2)	-	27.7(1)	-	2.7(2.2)	-	19.1(1)	6.3(0.6)	11.8(0.7)	-	-	10.4(0.9)	13.6(1.9)	2.8(0.6)	2.8(0.1)	3
L. tucsonensis	3.1(0.1)	-	28.2(0.6)	0.9(0.1)	-	-	21.3(0.1)	13.8(0.4)	5.7(0.2)	-	-	16.7(0.2)	5.6(0.3)	1.4(0.1)	2.3(0.8)	3
L. wadsworthii	1.5(0.2)	-	40.8(1.5)	1.1(0.1)	-	-	12.8(0.1)	6.8(0.6)	2.9(1.1)	-	-	26.3(0.6)	4.7(0.3)	2.2(0.1)	1.5(0.8)	3
L. worsliensis	5.9(0.5)	-	13.9(0.4)	1.5(0.1)	-	-	19.6(1.6)	30.9(1.2)	11.9(1.7)	-	-	6.5(0.2)	-	2(0.1)	8.2(1.5)	3

Table 22 Variation in nonhydroxy fatty acid composition with growth on different batches of BCYE medium.

Legionella species	Mole % nonhydroxy fatty acid composition by gas-liquid chromatography															Media/Batch
	i14:0	i15:0	a15:0	n15:1	n15:0	i16:1	i16:0	n16:1	n16:0	a17:1	i17:0	a17:0	cyc 17	n17:0	n18:0	
Part A																
L.pneumophila SG 2	8.8	-	15.9	-	-	3.8	46.4	8.4	3.9	-	-	6.1	6.7	-	-	BCYE No.1.1
" "	8.1	-	14.6	-	-	3.6	48.5	7.9	4.1	-	-	6.8	6.4	-	-	BCYE No.9.1
Part B																
L.pneumophila SG 3	7.3	-	15.4	-	-	4.6	45.2	8.1	3.0	-	-	7.8	8.6	-		-BCYE No.12.1
" "	8.1	-	14.6	-	-	3.6	48.5	7.9	4.1	-	-	6.8	6.4	-		-BCYE No.20.1
Part C																
L.pneumophila SG 6 (Chicago-2)	7.1	-	16.1	-	-	5.1	45.1	8.2	2.8	-	-	8.7	6.9	-		-BCYE No.8.1
" "	7.0	-	16.1	-	-	5.2	47.6	7.2	2.1	-	-	9.4	5.4	-		-BCYE No.15.1
" "	7.0	-	16.0	-	-	5.0	47.8	7.3	2.2	-	-	9.4	5.3	-		-BCYE No.15.1
" "	6.9	-	16.3	-	-	4.9	47.3	7.4	2.3	-	-	9.5	5.4	-		-BCYE No.15.1

Table 23 Mole % nonhydroxy fatty acid composition by gas-liquid chromatography of 86/35784 and L.quinlivanii.

Legionella species	Mole % nonhydroxy fatty acid composition by gas-liquid chromatography														No. of analyses
	i14:0	i15:0	a15:0	n15:1	n15:0	i16:1	i16:0	n16:1	n16:0	a17:1	i17:0	a17:0	cyc 17	n17:0	n18:0	
86/35784 (BCYE-grown)	2.1 (0.9)	-	19.5(2.1)	3.2(0.4)	1.5 (0.2)	-	24.2(2.1)	19.6(1.0)	8.8(0.7)	-	1 (0.1)	12.6(1.4)	-	2.4(0.4)	1.7(0.3)	3
86/35784 (Oxoid-grown)	2.4 (0.4)	-	19.8(0.7)	3.3(0.1)	1.3(0.1)	-	29.2(1.7)	20.7(0.6)	8.0(0.3)	-	0.8(0)	11(0.3)	-	1.7(0.1)	1.2(0.2)	3
(L.quinlivanii)	1.4(0.5)	-	18.3(2.8)	3.2(0.7)	1.9(0.5)	-	17.6(2.2)	22.3(2.9)	11.3(3.0)	-	1.7(0.2)	15.6(1.0)	-	2.9(0.3)	2.0(0.6)	7

Figures are presented as mean values with the standard deviation shown in brackets.

cultures and derivitisation and analysis of fatty acids of the Legionellaceae had been established, a library of FA content for each of the 42 species (including proposed and possible new species Wilkinson et al., 1990) was constructed (table 21). L.adelaidensis (strain designation F/636) and six possible new species (Wilkinson et al., 1990) are shown in table 20 (their GLC profiles are shown graphically in figures 30 to 78).

A further investigation was performed to discover whether the 3 DNA groups of L.pneumophila described by Brenner et al. (1988) could be differentiated also by their FA profiles. All L.pneumophila strains allocated to one of the three DNA groups as defined by Brenner et al. (1988) and their $\bar{x}$ and S.D. values for FA's recalculated. Although quantitative differences in FA's were observed (e.g. a15:0 and n16:0 values, table 24) between strains of each of the 3 DNA groups, these were not substantial. The addition or subtraction of one S.D. from each $\bar{x}$ value for a particular fatty acid allowed mole % values to overlap between strains of each DNA group. Therefore, it was not practicable to draw any further conclusions other than DNA groups defined by Brenner et al. 1988 analysed for their FA content could not be distinguished on this basis.

3.6.8 Fatty acid variation in L.pneumophila

It was observed that type strains representing three serogroups of L.pneumophila; SG4, SG5 and serogroup

Key for figures 30-78 where FA's represented by peaks are labelled with letters for their identification.

Notation	Key	Notation	Key
i14:0	a	a17:1	j
i15:0	b	i17:0	k
a15:0	c	a17:0	l
n15:1	d	cyc 17	m
n15:0	e	n17:0	n
i16:1	f	n18:0	o
i16:0	g	n19:0	p
n16:1	h	n20:0	q
n16:0	i		

GLC profiles of FAMES of the Legionellaceae

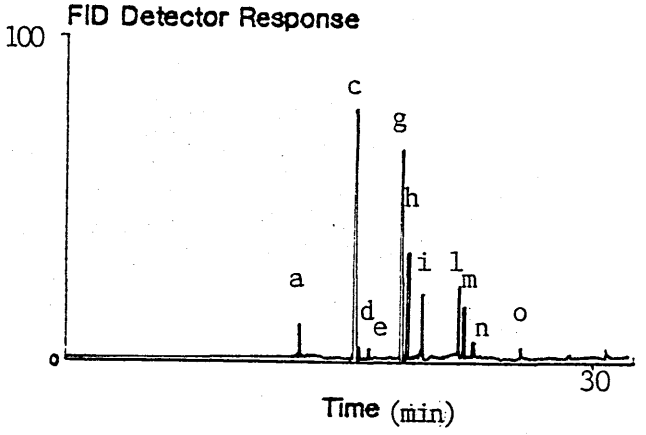


Figure 30

L. anisa

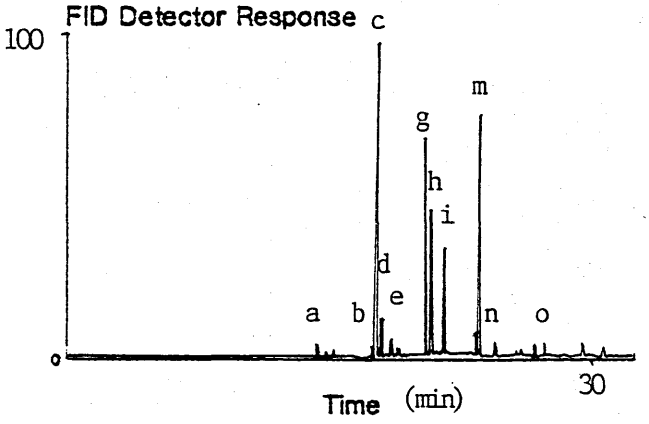


Figure 31

L. birminghamsis

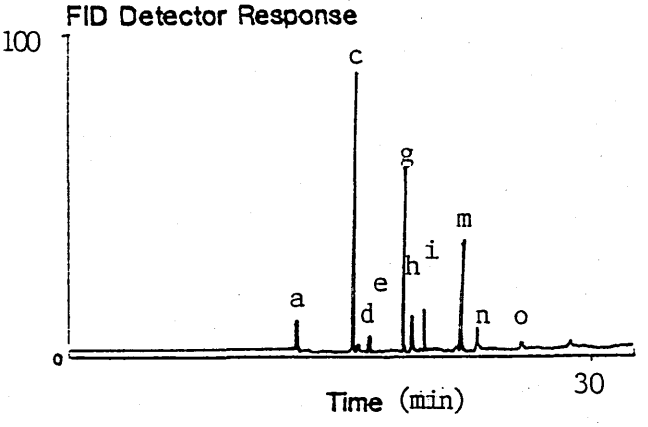


Figure 32

L. bozemanii SG 1

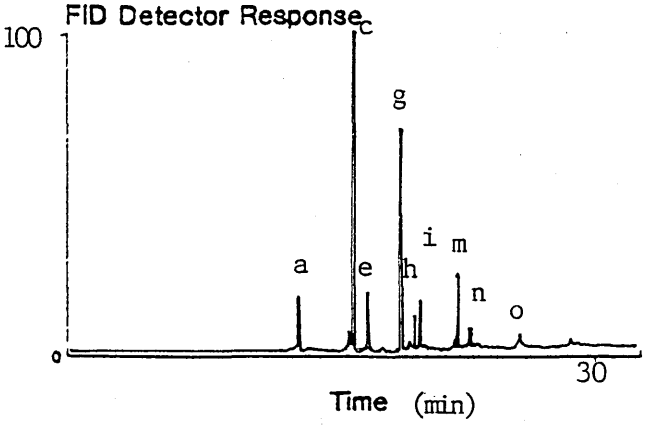


Figure 33

L. bozemanii SG 2

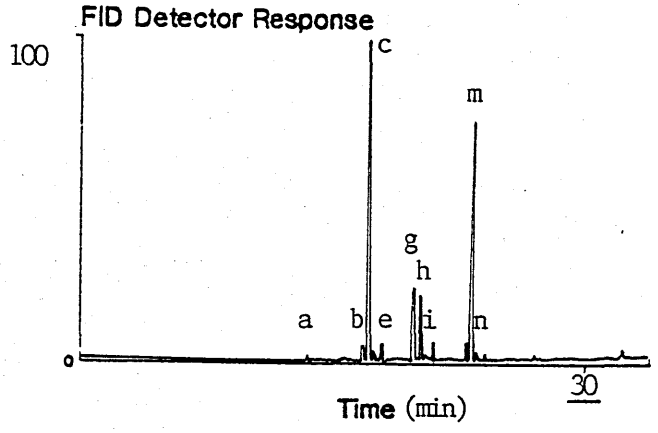


Figure 34

L. brunensis

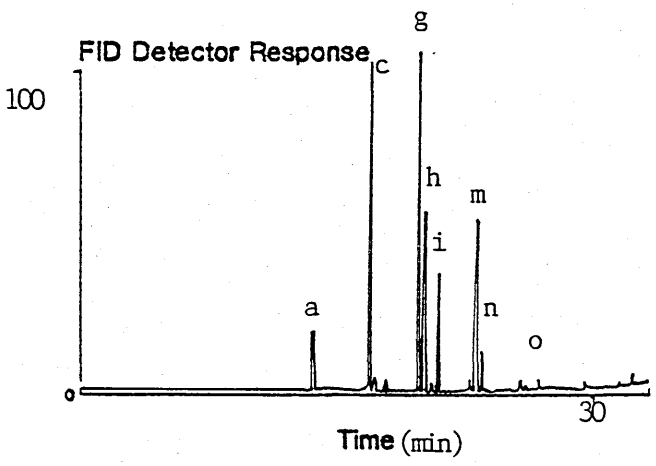


Figure 35

L. cherrii

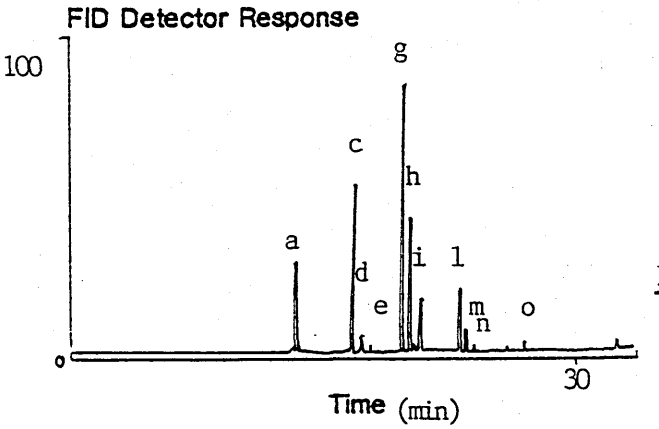


Figure 36

L. cincinnatiensis

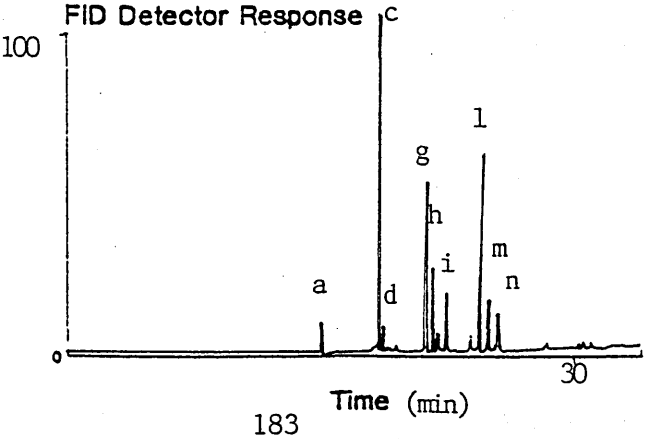


Figure 37

L. dumoffi

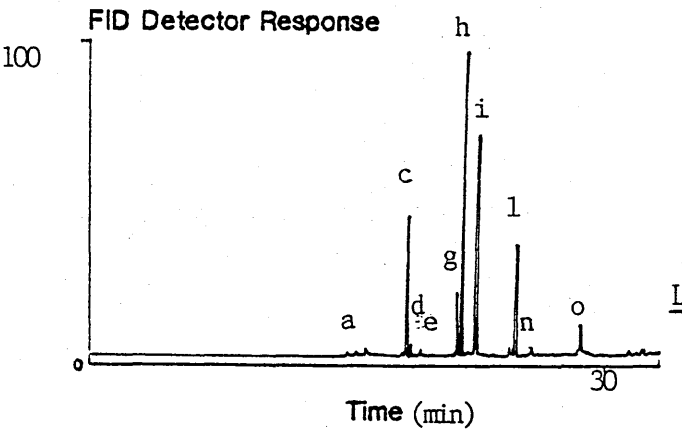


Figure 38

L. erythra

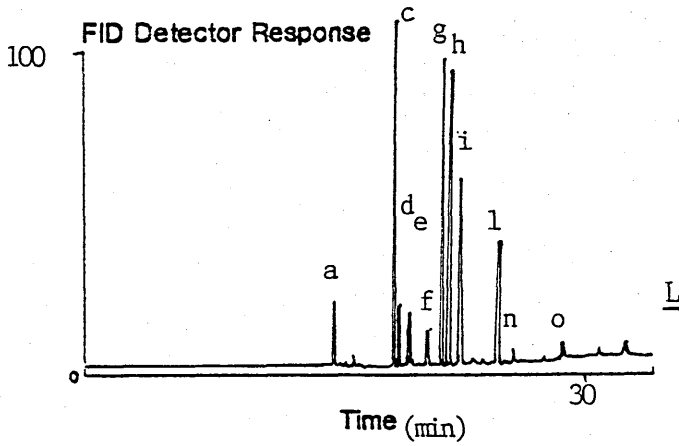


Figure 39

L. feeleii SG 1

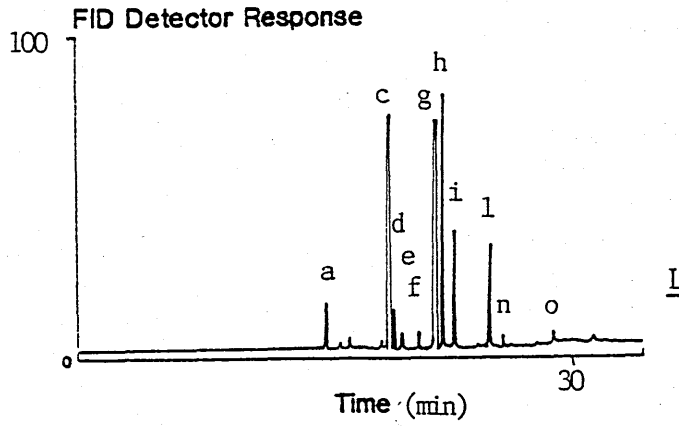


Figure 40

L. feeleii SG 2

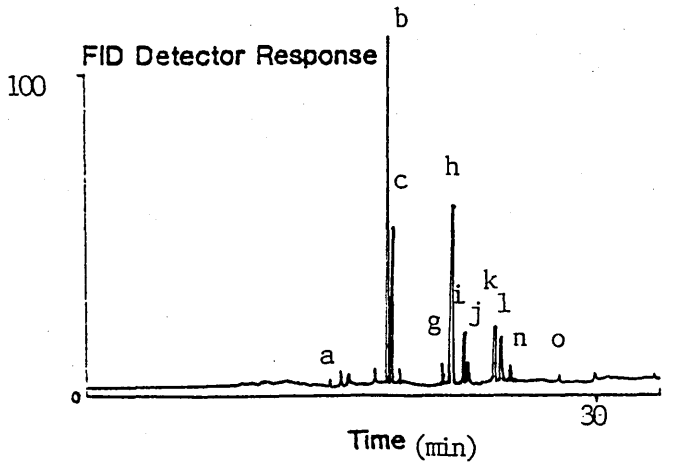


Figure 41

L. geestiae

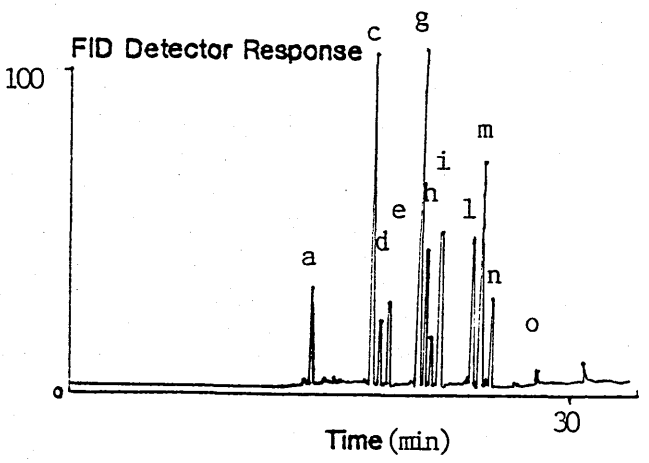


Figure 42

L. gormanii

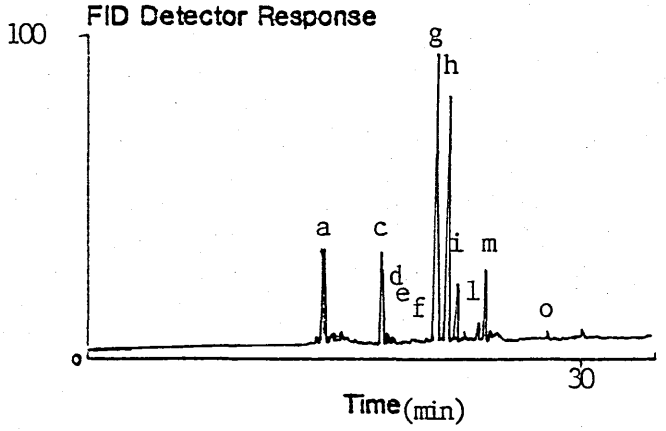


Figure 43

L. gratiana

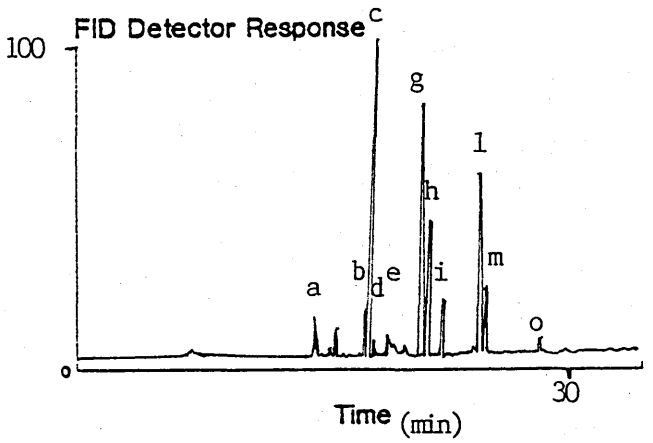


Figure 44

L. hackeliae SG 1

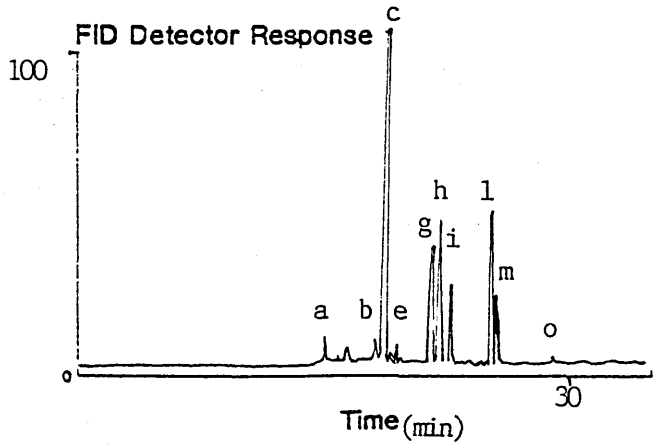


Figure 45

L. hackeliae SG 2

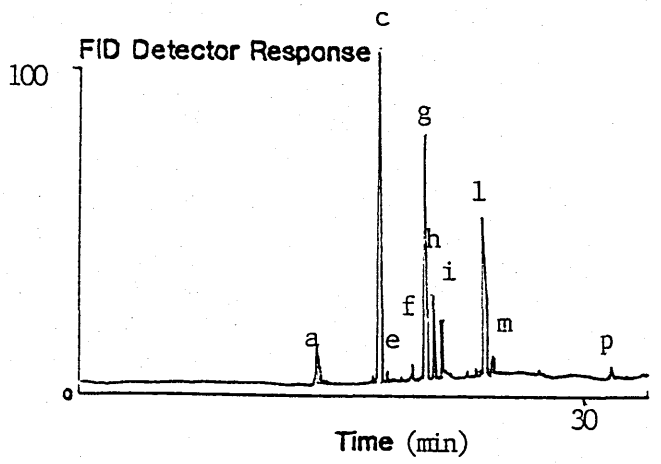


Figure 46

L. israelensis

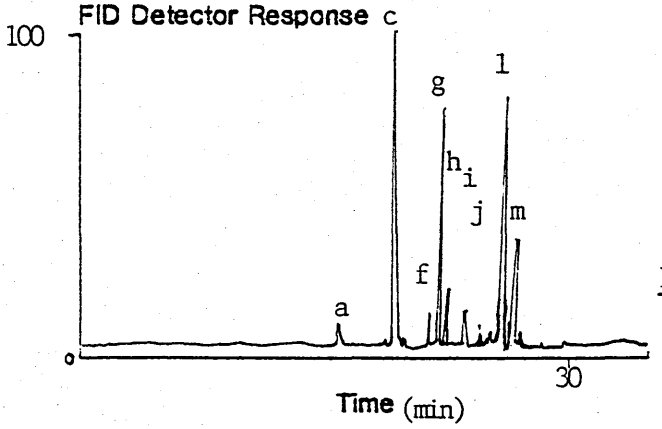


Figure 47

L. jamestowniensis

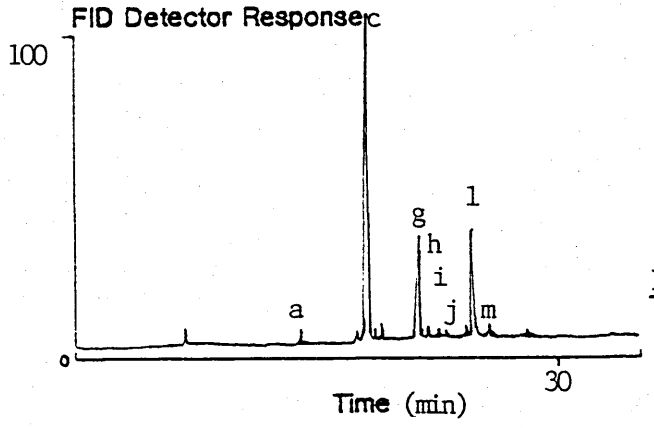


Figure 48

L. jordanis

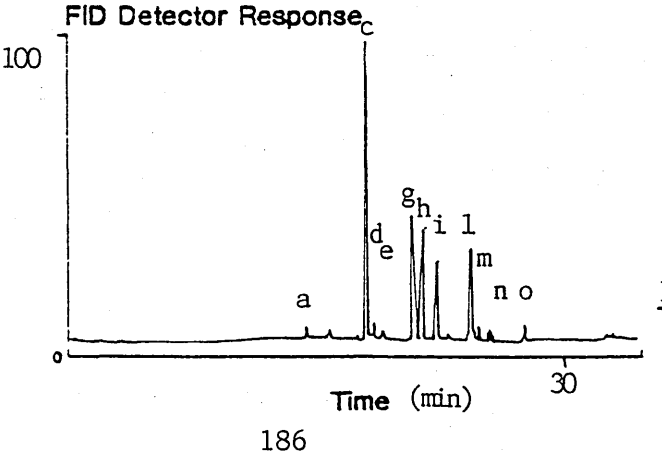


Figure 49

L. londiniensis

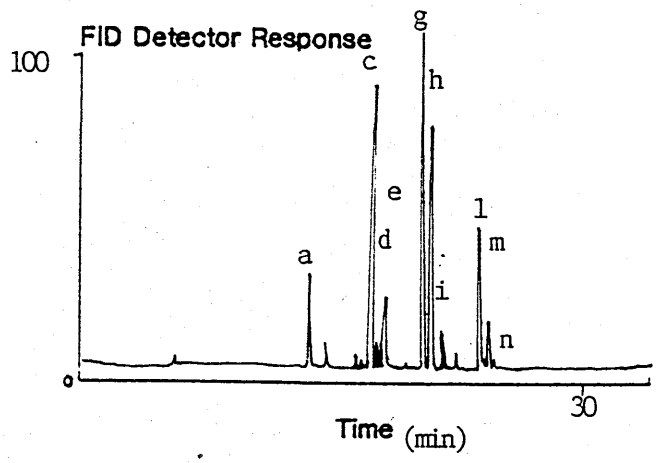


Figure 50

L. longbeachae SG 1

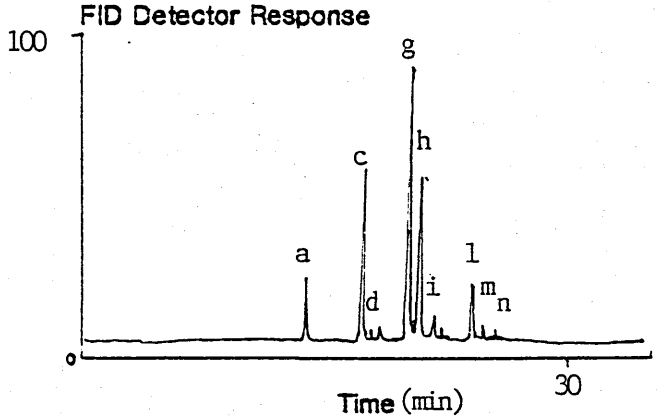


Figure 51

L. longbeachae SG 2

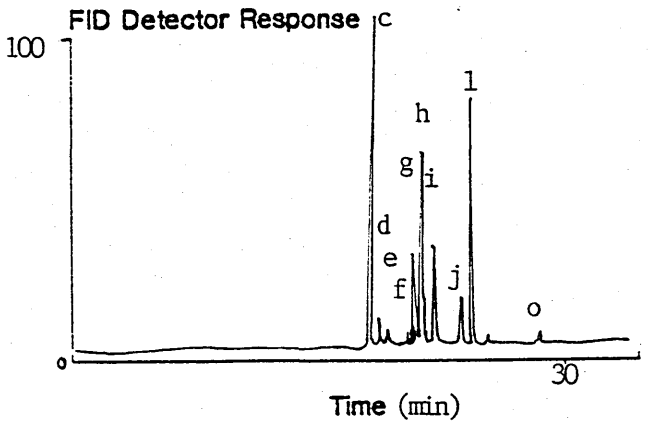


Figure 52

L. maceachernii

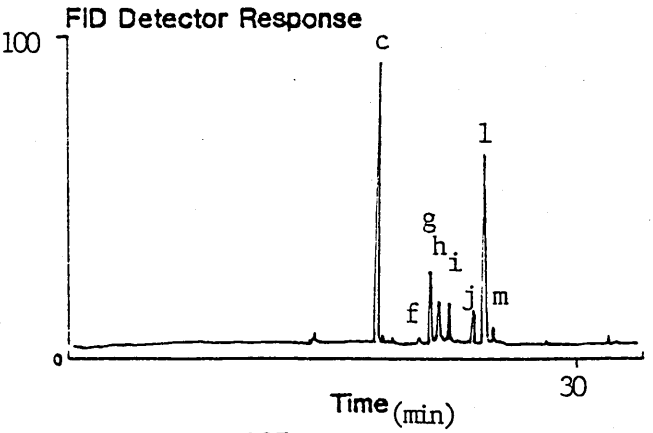


Figure 53

L. micdadei

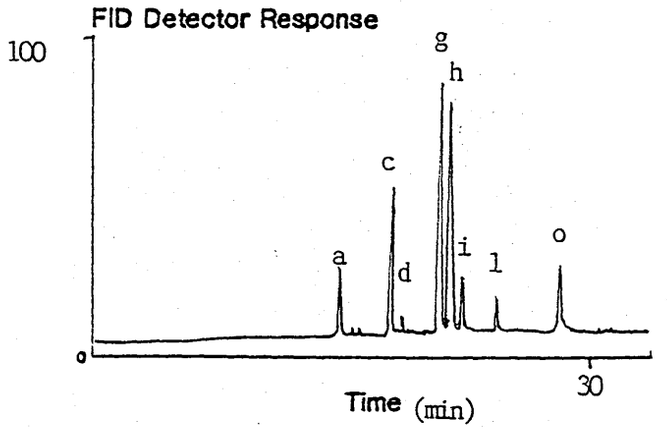


Figure 54

L. moravica

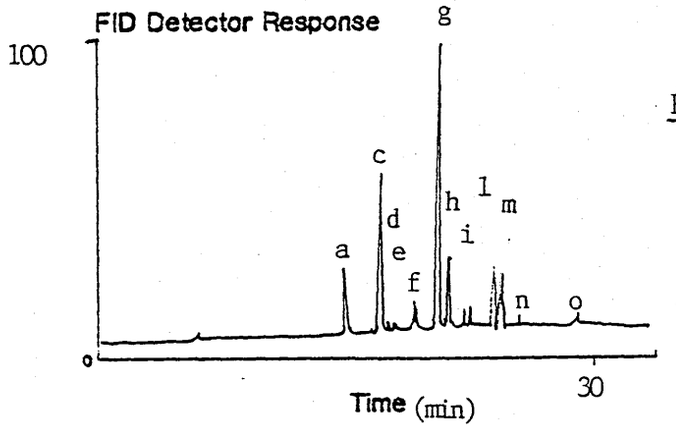


Figure 55

L. nautarum

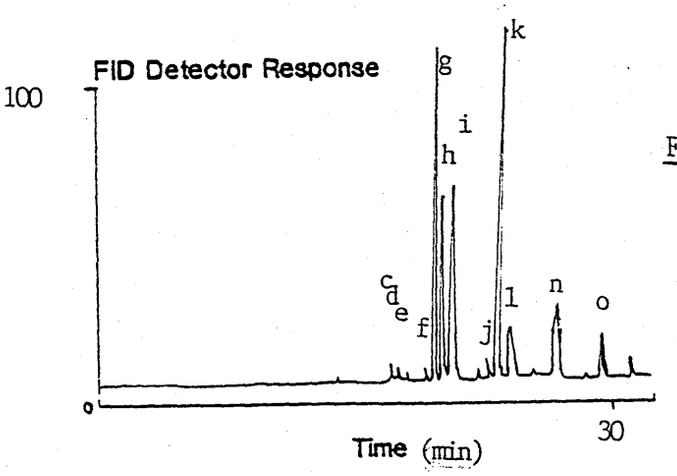


Figure 56

L. oakridgensis

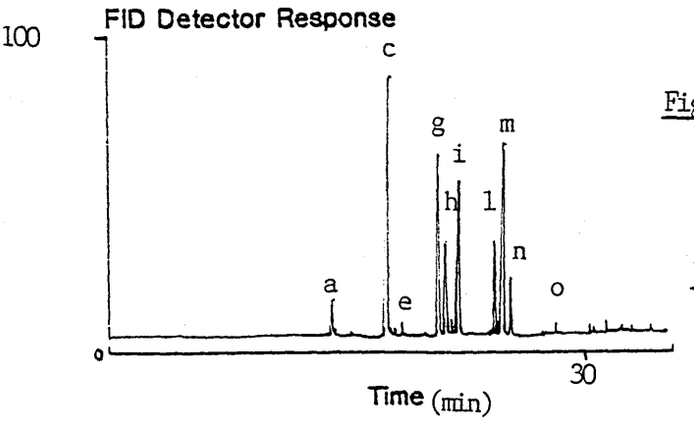


Figure 57

L. parisiensis

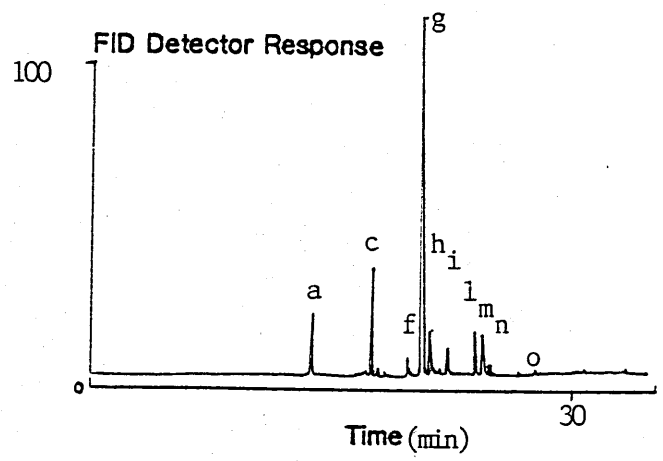


Figure 58

L. pneumophila SG 1
(Knoxville-1)

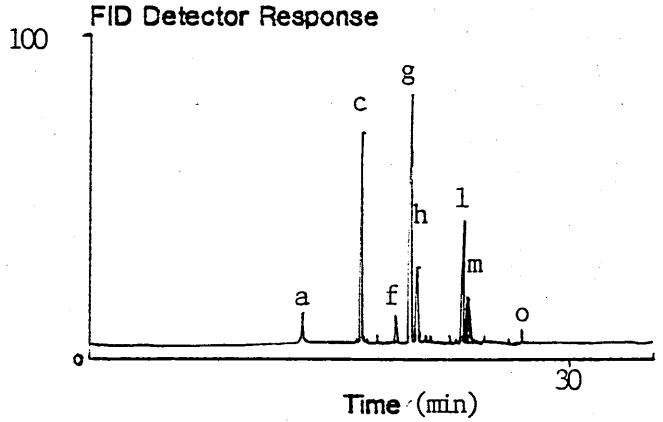


Figure 59

L. pneumophila SG 4/5

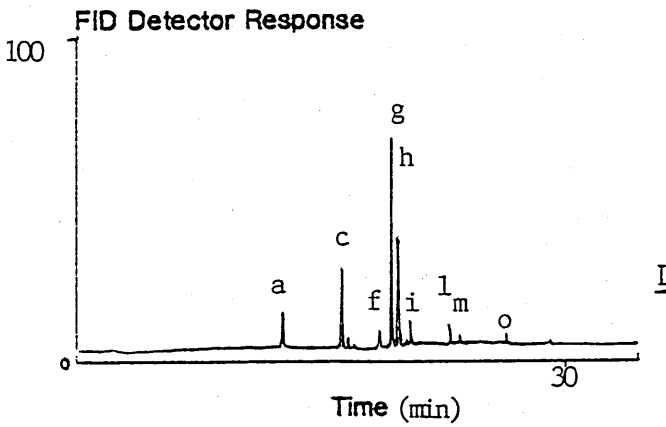


Figure 60

L. pneumophila SG 10

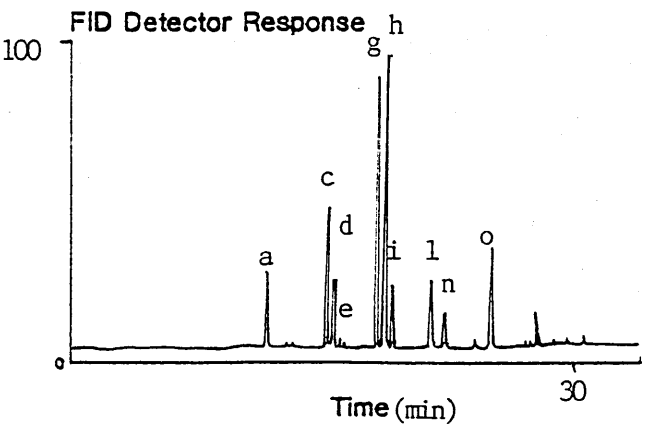


Figure 61

L. quateriensis

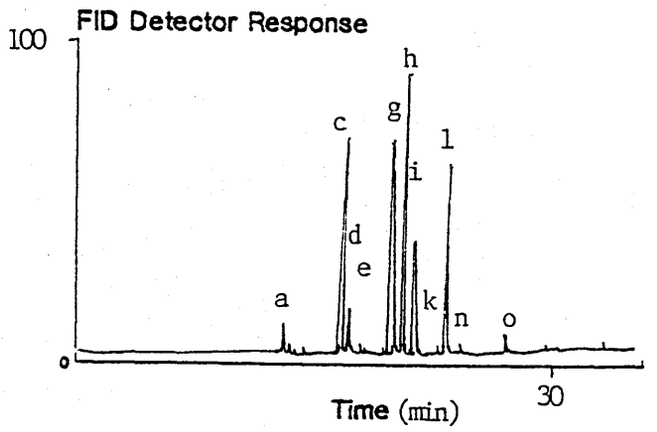


Figure 62

L. quinlivanii
1442-AUS-E

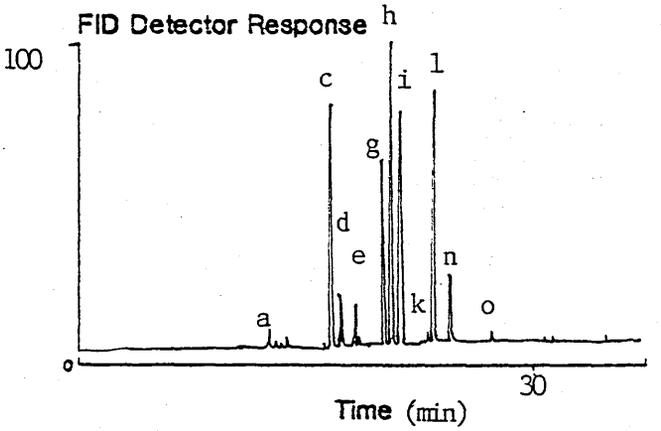


Figure 63

L. quinlivanii
1452-AUS-E

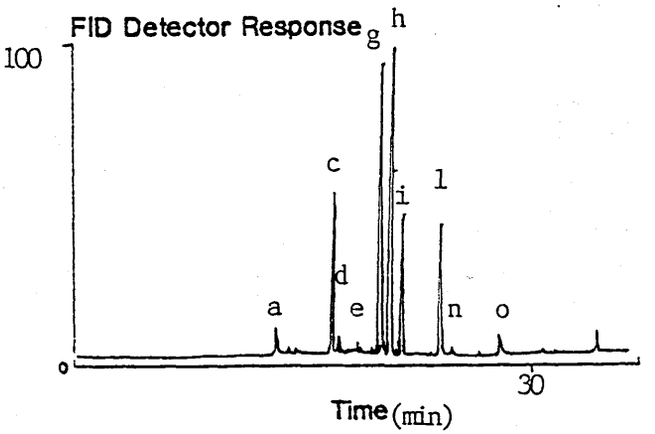


Figure 64

L. rubrilucens

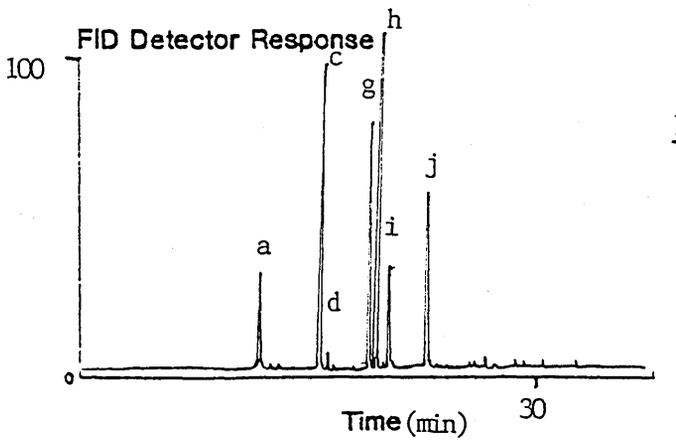


Figure 65

L. sainthelensi

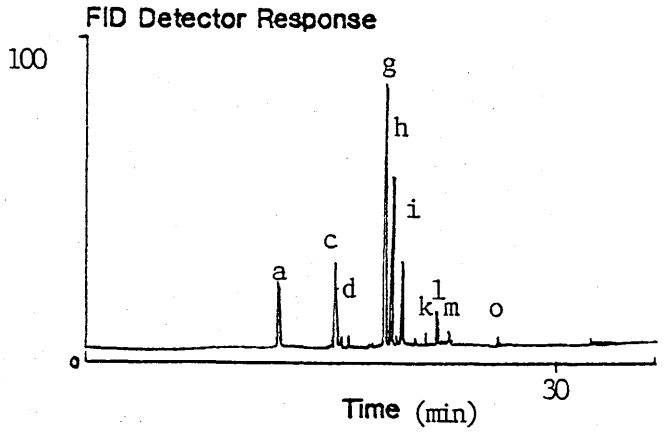


Figure 66

L. santicrucis

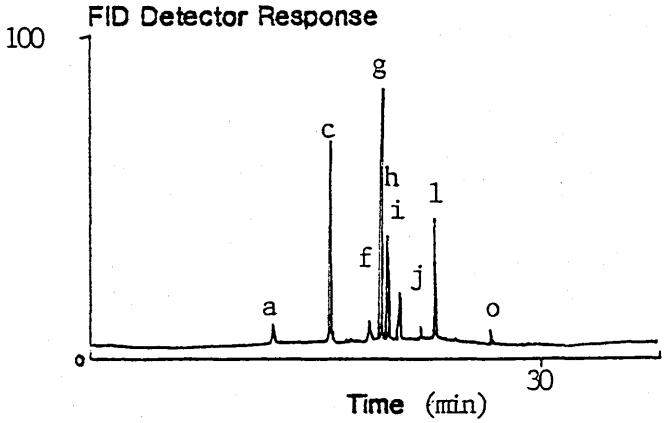


Figure 67

L. spiritensis

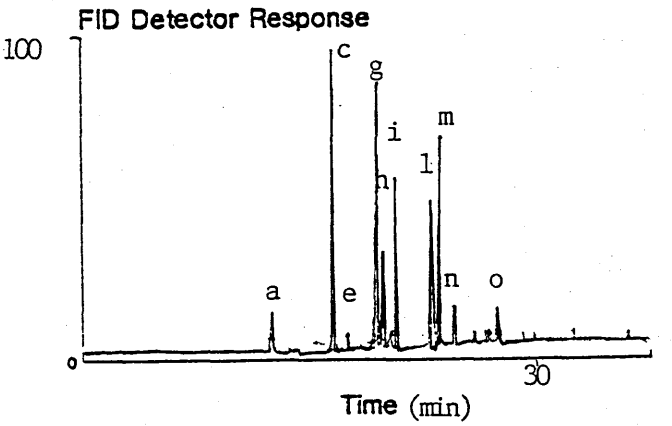


Figure 68

L. steigerwaltii

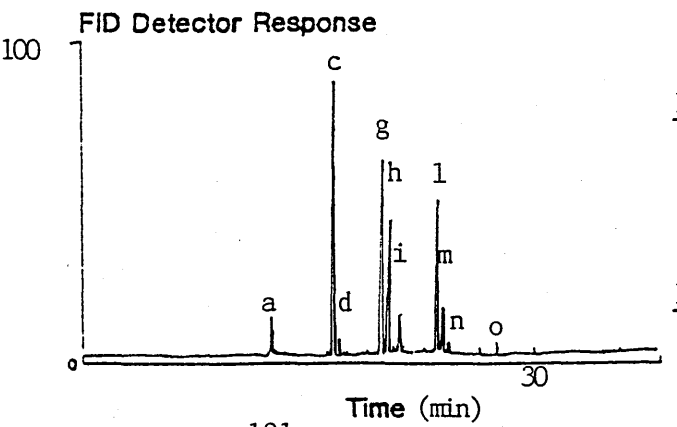


Figure 69

L.tucsonensis

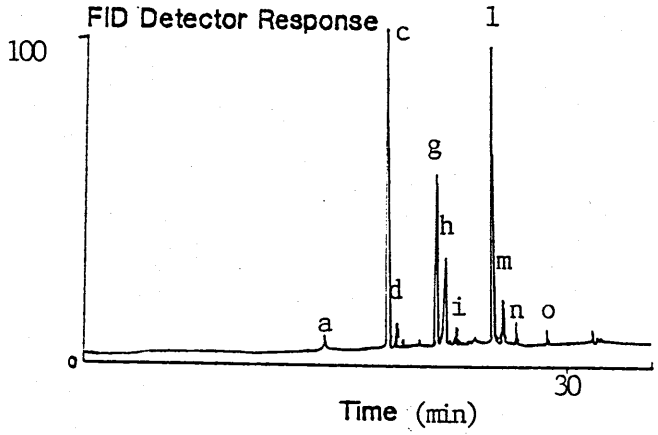


Figure 70

L. wadsworthii

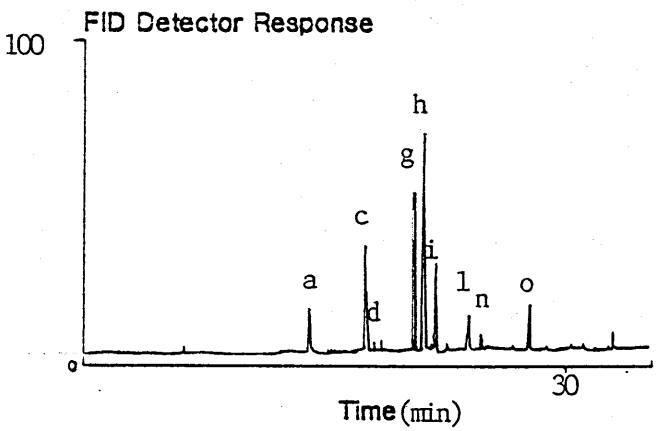


Figure 71

L. worsliensis

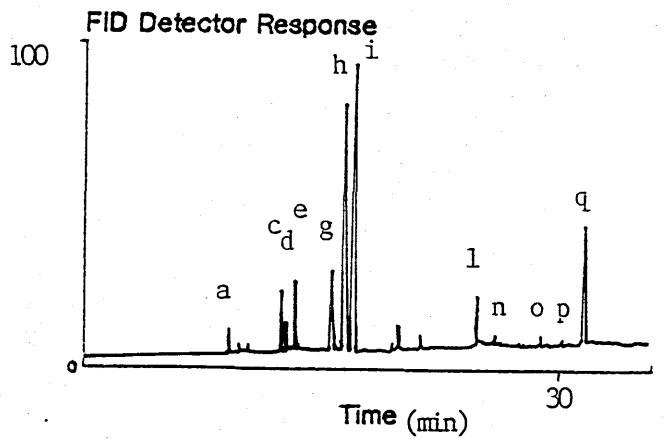


Figure 72

L.adelaidensis

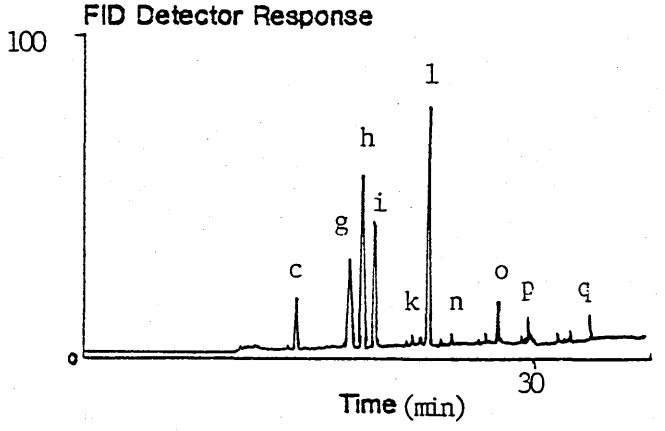


Figure 73

A/36

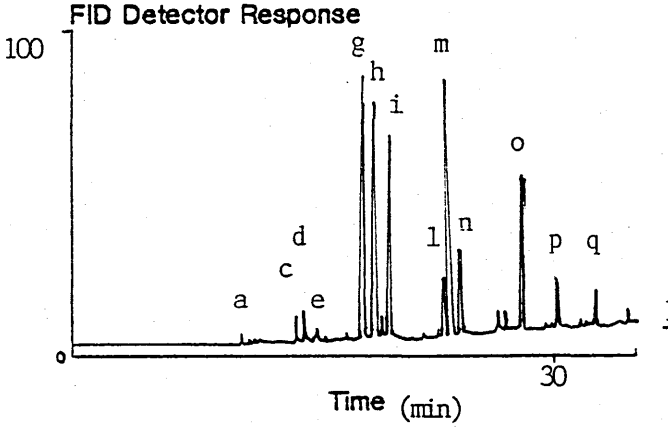


Figure 74

B/86

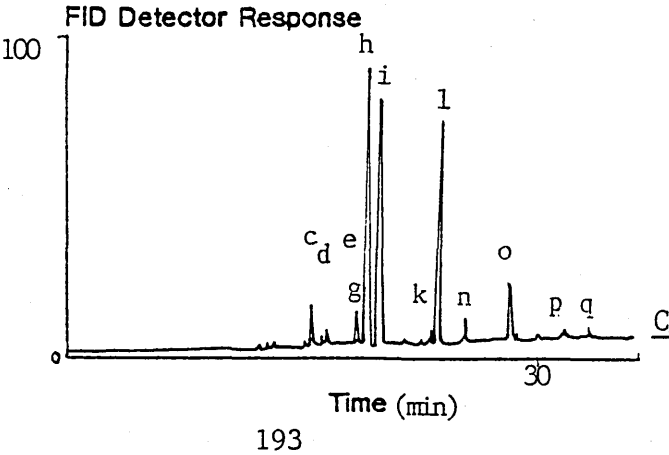


Figure 75

C/449

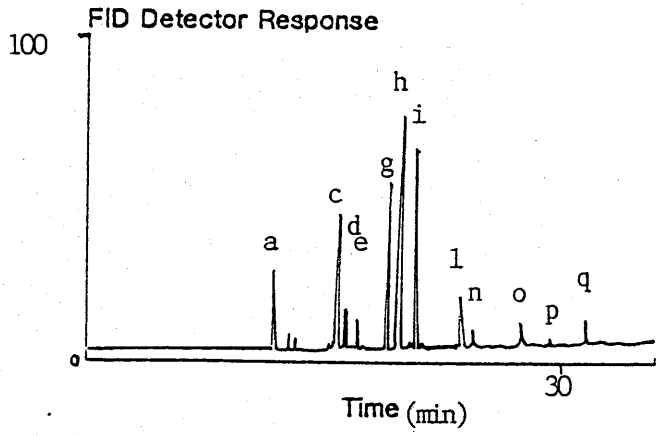


Figure 76

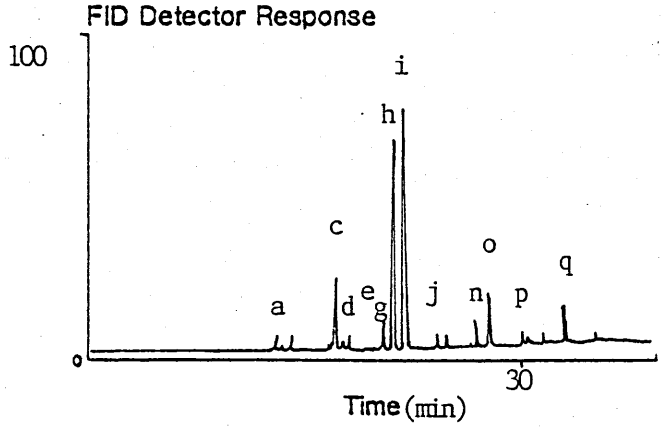


Figure 77

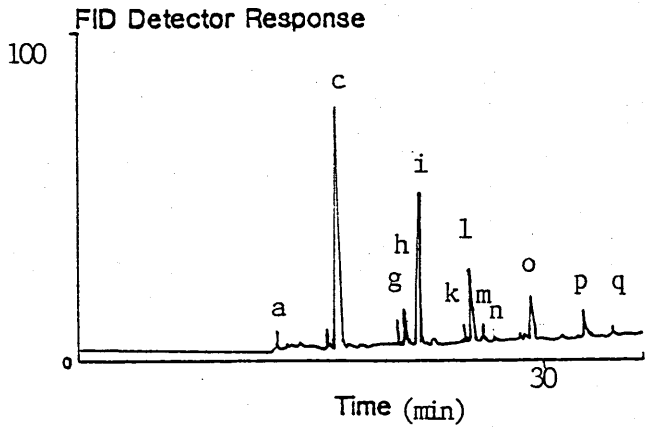


Figure 78

Table 24 Mole % nonhydroxy fatty acid composition of the 3 DNA groups of L.pneumophila Brenner et al., (1990).

Legionella species	Mole % nonhydroxy fatty acid composition by gas-liquid chromatography															No. of analyses
	i14:0	i15:0	a15:0	n15:1	n15:0	i16:1	i16:0	n16:1	n16:0	a17:1	i17:0	a17:0	cyc 17	n17:0	n18:0	
Members of Legionella pneumophila DNA group 1	6.7(1)	-	15.6(3.3)	-	-	4.1(1.1)	42.5(5)	8.1(0.4)	5.9(4.1)	-	-	8.6(1.8)	6.7(2.8)	1.7(0.4)	3(1)	40
Members of Legionella pneumophila DNA group 2	5.4(1.1)	-	23.8(3.7)	-	-	4.1(1.2)	34.2(7.5)	5(2.6)	8.7(4.3)	-	-	13.2(2.6)	6.6(1)	1.3(0.5)	1.7(0.4)	6
Members of Legionella pneumophila DNA group 3	6.1(1.2)	-	17.5(4.6)	-	-	3.9(1.1)	40.3(5.9)	6.1(3.8)	2.9(1.6)	-	-	11(2.2)	7.9(1.7)	1.3(0.5)	1(1.1)	3

Figures are presented as mean values with the standard deviation shown in brackets.

Lansing-3 (table 25) produced higher than the $\bar{x}$ values (7 mol%) for the FA a15:0 than in other serogroups of L.pneumophila (table 21). L.pneumophila SG's 4 and 5 were similar to each other in producing up to 26 mole % of a15:0. The un-numbered serogroup Lansing-3 also produced up to 24.5 mole % of the FA, a15:0. In addition, L.pneumophila SG10 showed greater amounts of n16:1 than observed in other serogroups of L.pneumophila (table 25).

Where environmental strains representing these serogroups (identified by cultural characteristics and serological evidence only) have been available, their FA's have been analysed and mole % of FA's calculated (table 25).

The serogroup 5 strains (89/29805 and 89/03289) produced unremarkable FA profiles with essentially no differences noted when compared with serogroups of L.pneumophila (table 22) which were not 4, 5, 10, or Lansing-3. However, U7W, a SG 5 strain, produced a FA profile similar to serogroups of L.pneumophila other than 4, other 5's, 10 and Lansing -3. A feature of this particular serogroup (5) may be its variable FA composition with respect to the fatty acids described.

Strain 88/42151 (table 25) showed increased amounts of the FA n16:1 of a quantity similar to that of the Leiden-1 type strain and may be a distinguishing feature of this serogroup. This needs to be substantiated by the isolation and analysis of other L.pneumophila SG10 strains.

Table 25 Mole % nonhydroxy fatty acid composition of L.pneumophila SG 4, 5, 10 and Lansing-3.

Legionella species	Mole % nonhydroxy fatty acid composition by gas-liquid chromatography														
	i14:0	i15:0	a15:0	n15:1	n15:0	i16:1	i16:0	n16:1	n16:0	a17:1	i17:0	a17:0	cyc 17	n17:0	n18:0
89/03289 (L.pneumophila SG5)	7.1	-	14.1	-	-	3.2	40.3	10.7	5.2	-	-	7.9	11.3	-	-
89/29805 (L.pneumophila SG5)	6.5	-	14.7	-	-	3.6	40.1	10.8	6.0	-	-	8.5	9.8	-	-
L.pneumophila SG5 (U7W)	7.4	-	19.9	-	-	3.3	38.5	8.6	4.4	-	-	8.8	9.1	-	-
L.pneumophila SG5 (U8W)	5.2	-	23.6	-	-	2.9	35.9	9.8	3.5	-	-	13.1	5.9	-	-
L.pneumophila SG5 (MICU B)	5.6	-	24.2	-	-	2.8	33.4	9.0	5.3	-	-	11.2	8.6	-	-
89/42151 (L.pneumophila SG10)	5.6	-	13.7	-	-	4.0	33.9	15.1	8.4	-	-	7.9	6.0	1.9	3.5
L.pneumophila SG10 (Leiden-1)	6.4	-	14.5	-	-	4.1	35.4	20.6	5.5	-	-	7.8	5.7	-	-
L.pneumophila SG Lansing-3	6.1	-	24.5	-	-	3.4	36.1	6.6	3.3	-	-	11.8	8.1	-	-
L.pneumophila SG4 (Los angeles-1)	4.8	-	26	-	-	4.5	29.8	12.6	3.5	-	-	15.4	3.3	-	-
"	4.7	-	26.5	-	-	4.3	29.6	12.8	3.3	-	-	15.7	3.1	-	-

The other serogroups (SG 4 and Lansing-3) were not compared with environmental isolates.

3.6.9 Confirmation of fatty acids by GC-MS

Although the peaks produced on test chromatograms can provisionally be identified by comparison of retention times (R.T.) with those FA's in standard mixtures (table 26). Confirmation of identity can be by mass spectrometry. However, this is not practicable for every peak of every chromatogram produced in view of the specialised equipment and the time involved.

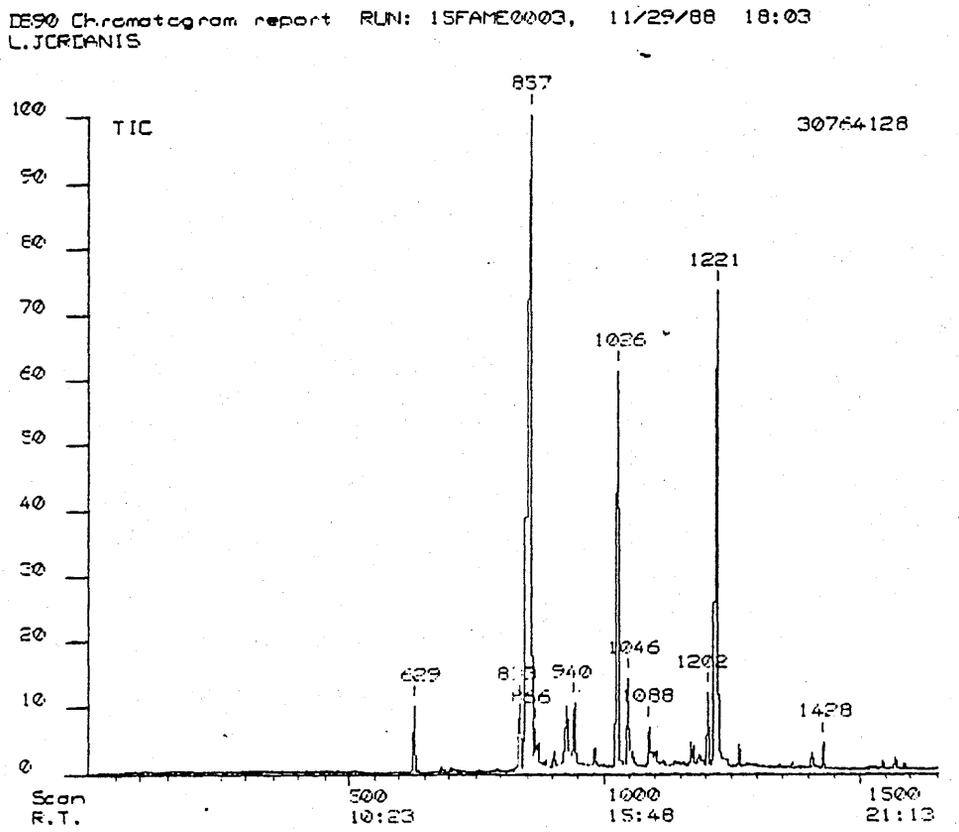
Fatty acids were extracted, hydrolysed and methylated as previously described (section 2.16.5) from five different legionellae for analysis by GC-MS. These were L.jamestowniensis, L.jordanis, L.oakridgensis, L.pneumophila and an environmental isolate, 86/35784. On GC-MS apparatus each produced a profile identical with that obtained when extracts of the same organisms were analysed on the 50m CPSil 5CB column (Chrompack) except that retention times (R.T.) for each peak were much reduced on the 25m SGE BP 1 column (Supelco) of the GC-MS apparatus.

This was expected as both columns separated volatile components of a mixture due to the different boiling points of these components. Figure 79 shows the GLC profile of L.jordanis obtained by GC-MS with the x-axis labelled with both the scan number and the R.T. at which each peak is eluted from the GC and analysed by the mass

Table 26 Table of retention times of FAME standards obtained by gas-liquid chromatography.

Methyl ester of fatty acid (Standards)	Shorthand designation	Retention Times (min)
Bacterial acid Methyl esters CP Mix Cat No. 4-7080 Supelco Inc.		
Me. undecanoate	n11:0	8.533
Me. 2-hydroxydecanoate	2-OH 10:0	8.758
Me. dodecanoate	n12:0	10.900
Me. tridecanoate	n13:0	13.558
Me. 2-hydroxydodecanoate	2-OH 12:0	13.883
Me. 3-hydroxydodecanaote	3-OH 12:0	14.617
Me. tetradecanoate	n14:0	16.333
Me. 13-methyltetradecanoate	i-15:0	18.117
Me. 12-methyltetradecanoate	a-15:0	18.350
Me. pentadecanoate	n15:0	19.117
Me. 2-hydroxytetradecanoate	2-OH 14:0	19.508
Me. 3-hydroxytetradecanoate	3-OH 14:0	20.233
Me. 14-methylpentadecanoate	i16:0	20.858
Me. cis-9-hexadecanoate	n16:1	21.217
Me. hexadecanoate	n16:0	21.817
Me. 15-methylhexadecanoate	i-17:0	23.500
Me. cis-9,10-methylenehexadecanoate	cyc 17	24.025
Me. heptadecanoate	n17:0	24.433
Me. 2-hydroxyhexadecanoate	2-OH 16:0	24.883
Me. cis-9,12-octadecadienoate	n18:2	26.042
Me. cis-9-octadecanoate	n18:1	26.250
Me. trans-9-octadecanoate &	n18:1	
Me. cis-11-octadecanoate	n18:1	26.417
Me. octadecanoate	n18:0	26.917
Me. cis-9,10-methyleneoctadecanoate	cyc 19	28.992
Me. nonadecanoate	n19:0	29.333
Me. eicosanoate	n20:0	31.733
Cross-reference Standards (Sigma)		
Me. undecanoic acid	n11:0	8.502
Me. pentadecanoic acid	n15:0	19.085

Figure 79 GLC profile of FAME's of L.jordanis obtained by GC-MS.



Key

Scan - Scan No.

R.T. - Retention time

TIC - % Relative abundance

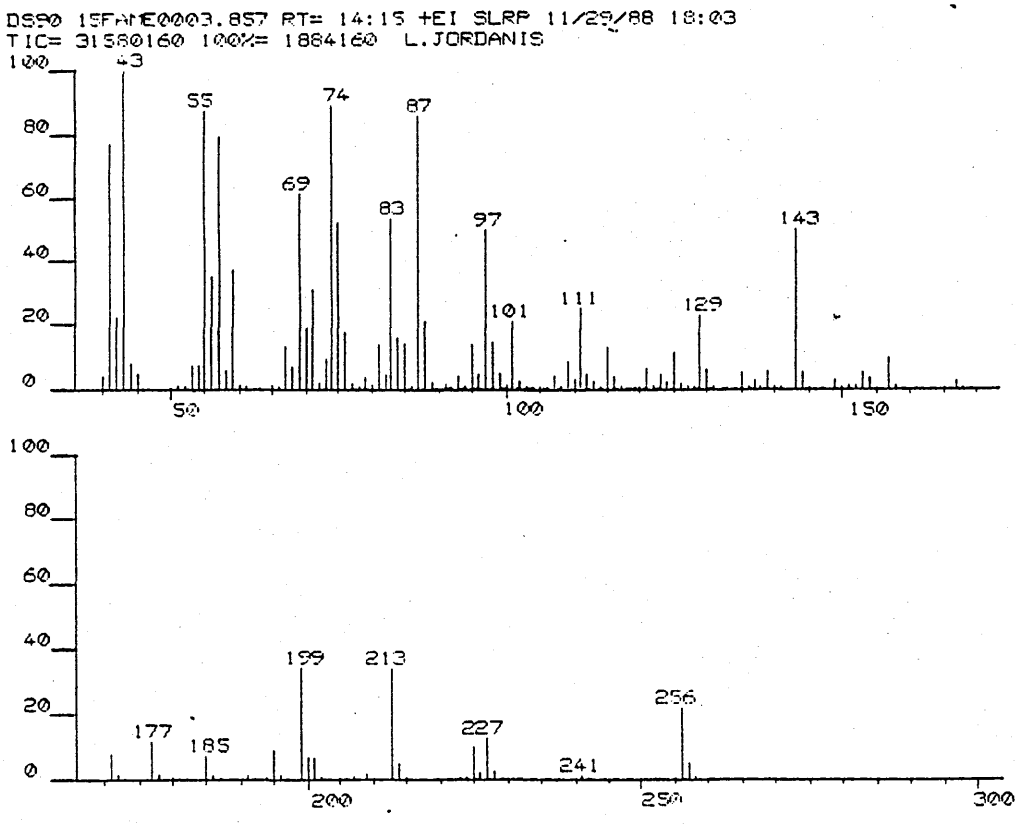
spectrometer. The 5 legionellae analysed by GC-MS, between them, demonstrated every nonhydroxy FA so far found in the Legionellaceae. Examples of the results produced by GC-MS analysis are shown in figures 80, 81 and 82. These show the mass spectra of three of the major peaks observed in the FA profile of L.jordanis, a15:0, i16:0 and a17:0 observed at scan nos. 857, 1026 and 1221 and whose R.T.'s are 14min 15s, 16min 5s and 18min 12s respectively. These fragmentation patterns with their molecular ions at mass numbers 256, 270 and 284 are typical of these fatty acids. The difference between these figures equate to the mass of a $-CH_2$ group i.e. 14.

Difficulties exist in the structural identification of some FA's even by GC-MS. Methyl esters of iso (non methylated FA is shown in figure 83) and normal FA (non methylated FA shown in figure 84) produce nearly identical mass spectra.

Similarly unsaturated (figure 85) and cyclopropane FA's (figure 86) cannot be distinguished easily, nor can the position of the double bonds and cyclopropane rings be established. However the preparation of picolinyl esters, followed by mass spectrometry allows the structure of all types of fatty acid compound to be confirmed in a single experiment (Harvey, 1982), but this was not investigated.

However it has been shown using this technique that of the FA's extracted from the legionellae, n16:1 is always

Figure 80 Mass spectra of the FA a15:0 from L.jordanis by GC-MS showing its fragmentation pattern and molecular ion at m/e 256.

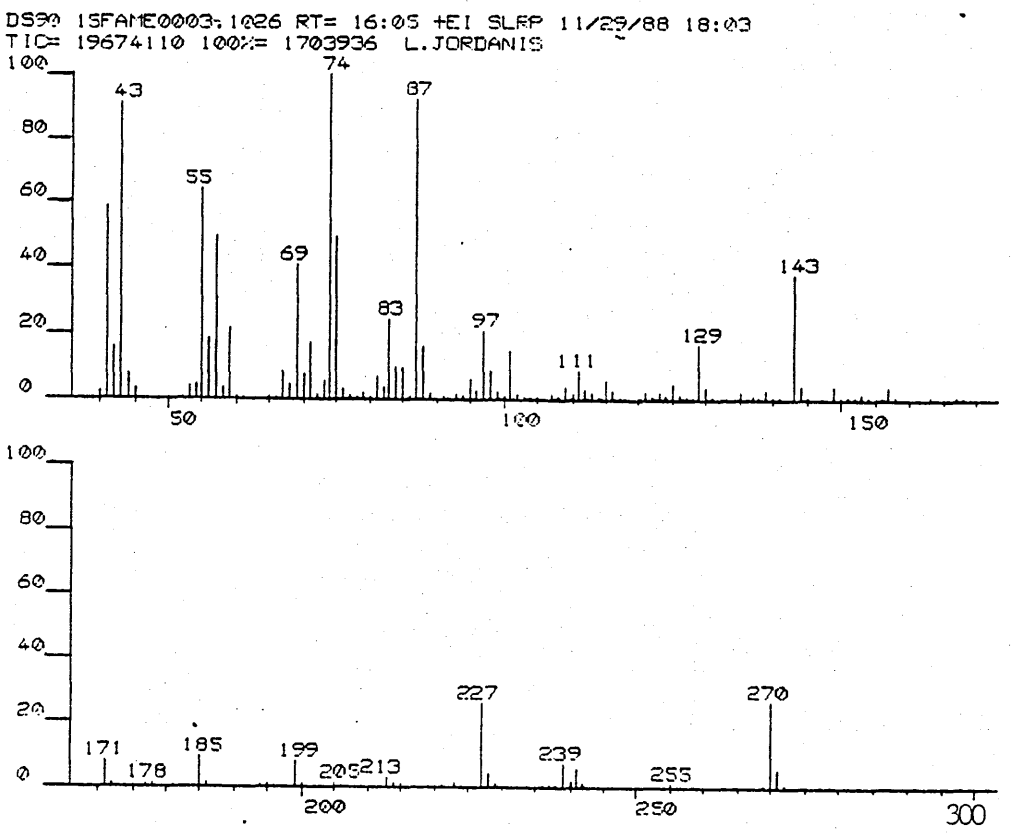


Key

X-axis - mass charge ratio (m/e)

Y-axis - % Relative abundance

Figure 81 Mass spectra of the FA i16:0 from L.jordanis by GC-MS showing its fragmentation pattern and molecular ion at m/e 270.

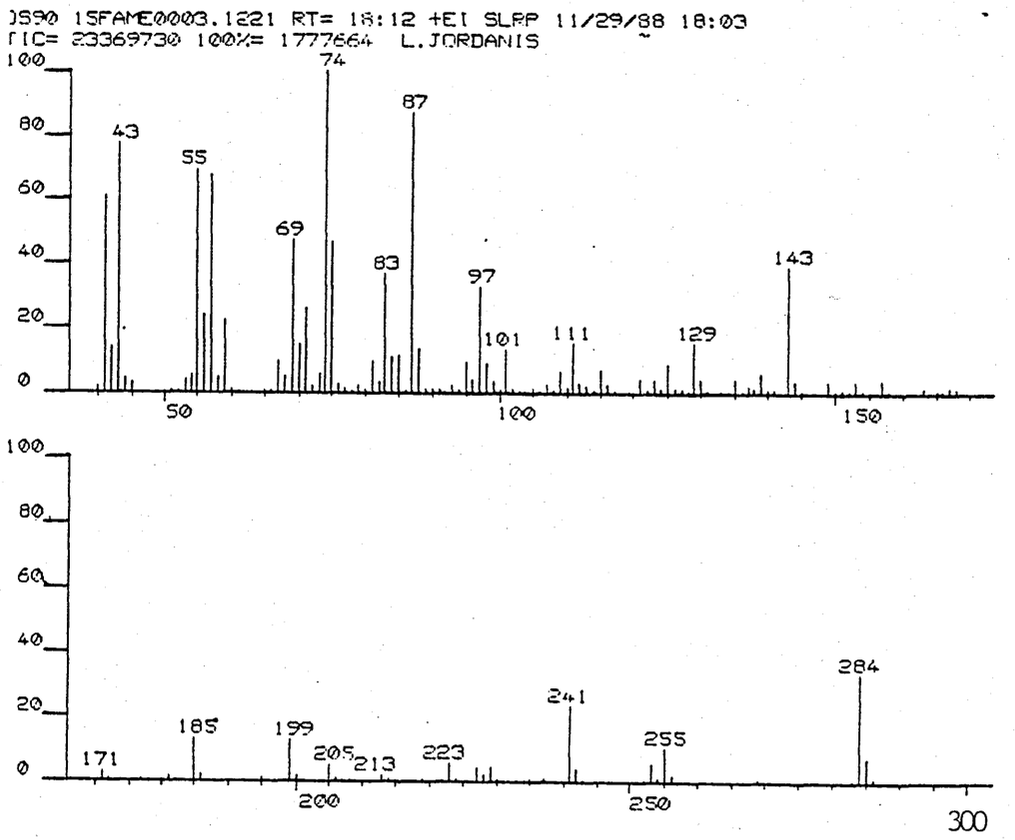


Key

X-axis - mass charge ratio (m/e)

Y-axis - % Relative abundance

Figure 82 Mass spectra of the FA a17:0 from L.jordanis by GC-MS showing its fragmentation pattern and molecular ion at m/e 284.

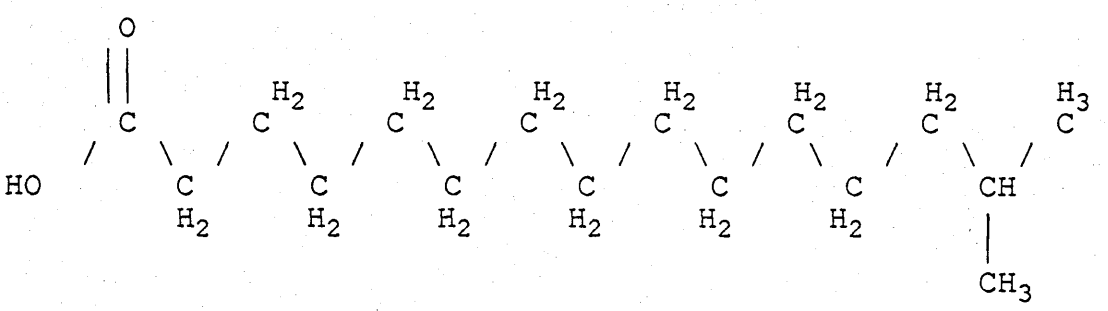


Key

X-axis - mass charge ratio (m/e)

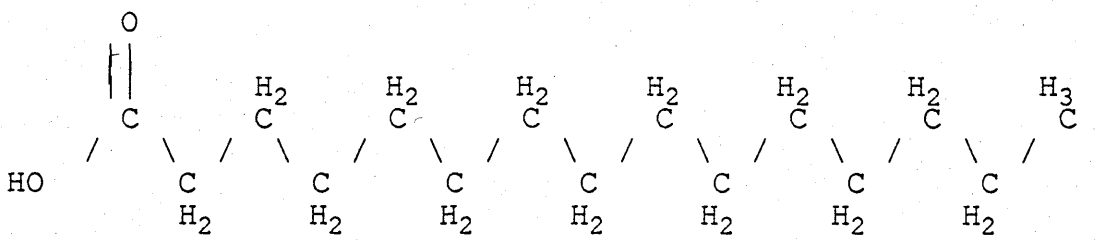
Y-axis - % Relative abundance

Figure 83 Structures of Bacterial Fatty Acids



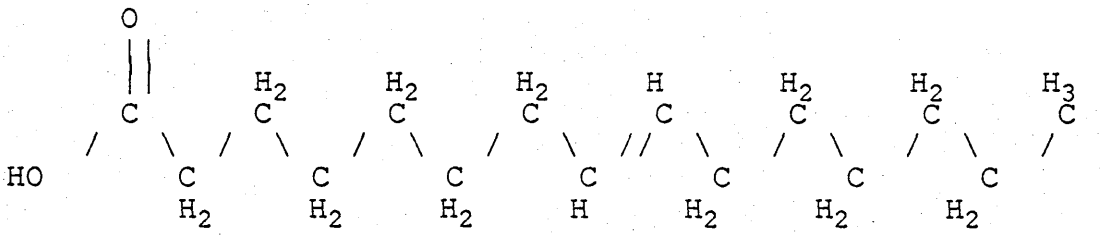
14-methyl pentadecanoic acid (iso-hexadecanoic acid: i16:0)

Figure 84



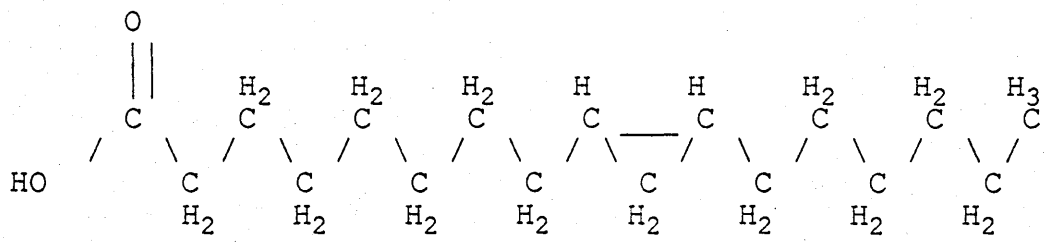
hexadecanoic acid (n16:0)

Figure 85 Structure of bacterial fatty acids cont.



9-hexadecanoic acid (n16:1)

Figure 86



9,10 methylene hexadecanoic acid (cyclopropane heptadecanoic acid: cyc 17)

9-hexadecanoic acid, and that the cyclopropane 17 (cyc 17) is 9,10-methylene hexadecanoic acid (Wait et al. 1988).

3.7 Ubiquinones analysed by reversed-phase thin layer chromatography (RPTLC)

Figure 87 shows the relationship between the number of isoprenoid units and the relative mobility (R_F) of ubiquinones on RPTLC plates. To obtain qualitative information some plates were examined under U.V. light (254 nm) and areas of U.V. absorption representing ubiquinones were observed. Although absorption patterns were seen, it was difficult to estimate relative concentrations of ubiquinones. The phosphomolybdate spray, although not specific for ubiquinones, did yield more detail in the areas where ubiquinones were expected from Rf values. This greater sensitivity and the resultant semi-permanent RPTLC plate made results easier to read and record. This outweighed the disadvantage of other lipids being stained by the phosphomolybdate (figure 88).

The method used for the saponification of the extracts removed many of the other lipids observed on the RPTLC plate (figure 89) which chromatographed beyond the area where ubiquinones were resolved. Results by this method were compared, for 10 strains, with direct extraction. One strain (L.maceachernii) showed less Q-10 in the saponified extract (figure 89, lane D) than in the direct extraction (figure 90, lane D). The direct extraction procedure took less time

Figure 87 Plot of the R_f values on RPTLC (log scale) of ubiquinones against the number of isoprenoid units.

A - purified standards, Q-6 and Q-10 (Sigma)
B - ubiquinones extracted from L.pneumophila SG 1
Q - No. of isoprenoid units

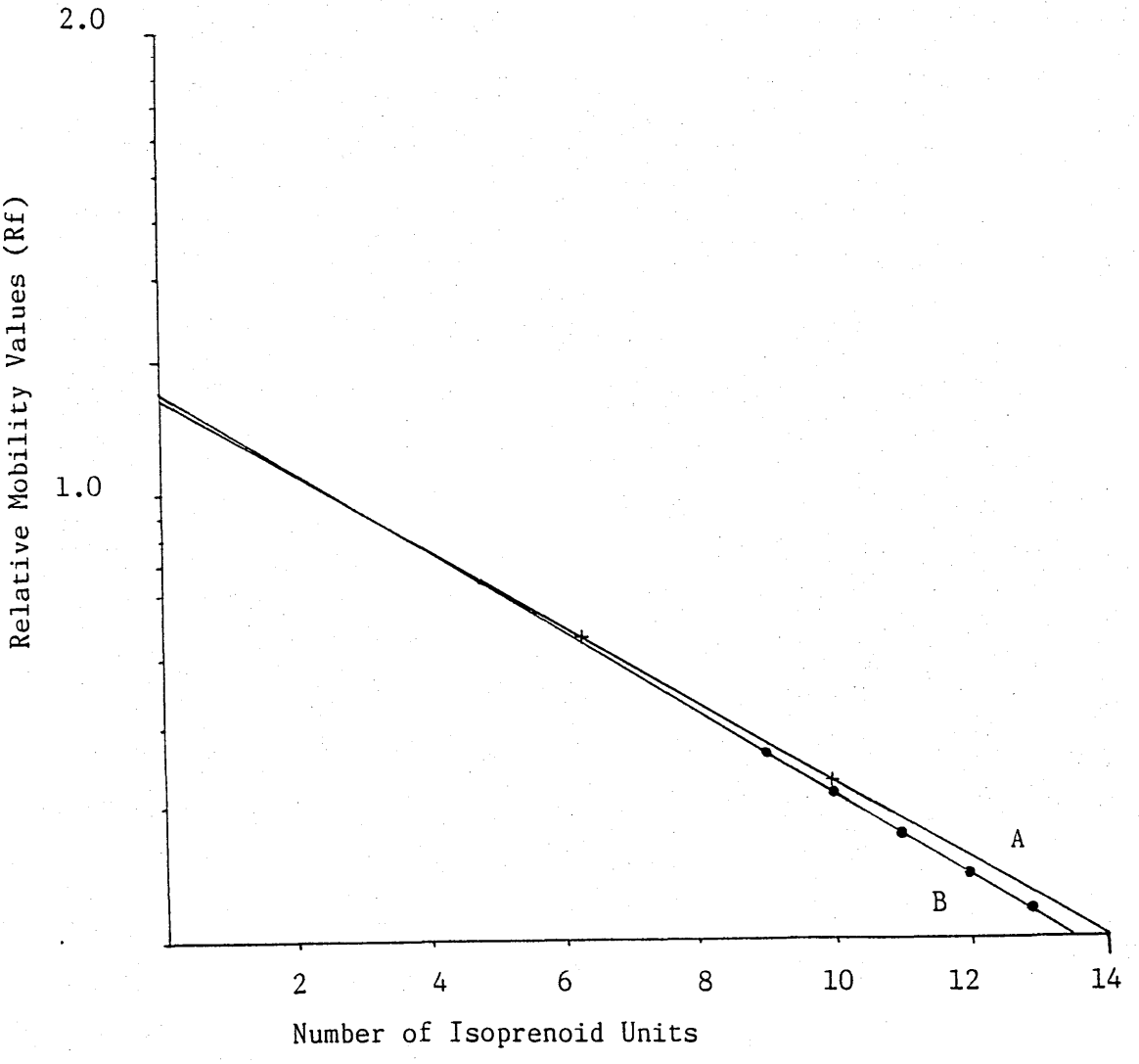


Figure 88 RPTLC plate of profiles of ubiquinones extracted by the direct extraction method from 14 type strains of L.pneumophila.

Lanes A-N were loaded with extracts from SG's 1-14 respectively, lane E; SG 5 [Dallas 1E strain] together with Q-6 (lane O) and Q-10 (lane P) standards (Sigma).

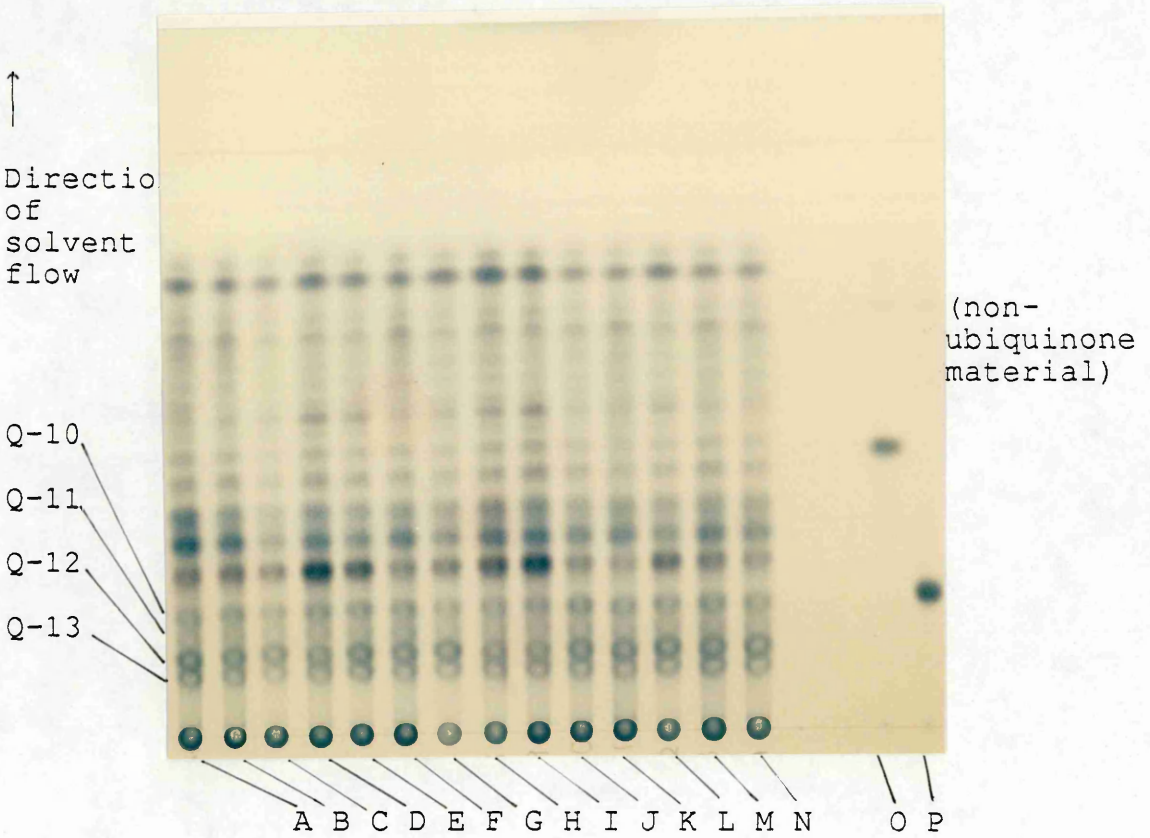
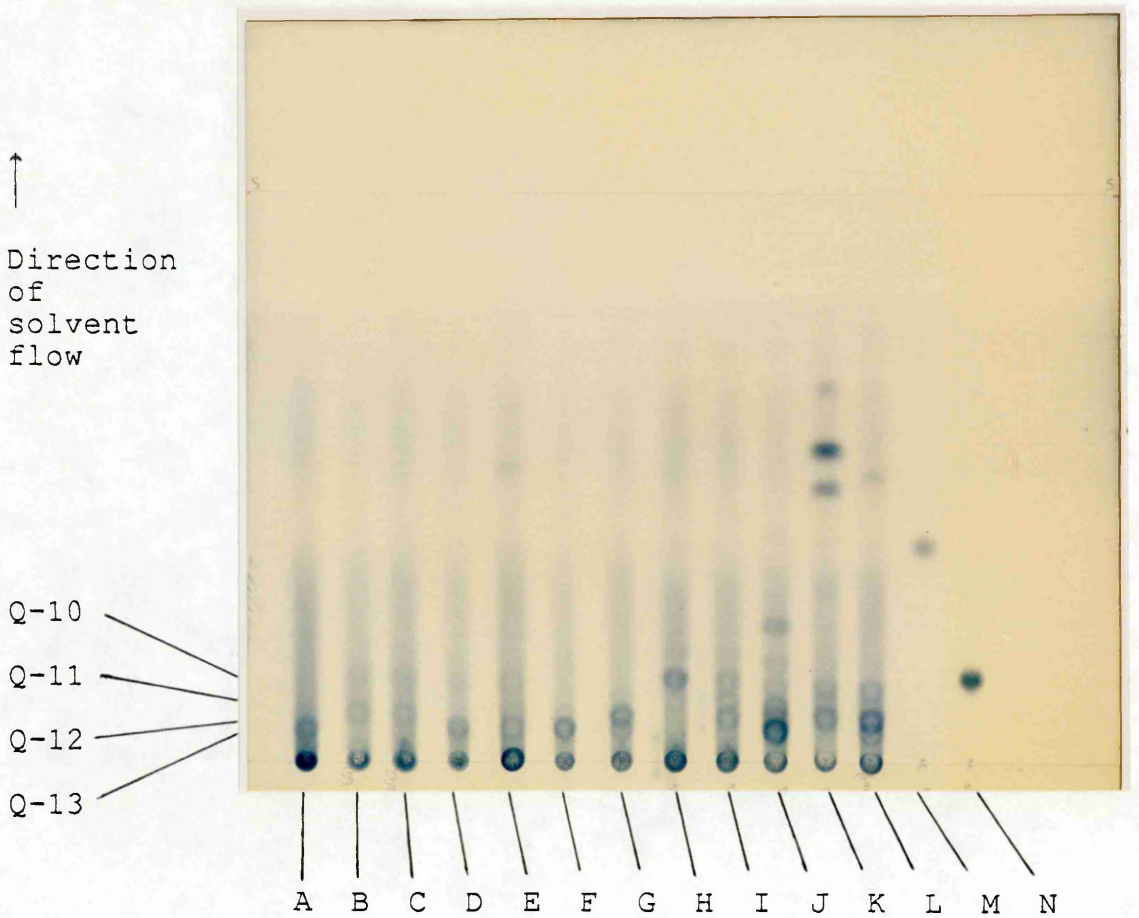


Figure 89 RPTLC plate of Legionella ubiquinone profiles (where all extracts were saponified).

Legionella species were loaded as follows: L.israelensis (lane A); L.longbeachae SG 1 (lane B); L.longbeachae SG 2 (lane C); L.maceachernii (lane D); L.micdadei (lane E); L.moravica (lane F); L.nautarum (lane G); L.oakridgensis (lane H); L.parisiensis (lane I); L.quateriensis (lane J); L.rubrilucens (lane K); L.pneumophila SG 1 (Knoxville-1, lane L); and two ubiquinone standards (Sigma) Q-6 (lane M) and Q-10 (lane N).



and thus was chosen to compile the library of ubiquinone profiles of the Legionellaceae.

Table 27 was compiled from the results of ubiquinone analyses by RPTLC of 36 Legionella species. All analyses by RPTLC were repeated at least twice to confirm ubiquinone profile stability, with identical results being obtained for each Legionella strain tested. All strains analysed contained ubiquinones with 10-13 isoprenoid units in their side chains. Fourteen of the strains had trace to small amounts of Q-14, but L.feeleii SG 1 & 2 and "L.geestiae" had substantial amounts of this ubiquinone. Q-9 was found in 27 of the strains analysed including L.longbeachae SG 1 and 2, and seven strains of L.quinlivanii (table 27). However, Q-9 was more difficult to detect on a RPTLC plate since it ran close to Q-10 and other lipid components. Visually, the other ubiquinones were more easily distinguished.

There were no qualitative or quantitative differences detectable in the ubiquinone profiles of the L.pneumophila strains analysed (figure 88) but in contrast many differences, both qualitative and quantitative were evident when profiles of some of the other Legionella species were examined (figures 90 and 91).

The ubiquinone profiles of 78 Legionella including possible new species (Wilkinson et al., 1990) allowed each strain to be placed in one of five groups designated A-E (table 27). This system of grouping legionellae according to their

Figure 90 RPTLC of Legionella ubiquinone profiles extracted by the direct extraction method of 11 Legionella species.

These were loaded as follows: L.londoniensis (lane A); L.longbeachae SG 1 (lane B); L.longbeachae SG 2 (lane C); L.maceachernii (lane D); L.micdadei (lane E); L.moravica (lane F); L.nautarum (lane G); L.oakridgensis (lane H); L.parisiensis (lane I); L.quateriensis (lane J); L.rubrilucens (lane K); L.pneumophila SG 1 (Knoxville-1, lane L); and two ubiquinone standards (Sigma) Q-6 (lane M) and Q-10 (lane N).

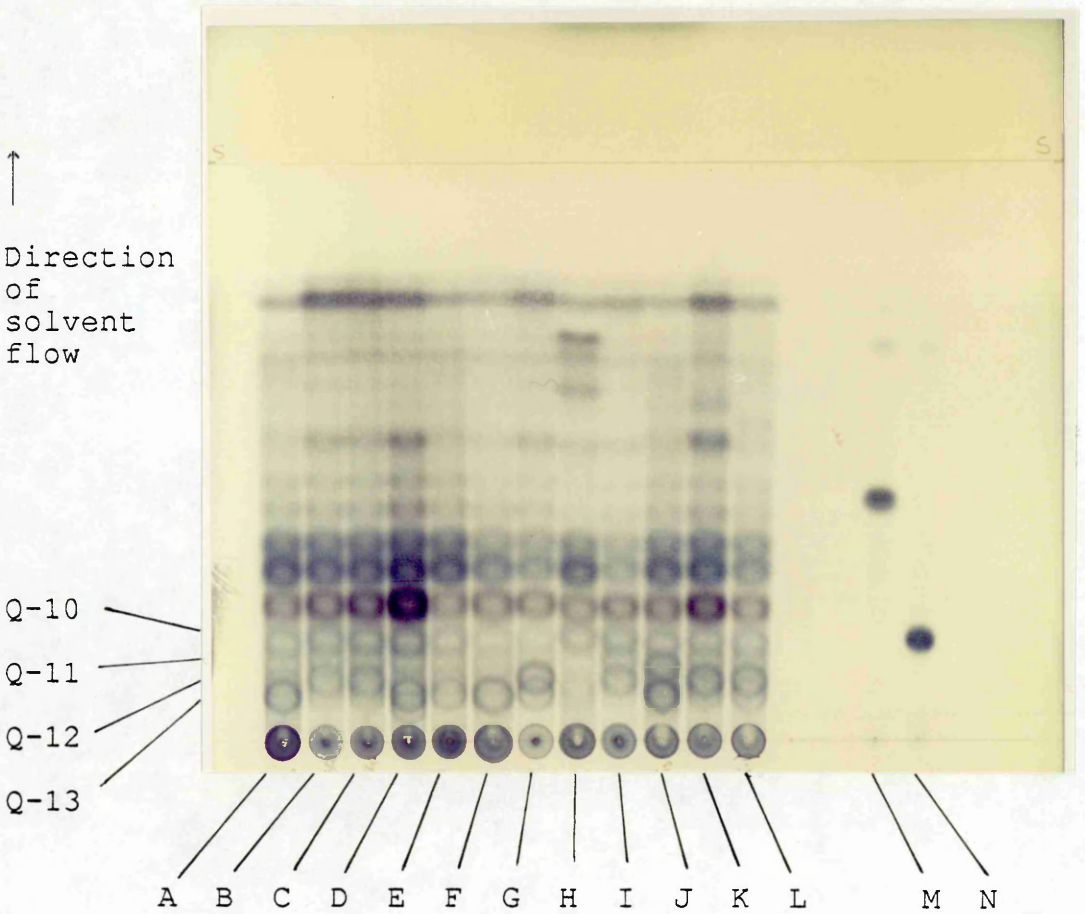


Figure 91 RPTLC plate of ubiquinone profiles extracted by the direct extraction method of 12 strains of Legionella.

These were loaded as follows: L.dumoffii (lane A); L.erythra (lane B); L.feeleii SG 1 (lane C); L.feeleii SG 2 (lane D); "L.geestiae" (lane E); L.gormanii (lane F); L.hackeliae SG 1 (lane G); L.hackeliae SG 2 (lane H); L.israelensis (lane I); L.jamestowniensis (lane J); L.jordanis (lane K); L.pneumophila SG 1 (Knoxville-1) (lane L) together with two ubiquinone standards, Q-6 (lane M) and Q-10 (lane N).

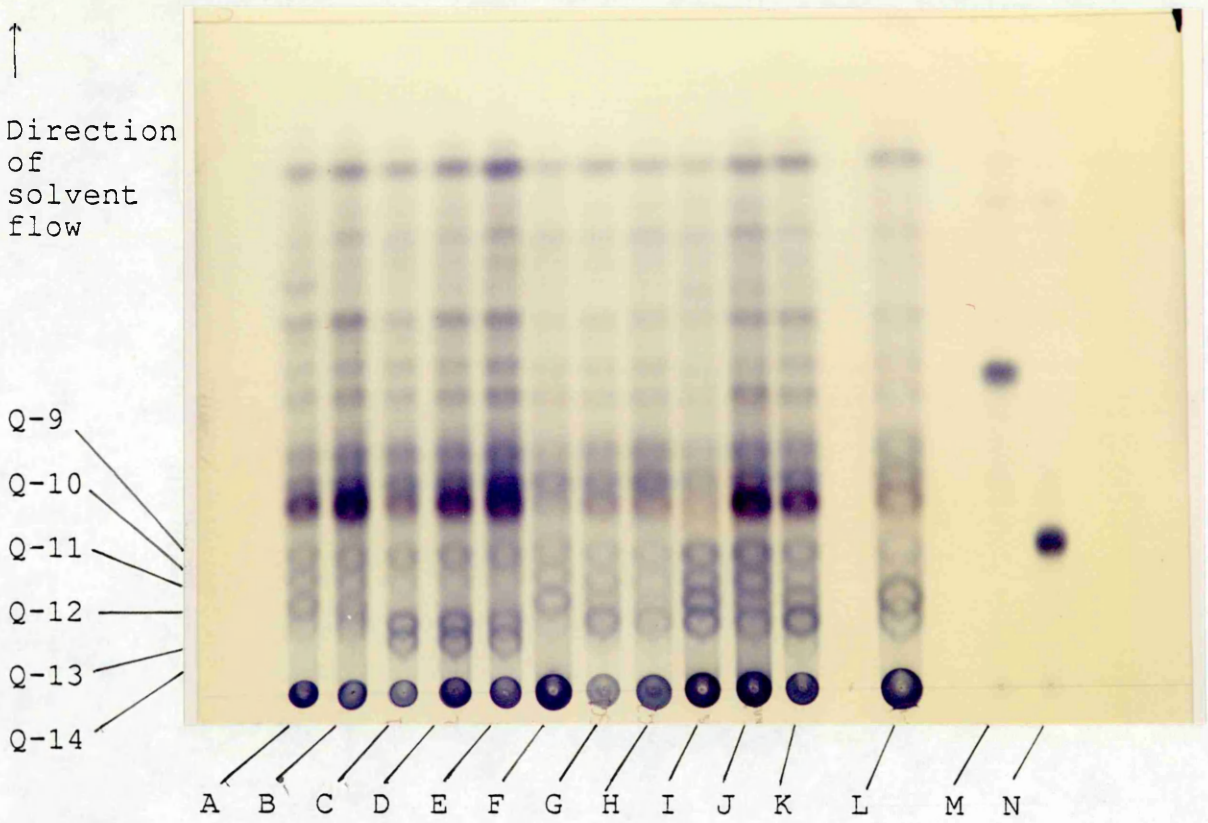


Table 27 Ubiquinone profiles of the Legionellaceae

SPECIES	Ubiquinone content Q-9	Q-10	Q-11	Q-12	Q-13	Q-14	Group
L.adelaidensis	-	4	4	4	1-2	-	E
L.anisa	tr-1	4	2	3	2	-	D
L.birminghamensis	1	2-3	4	2	1	-	C
L.bozemanii SG1 & 2	-	3-4	2	4	1-2	-	E
L.brunensis	tr	2	3	2	4	tr-1	A
L.cherrii	tr-1	4	2	4	2	-	E
L.cincinnatiensis	-	4	2	2	2	-	D
L.dumoffii	1	3	3	4	2	-	B
L.erythra	1	3-4	3	2-3	2-3	tr	D
L.feeleii SG1 & 2	-	2	1	1	4	3-4	E
"L.geestiae" (1308)	1	2-3	1	1	4	4	E
L.gormanii	-	2-3	2	4	1-2	-	B
L.hackeliae SG1 & 2	-	2-3	2-3	2	4	1	A
L.israelensis	tr	3	3	4	4	tr	E
L.jamestownsiensis	tr	4	3	2-3	2-3	-	D
L.jordanis	-	3	2	3	4	1	A
"L.londoniensis"(1224)	1	3	2	1-2	4	tr	A
L.longbeachae SG1 &2	1	3	2	3-4	1	-	E
L.maceachernii	1	4	1-2	1-2	4	1-2	E
L.micdadei	1	4	2	2	4	-	E
L.moravica	1	2	1	1	4	1	A
"L.nautarum" (1477)	-	1	1	4	2	-	B
L.oakridgensis	-	4	1-2	1	2	-	D
L.parisiensis	-	3-4	3-4	4	1	-	E
L.pneumophila ^	-	2	1	4	2-3	-	B
"L.quateriensis"(1335)	1	2	3	2	4	tr-1	A
L.quinlivanii	1	2	2	4	1-2	-	B
L.rubrilucens	-	2	2	4	1	-	B
L.sainthelensi	-	3-4	3	3-4	2	-	E
L.santicrucis	1	4	3	3	2-3	-	D
L.spiritensis	-	1	1-2	1	4	tr	A
L.steigerwaltii	-	2	2	4	1	-	B
L.tucsonensis	1	3	3	4	2	-	B
L.wadsworthii	-	4	2-3	2-3	1	-	D
"L.worsleiensis"(1347)	-	1-2	1	3	4	-	A
* A/36	-	2	3	4	1	-	B
* B/86	-	4	2	1	3	-	D
* C/449	-	3	4	2-3	1-2	-	C
* D/500	-	2	2	4	2	-	B
* D/532	-	3	2	4	2	-	B
* E/594	-	2	1	1-2	4	-	A

KEY

"..." Proposed new species
* Possible new species
^ SG 1-14 and SG Lansing-3
tr-trace amounts detected
1,2,3,4- arbitrary value to estimate quantity of Q

predominant ubiquinone is simple both in terms of definition of a group and of allocation of strains to a group. It should not be confused with that of Lambert and Moss (1989) where 23 Legionella species were placed in groups according to their ubiquinone profiles as analysed by HPLC. The 5 groups dividing the Legionellaceae are distributed as follows; group A, 9 species (21%); group B, 12 species (28 %); group C, 2 species, (5%); group D, 9 species (21 %) and group E, 11 species, (25%). Legionella species assigned to any one group may be further differentiated if necessary on the relative amounts of ubiquinones other than that which is dominant.

All the strains of L.pneumophila including L.pneumophila subspecies pneumophila (DNA group 1), L.pneumophila subspecies fraseri (DNA group 2) and L.pneumophila subspecies pascullei (Brenner et al. 1988) produced ubiquinone profiles which were indistinguishable.

These were assigned to group B (table 27) as Q-12 was present in the highest relative quantity. Groups A, C, and D contained species whose highest relative ubiquinone concentration was Q-13, Q-11 and Q-10 respectively. Group E contained species where no one ubiquinone predominated i.e. two or more ubiquinones were observed in the highest quantity.

Ubiquinone profiles analysed by RPTLC may also be used to discriminate between the blue/white autofluorescent

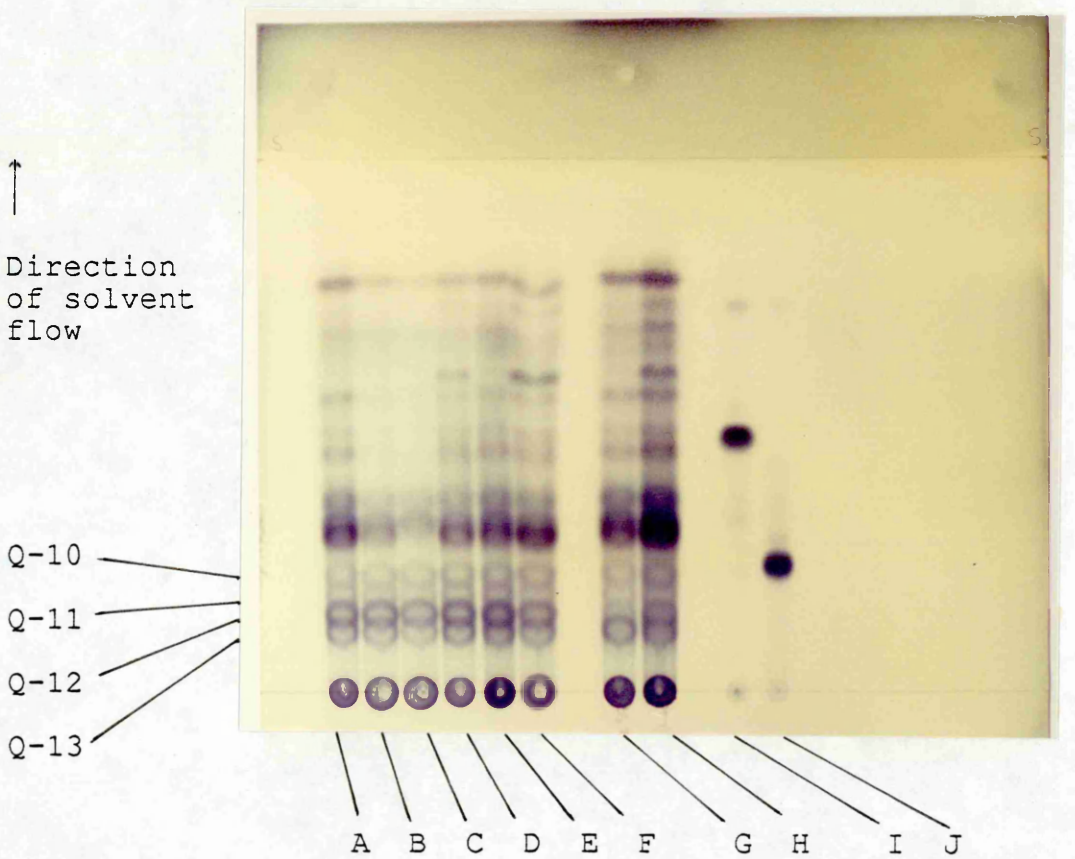
and the red autofluorescent species where these may be difficult to identify serologically. Of the eight currently described blue/white autofluorescent species, L.dumoffii, L.gormanii, L.steigerwaltii, and L.tucsonensis were designated members of group B by their ubiquinone profiles and could be differentiated from L.bozemanii, L.cherrii and L.parisiensis of group E and L.anisa of group D. The two red autofluorescent species L.erythra and L.rubrilucens could also be differentiated as they were assigned to groups D and B respectively.

The reproducibility of ubiquinone profiles was investigated in two legionellae, a human pathogenic strain L.pneumophila SG 1 (Knoxville-1 strain) and an environmental strain "L.geestiae". The relative amount of each component was constant whether cultures were incubated for 48, 96, 192 or 240 h at 37°C and also whether grown on Oxoid or in-house BCYE.

RPTLC has been used to analyse strains other than the stock strains shown in table 1. Six legionellae isolated from the environment reacted with unabsorbed L.hackeliae SG1 rabbit antiserum (IFA titre = 512, positive control with the homologous organism 1024). Their ubiquinone profiles (figure 92), matched closely those of L.pneumophila and not those of L.hackeliae. Further studies of fatty acids by GLC supported these findings and the organisms were subsequently identified using serogroup

Figure 92 RPTLC plate of ubiquinone profiles from six legionellae isolated from environmental samples.

The plate was loaded as follows: extracts from environmental samples, (lanes A-F) ; and two type strains, Lane G, L.hackeliae SG 1; lane H, L.pneumophila SG 1 (Knoxville-1); lane I Q-6 standard (Sigma) and lane J, Q-10 standard (Sigma).



specific rabbit antisera to be L.pneumophila SG 1 (2 strains), SG6 or SG12.

Comparisons of ubiquinone profile for 10 Legionella species have been made with those of two other groups of workers who used HPLC (table 28). One group (Collins and Gilbart 1983, Gilbart and Collins, 1985), expressed their HPLC peaks of ubiquinones as percentages of the total ubiquinone content. The other group (Lambert and Moss, 1989) reported their relative concentrations of ubiquinones based on a visually estimated scale of 1-4. As the method of presentation between these workers differed, comparison of results was achieved by applying the simple method of grouping based on the recognition of predominant ubiquinone for 10 Legionella species. Where only the predominant ubiquinones were used to group the legionellae, the method of RPTLC compared well with the results produced by HPLC. However, it was noted that some differences were observed between the quantities of ubiquinones of some legionellae both between RPTLC and HPLC results and also between HPLC results where these were carried out in different laboratories. We observed greater amounts of Q-10 in 3 out of 10 of these species (L.jordanis, L.longbeachae and L.micdadei) than reported by other workers. The reason for this is unclear but the possibility that it could be due to a lipid component of R_f value similar to that of Q-10 warrants further investigation including mass spectrometry.

Table 28 A summary of the groups into which 10 Legionella species fall when analysed by three groups of workers using HPLC or RPTLC to determine ubiquinone profiles.

Species	Collins and Gilbart 1983, 1985 [HPLC]	Lambert and Moss 1989 [HPLC]	Mitchell and Fallon 1990 [RPTLC]
L.bozemanii	E	E	E
L.dumoffii	B	E	B
L.gormanii	B	E	B
L.jordanis	A	E	A
L.longbeachae	B	E	E
L.micdadei	A	A	A
L.oakridgensis	D	D	D
L.pneumophila	B	B	B
L.sainthelensi	B	E	E
L.wadsworthii	D	D	D

Letters A-D represent the groups into which Legionella species have been compiled based on the predominant ubiquinone(s) in each of their profiles (Mitchell and Fallon, 1990)
Predominant ubiquinone: group A, Q-13; group B, Q-12; group C, Q-11; group D, Q-10; group E, species with two or more ubiquinones present in equally high cocncentration.

Differences between analytical methods may account for other minor differences noted between laboratories.

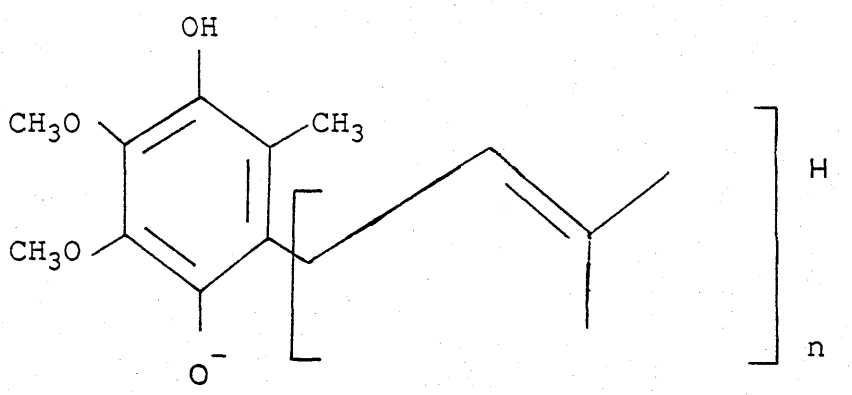
3.7.1 Confirmation of ubiquinones by FAB-ms

The contents of hexane-soluble extracts were complex and although these crude extracts were analysed on RPTLC plates and the ubiquinones tentatively identified by comparing R_f values of the resolved spots to that of known ubiquinone commercial standards (Q-6, Q-10, Sigma) and a previously defined ubiquinone profile of L.pneumophila SG 1, it was desirable to provide confirmation of the presence of ubiquinones in some of these extracts. Mass spectrometry using a direct insertion probe is often employed to identify ubiquinones where a base peak at m/z 235 (mass/charge ratio) is observed, with a further intense peak at m/z 197 and strong molecular ions at even mass numbers corresponding to the molecular weight of each ubiquinone species are present (e.g Q-11, Q-12 and Q-13 have molecular ions at m/z 930, 998 and 1066 respectively). Here, an alternative technique was used - negative-ion Fast Atom Bombardment (FAB) mass spectrometry. This technique has not previously been applied to the identification of ubiquinones, but from the results produced, gave unequivocal data and quite good sensitivity (Wait, personal communication , Wait and Hudson, manuscript in preparation). The complexity of the crude extract would have caused problems in the interpretation of data produced by FAB-ms and so a preparative step to remove

other non-ubiquinone components was included (section 2.16.3).

Figure 93 shows the data produced for a ubiquinone standard (Sigma), Q-10 with a molecular ion at 863; a value indicating that the ubiquinone has been reduced.

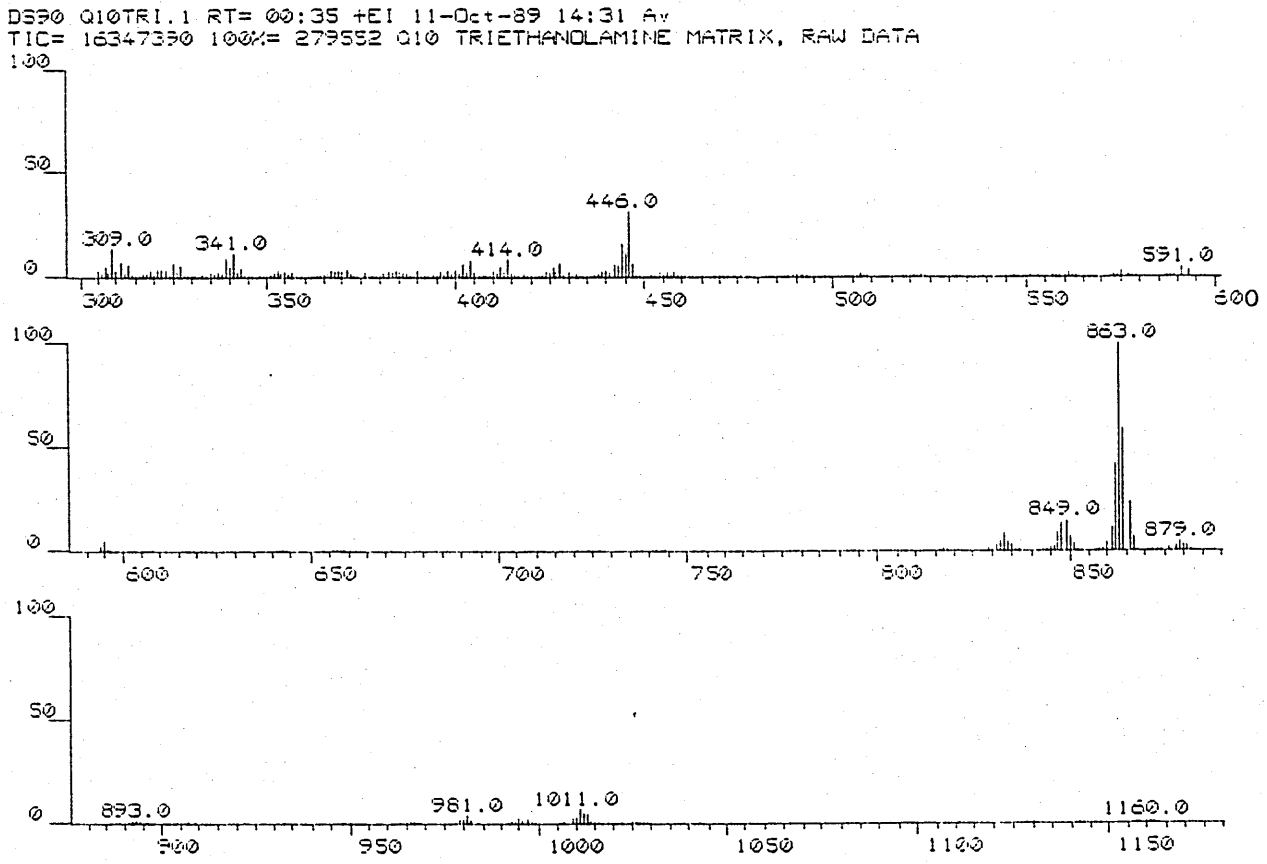
Normally under negative ion conditions deprotonated molecular ions (i.e. $[M-H]^-$) are observed. In the case of ubiquinones in triethanolamine, deprotonated molecular ions of the reduced quinones (shown below) are observed so that Q-6 and Q-10 standards produce molecular ions at m/z (mass/charge) 591 and 863 respectively. The technique was applied to TLC-purified extracts of L.jamestowniensis and intense signals at m/z 1067 and 999 (figure 94)



Structure of reduced ubiquinone (Q-n)

corresponding to reduced protonated molecular ions of Q-13 (M.W. = 1066) and Q-12 (M.W. = 998) were observed.
Loss of methyl groups from the species generates the ions

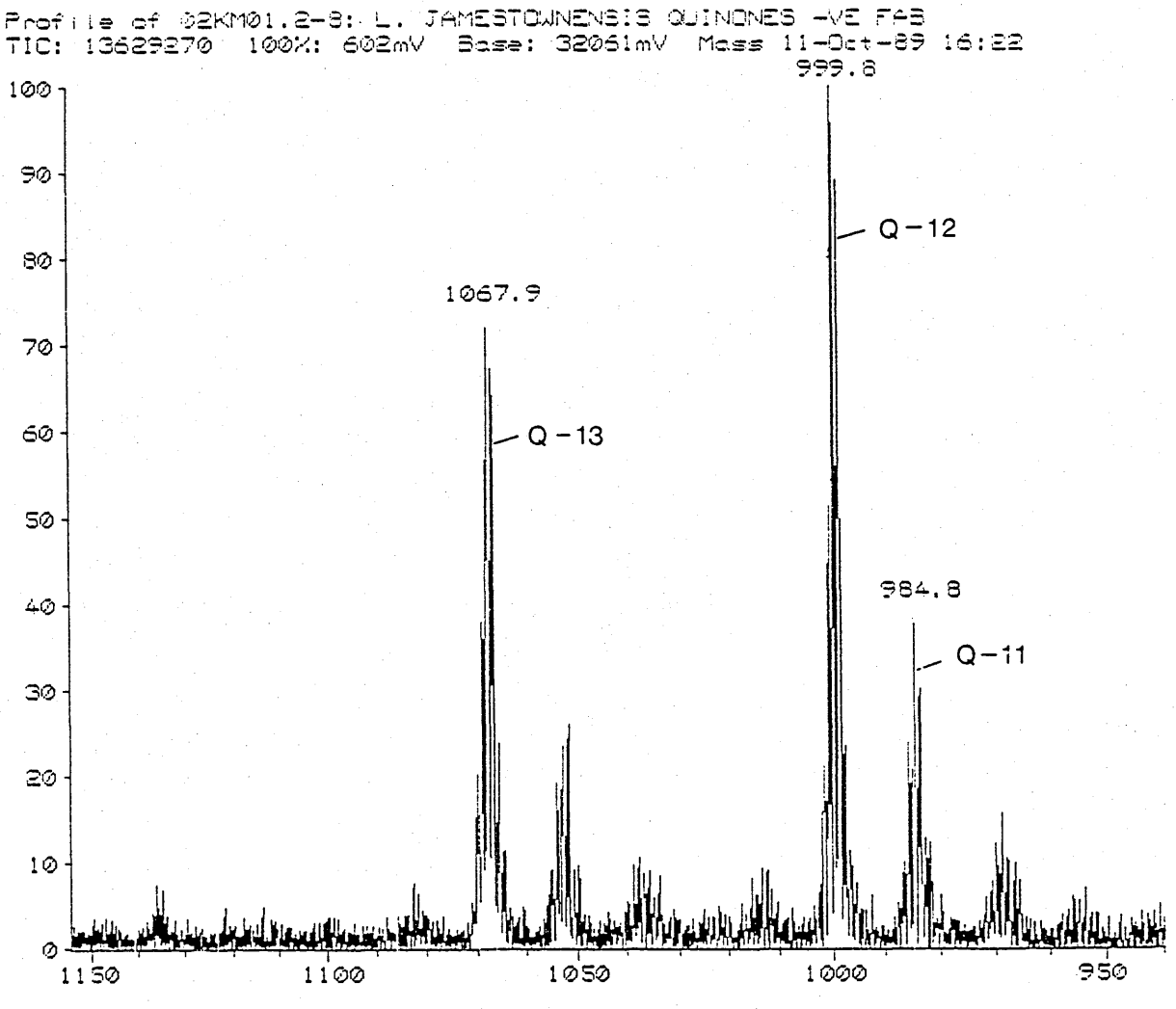
Figure 93 Mass spectra of a Q-10 ubiquinone (Sigma) standard analysed under negative-ion FAB conditions.



Key

X-axis - m/e ratio
Y-axis - % Relative abundance

Figure 94 Mass spectra of TLC-purified ubiquinones of L.jamestowniensis analysed under negative-ion FAB conditions.



Key

X-axis - m/e ratio
Y-axis - % Relative abundance

m/z 1052 and 984. A weaker signal at m/z 931 corresponded to Q-11 (M.W. = 930).

Ubiquinones, Q-10, Q-11, Q-12 and Q-13 were also detected in the TLC-purified extract of L.pneumophila SG 1 (Knoxville-1) by FAB-MS with very weak signals at m/z ratios corresponding to Q-9 and Q-14. Therefore, care must be taken in handling ubiquinone material to prevent its loss during purification before mass spectrometry.

3.8 Identification of Legionella isolates from the environment and from humans

The purpose of a library of FA profiles of type strains of each species of the Legionellaceae was to allow the FA profiles of environmental and clinical isolates to be compared with those of the library as an aid to identification. Possible new Legionella serogroups or species could also be detected.

3.8.1 Environmental isolates

Table 29 shows the FA data of twelve environmental Legionella isolates identified by serological methods.

All FA's of the first eight strains, except one, are within the range $\bar{x}$ +/- one S.D.. This FA, i14:0 of 87/31871 is only 1.9 % greater than the upper limit (i.e. $\bar{x}$ + one S.D.) of the same FA determined from 49 analyses (table 21) of 24 strains of L.pneumophila (table 1, Materials and Methods).

Six of these strains (88/42791, 88/42787, 88/42784,

Table 29 Mole % nonhydroxy fatty acid composition by gas-liquid chromatography of 12 legionellae isolated from the environment.

Legionellas isolated from the environment	Mole % nonhydroxy fatty acid composition by gas-liquid chromatography																
	i14:0	i15:0	a15:0	n15:1	n15:0	i16:1	i16:0	n16:1	n16:0	a17:1	i17:0	a17:0	cyc 17	n17:0	n18:0	n19	n20
87/31871 (L.pneumophila)	9.2	-	14.5	2.7	-	2.4	45.6	8.9	3.2	-	-	6.1	5.1	-	1.3	-	-
P185 (L.pneumophila SG9 (?))	4.9	-	11.7	2.1	-	3.3	32.3	14.1	6.4	-	-	7.6	7.9	3.5	1.4	4.7	-
88/42791 (Bellingham)	7.6	-	15.5	-	-	3.9	41.3	9.3	3.4	-	-	8.2	7.9	-	2.8	-	-
88/42787 (Olda)	6.3	-	16.8	-	-	4.9	40.3	8.2	7.0	-	-	5.7	7.2	1.3	2.8	-	-
88/42784 (Olda)	7.1	-	13.5	-	-	2.7	39.8	8.7	6.0	-	-	10.1	7.0	2.8	2.3	-	-
88/42785 (L.pneumophila SG6)	6.8	-	13.6	-	-	3.6	38.4	11.7	6.0	-	-	7.2	6.1	3.3	3.1	-	-
88/42788 (L.pneumophila SG6)	7.3	-	14.4	-	-	3.5	42.3	11.1	4.8	-	-	7.9	3.2	2.6	2.7	-	-
88/42783 (L.pneumophila SG12)	6.0	-	13.8	-	-	4.0	38.7	8.4	7.9	-	-	9.6	8.4	1.0	3.2	-	-
89/42869 (L.longbeachae SG2)	15.3	-	12.1	5.1	2.5	-	29.1	18.4	8.6	-	-	-	7.5	-	1.3	-	-
89/13077 (L.hackeliae SG1)	1.2	-	38.2	1.0	-	-	14.5	4.8	4.8	1.2	1.8	24.0	7.1	-	1.2	-	-
89/20794 (L.quateriensis)	6.4	-	5.2	4.1	-	-	22.9	39.8	6.0	-	-	2.2	1.6	-	11.7	-	-
88/1058 (L.micdadei)	-	-	38.8	2.4	4.4	1.7	11.9	7.2	6.4	4.9	-	19.7	2.7	-	-	-	-

88/42785, 88/42788 and 88/42783) have previously been studied to determine their ubiquinone profiles (Mitchell and Fallon, 1990) as they cross-reacted with unabsorbed rabbit antiserum raised to L.hackeliae SG1.

GLC of their respective FA's produced profiles indistinguishable from those of L.pneumophila type strains as did the ubiquinone profiles. The identification by IFAT with specific antisera confirmed their identity as L.pneumophila SG1 (three strains), SG6 (two strains) and SG12 (one strain).

The last four strains in table 29 had profiles similar to those of known type strains. However, some differences between the isolate and the type strains were obvious. The isolate identified serologically as L.longbeachae SG 2, 89/42869, showed twice the amount of i14:0, 7% less a15::0 and no a17:0, compared with 9.2% of a17:0 in the type strain, Tucker-1. By RPTLC analysis of its extracted ubiquinones, it produced a profile indistinguishable from that of L.longbeachae (but was also similar those of L.cherrii, L.oakridgensis and L.sainthelensi, also members of group E). The isolate, 89/13077, serologically identified as L.hackeliae SG 1 showed 2% less i14:0, no n15:0 and n15:1, 4.6% less n16:1 and 8.5% more a17:0 than the type strain, Lansing-2. The ubiquinone profile of 89/13077 was indistinguishable from that of the type strain of L.hackeliae SG 1 (in addition to other legionellae

including L.quateriensis also of group A). Isolate-89/20794, serologically identified as L.quateriensis produced a FA profile with half the a15:0, 2% less n15:1, no n15:0, 9% more n16:1, 3% less a17:0 and no n17:0 than found with the type strain (proposed new species no. 1335, see table 1). Isolate-88/1058 was serologically identified as L.micdadei and was the organism responsible for the biggest outbreak of Pontiac fever outside North America (Goldberg et al., 1989 Fallon and Rowbotham, 1990) which occurred at Lochgoilhead on the west coast of Scotland in January 1988. Its FA profile, when compared to that of the type strain (figures 95 and 96, p236), Tatlock, showed detectable amounts i.e. >0.5% of n15:0 and n15:1, 9% less a17:0 and 1.2% less cyc 17 (table 29). Its ubiquinone profile determined by RPTLC was identical to that of the L.micdadei type strain (table 27).

FA and ubiquinone profiles were examined for strain ML-76 without any previous information being available about this strain. Its ubiquinone profile was indistinguishable from the ubiquinone profile of L.spiritensis (Mt. St. Helens-9). This was easily identified as it had Q-13 as its major ubiquinone present in very high concentration compared with other ubiquinones in the profile. Also, by FA analysis it appeared to show a profile identical with that of the type strain (table 30). It was later revealed that ML-76 was an environmental isolate and restriction fragment length polymorphisms (produced by digesting the extracted bacterial

Table 30 Mole % nonhydroxy fatty acid compositon of two Legionella spiritensis strains.

Legionella species	Mole % nonhydroxy fatty acid composition by gas-liquid chromatography														
	i14:0	i15:0	a15:0	n15:1	n15:0	i16:1	i16:0	n16:1	n16:0	a17:1	i17:0	a17:0	cyc 17	n17:0	n18:0
Part A															
L. spiritensis	1.7	-	18.7	0.9	-	3	26.2	16.9	7.4	2.6	-	19.9	0.5	0.8	1.3
ML-76	1.7	-	20.3	1.3	-	3	24	16.4	6.5	2.7	-	18.9	2.4	1.4	1.2
Part B															
L. spiritensis	2.5	-	19.5	0.8	0.5	4.7	31.8	14.7	6.3	2.6	-	14	0.4	0.7	0.8
ML-76	1.6	-	21.8	1.2	0.8	2.6	26.6	14.9	6.6	2.1	-	16.9	1	1	1

Part A : Figures published by Harrison et al. (1988)

Part B : Results of analyses obtained in this laboratory

ML-76 : Serovariant of L.spiritensis

DNA with the restriction enzyme Nci 1 followed by separation using agarose electrophoresis) showed the Legionella to be L.spiritensis, with this confirmed by DNA homology (Harrison et al., 1988).

3.8.2 Patient isolates

The FA profiles of two legionellae (table 31) isolated from different patients were investigated.

Isolate-88/24485 was from the sputum of a male patient and identified serologically as L.anisa. The FA profile differed quantitatively from type strain (table 21) where i14:0 was increased by 0.8 mole %, a15:0 by 3.4 mole %, i16:0 by 1.7 mole %, n16:1 by 2.5 mole %, a17:0 by 5.1 mole %. The following were present in decreased amounts; n16:0 by 4.6 mole % and cyc 17 by 4.7%. The only qualitative difference found was that the patient isolate produced less than 0.5% n15:0 which was therefore not included as part of the overall profile. The type strain of L.anisa produced 1.7 mole % of this FA.

Another patient strain, 85/46768, identified serologically as L.pneumophila SG 1. When its FA profile was compared with those compiled in table 19 for the same species, the only difference found was that the patient isolate produced no n17:0, 1.4 mole % less than a typical L.pneumophila strain. Quantitative differences are almost all eliminated if the environmental and patient isolates are compared to $\bar{x}$ +/- two times S.D. for each FA of the type

Table 31 Mole % nonhydroxy fatty acid composition by gas-liquid chromatography of two Legionella strains isolated from two patients' sputum samples.

Legionellas isolated from patients	Mole % nonhydroxy fatty acid composition by gas-liquid chromatography														
	i14:0	i15:0	a15:0	n15:1	n15:0	i16:1	i16:0	n16:1	n16:0	a17:1	i17:0	a17:0	cyc 17	n17:0	n18:0
88/24485 (L.anisa)	5.2	-	31.5	1.7	-	-	25.3	14.1	4.1	-	-	12.4	3.2	1.4	1.2
85/46768 (L.pneumophila SG 1)	7.2	-	13.6	1.8	-	5.2	42.8	8.3	3.5	-	-	7.6	7.5	-	2.5

strains.

In this laboratory, FA (table 21) and ubiquinone profiles (table 27) of either environmental or patient isolates can in most cases be readily matched to those of the type strains. This would support that the GLC of fatty acids and RPTLC of ubiquinones are indeed useful methods to aid in the identification of Legionella species.

3.8.3 86/35784: A new subspecies of L.quinlivanii

This environmental isolate identified as a Legionella species by routine bacteriological methods (i.e. by its growth on BCYE medium but not on horse blood agar, weak staining as Gram-negative rods, etc.) was extracted from the pan of a cooling tower at Glasgow Royal Infirmary.

Serological studies indicated that 86/35784 shared heat-stable antigens with L.sainthelensi and L.oakridgensis but also had a unique antigen (table 32).

In addition, when L.quinlivanii was isolated (Benson et al.,1989) and forwarded to this laboratory, it was used as an immunogen to produce polyclonal rabbit antiserum. This antiserum reacted in IFAT tests to 6 other strains of L.quinlivanii, the homologous organism and 86/35784. The IFA titres (table 33) showed only a weak reaction between the unabsorbed serum raised to strain 1442-AUS-E and 86/35784 compared to higher titres which varied when reacted to different strains of the same species. FA's were extracted from 86/35784, derivitised and analysed by GLC. Its mole %

Table 32 IFA titres of absorbed rabbit antisera reacted to *Legionella* organisms.

Antigens	Antiserum: *L.sainthelensi* absorbed with *L.oakridgensis*	Antiserum: *L.sainthelensi* also absorbed with 86/35784	Antiserum: *L.oakridgensis* absorbed with *L.sainthelensi*
L.sainthelensi	3200/6400	4096	<100
L.oakridgensis	<100	<100	25000
86/35784	2048/4096	16	4096

Antigens	Antiserum: *L.oakridgensis* also absorbed with 86/35784	Antiserum: 86/35784 absorbed with *L.sainthelensi*
L.sainthelensi	<100	128
L.oakridgensis	2048	128
86/35784	<16	4096

Table 33 Anti-L.quinlivanii rabbit antiserum reacted to doubling dilutions of different strains of L.quinlivanii and also isolate-86/35784 in IFAT.

Organism		1442-AUS-E antisera (IFA)
L.quinlivanii	1442-AUS-E	32k
"	1448-AUS-E	4096
"	1449-AUS-E	4096
"	1450-AUS-E	4096
"	1451-AUS-E	4096
"	1452-AUS-E	4096
"	2359-AUS-E	2048
	86/35784	64

FA content is shown in table 21 together with the FA content ($\bar{x}$ +/- one S.D.) of L.quinlivanii calculated from all seven strains. The GLC profiles of 86/35784 when grown on in-house and Oxoid BCYE are shown (figures 97 and 98 respectively).

It can be seen that the FA composition of 86/35784 is similar to that of L.quinlivanii (table 21). However, it differs significantly from L.oakridgensis and L.sainthelensi. 86/35784 has three times less i14:0 than L.sainthelensi whereas L.oakridgensis produces less than 0.5% i14:0. 86/35784 produces nine times more a15:0 than L.oakridgensis. There are also minor quantitative differences in FA profile between these three organisms.

When the ubiquinones are extracted from these organisms and analysed by RPTLC the profiles produced by 86/35784 and L.quinlivanii are identical both having Q-12 as their major ubiquinone and both therefore designated as group B. The other ubiquinones in each of their profiles are also present in similar quantities (according to the densities observed on the RPTLC plates). L.oakridgensis however, has Q-10 as its major ubiquinone and therefore belongs to group D. It is easily differentiated on this basis and is therefore not likely to be of the same species. L.sainthelensi is a member of group E, as both Q-10 and Q-12 are equally dominant and therefore again is easily differentiated from 86/35784 by its RPTLC profile. It is therefore not related at species level.

Key for figures 95-98 where FA's represented by peaks are labelled with letters for their identification.

Notation	Key	Notation	Key
i14:0	a	a17:1	j
i15:0	b	i17:0	k
a15:0	c	a17:0	l
n15:1	d	cyc 17	m
n15:0	e	n17:0	n
i16:1	f	n18:0	o
i16:0	g	n19:0	p
n16:1	h	n20:0	q
n16:0	i		

GLC profiles of FAMES of 4 Legionella strains

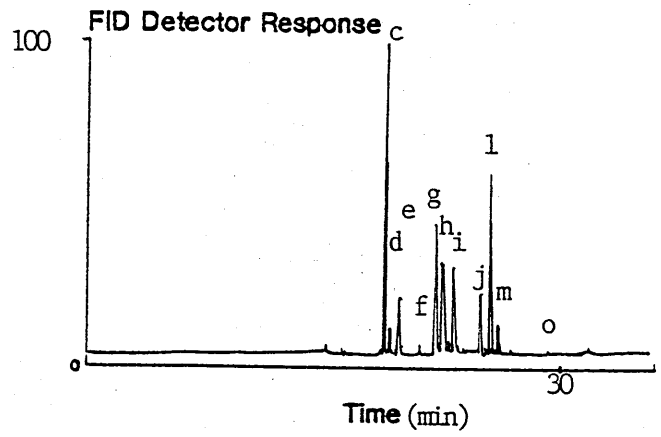


Figure 95

L. micdadei (88/1058)

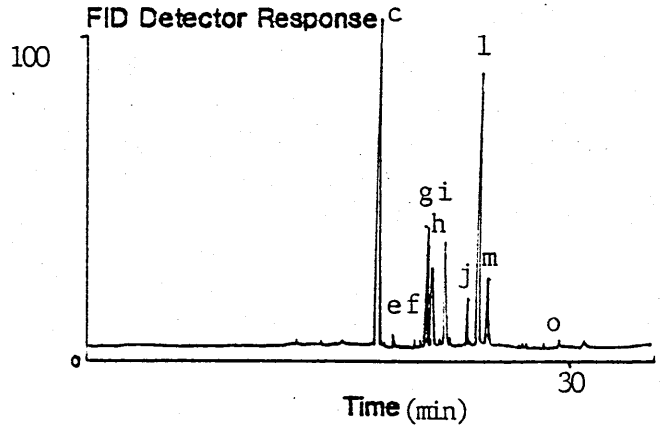


Figure 96

L. micdadei (Tatlock)

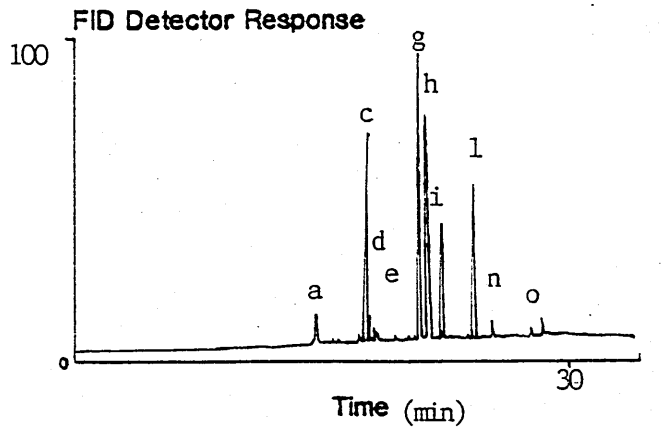


Figure 97

86/35784 (BCYE)

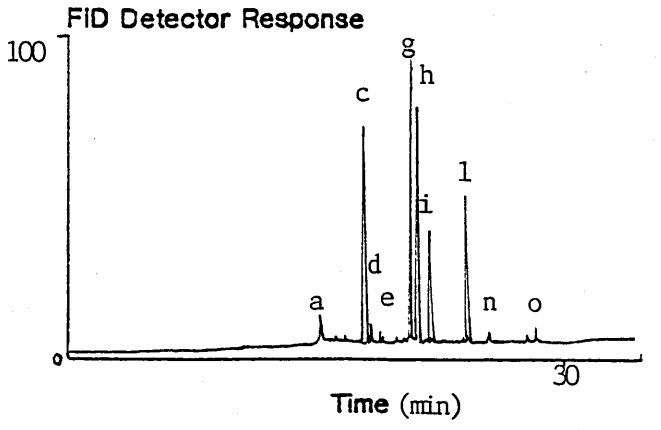


Figure 98

86/35784 (Oxoid)

Guinea pig antiserum to 86/35784 organisms reacted poorly with 6 strains of L.quinlivanii giving IFA titres <16 with four strains, 32 with one strain and <1000 with the type strain 1442-AUS-E (table 34). The titre with the homologous organism was 8192.

There was some indication from these studies that 86/35784 was serologically distinct each of the L.quinlivanii strains tested.

Quantitative DNA hybridisation (S1 nuclease method) studies were performed in which 36 Legionella strains were reacted with ^{32}P-labelled DNA from strain 86/35784 (figure 89).

DNA (^{32}P-labelled) from 86/35784 hybridised to unlabelled DNA of L.birminghamensis and L.steigerwaltii with % relatedness values of 17 and 7 % calculated, respectively (table 35).

The FA's and ubiquinones of these two organisms were compared with that of 86/35784 but even though L.steigerwaltii belonged to ubiquinone group B and produced a ubiquinone profile identical to that of 86/35784, its FA profile differed markedly in that L.steigerwaltii produced 30% more a15:0, 20% less i16:0 and 60% less n16:1. It also differed qualitatively in that it produced cyc 17 unlike 86/35784. L.birminghamensis contained Q-11 as its predominant ubiquinone and is the only member of group C (table 21). Its FA profile differed qualitatively and

Table 34 Anti-86/35784 guinea pig antisera reacted to strains of L.quinlivanii in IFAT.

Organism	86/35784 antisera (IFA)
86/35784	8192
L.quinlivanii 1442-AUS-E	< 1000
" 1448-AUS-E	< 16
" 1449-AUS-E	< 16
" 1450-AUS-E	< 16
" 1452-AUS-E	< 16
" 2359-AUS-E	32

Table 35 Quantitative DNA hybridisation (S1 nuclease method) carried out on the most reactive DNA's.

Source of unlabelled DNA	% Relatedness with labelled DNA from strain 86/35784
86/35784	100
L.steigerwaltii	7
L.birminghamensis	17
L.quinlivanii (1442-AUS-E)	68

Information kindly provided by Grimont, personal communication.

quantitatively from that of 86/35784.

Further DNA hybridisation experiments by the S1 nuclease method (Grimont *et al.*, 1980) revealed that unlabelled DNA from the type strain 1442-AUS-E of *L.quinlivanii* hybridised strongly (68%) with ^{32}P-labelled DNA of 86/35784 (table 35). This level of hybridisation is defined by Brenner *et al.* (1988) as that of a sub-species level.

DISCUSSION

4.1 Production of immunodiagnostic antisera to detect urinary antigen by ELISA

Rabbits were employed in these studies as they are widely used for the production of antisera, they usually respond well to immunisation and convenient volumes of blood can readily be collected. Mice and guinea pigs are less suited to this type of work as they do not satisfy the requirements in the way that rabbits do and although avid antibody has been raised in a goat (Berdal et al., 1979), these animals cannot be accommodated in our animal house. Innumerable immunisation protocols have been described in the literature but few have been based on comparative data.

Four protocols, two of which used both NZW and Half Lop breeds of rabbit have been employed to raise antibody for Legionella antigen detection. The most simple of these protocols is the method described by McKinney et al. (1979). The duration of six weeks is more acceptable than that of the other three, some of which last for many months even though we detected the production of avid antibody in some instances before their respective end points.

The method of McKinney et al. (1979), (see flowchart p45) utilising formolised whole cells plus adjuvant and stimulated very good IFAT responses from an initial intracutaneous immunisation followed three weeks later by an intramuscular injection and then by weekly intravenous immunisations where 2ml of 4×10^9 cells/ml without adjuvant

were used for each inoculation. This volume and density of cells at 40 IU may have been excessive as Mackie and McCartney (1989) advise that 0.5ml doses at this concentration are sufficient for immunising rabbits. It was notable that R's 8/89/, 9/89, 3/90 and 4/90 appeared to have circulating avid antibody detectable on the 35th day when blood was collected before the second intravenous immunisation. The titres by IFAT were high throughout the protocol, probably due initially to IgM from the primary response and then after immunoglobulin switching, due to the increase of IgG after further immunisation. However, avid antibody was not obtained from blood taken only 7 days after the bleed which showed avid antibody to be present. The reason for this is obscure but there are several possibilities:

1. It is conceivable that the class of IgG produced is important. If this is the case, it may also have some bearing on the explanation as to why avid antibody was eluted when test bleeds were purified by Protein-A whereas no avid antibody eluted after DEAE 52 purification of the same test bleeds (e.g. Nos. 67, 139). The technical department at Pharmacia could not guarantee the binding of both rabbit IgG subclasses to DEAE 52 but were more confident in the abilities of Protein-A to do so. Unfortunately, no anti-IgG class antibodies to detect rabbit IgG_1 or IgG_2 are commercially or privately available and

therefore this hypothesis could not be tested experimentally.

2. Another possibility is that the carbohydrate moeity (the heat-stable component) of Legionella LPS is a poor immunogen and is less likely to produce an appropriate antibody response. Carbohydrate analysis of L.pneumophila (Fox et al., 1990) revealed the presence of unusual and unique sugars including quinovosamine- 2-amino 2, 6 dideoxyglucose. It is very likely that quinovosamine, like the other 2, 6 dideoxyhexoses so far described in Gram-negative bacteria, occur as components of the "O" specific units of the repeat unit of LPS. These may be the components subsequently detected in the urine by avid antisera. If this is so, then "false" positive reactions observed with known "negative" urine may be explained by the detection of sugars of similar structure to those unique sugars found in L.pneumophila (quinovosamine is an isomer of fucosamine -detected in a number of bacterial species).

3. The cellular component found in positive urine may have been modified after exposure to host catabolic or metabolic modification whilst circulating in the human body, where its detection in urine after excretion requires an antibody of unusual affinity or avidity. Such modifications are not unknown. For example, the serum resistance of some gonococci is due to sialylation of the bacterial LPS by indigenous Sialyl Transferases in serum using CMP N-acetyl neuraminic

acid of host origin as the sialic acid donor (Parsons et al, 1989).

This immunisation schedule utilised complete Freund adjuvant which contains killed Mycobacterium tuberculosis and to be sure that no possible cross-reaction would be produced when IgG was used in the diagnostic environment, avid IgG was reacted with heat fixed M.tuberculosis cells which were then examined by IFAT. No fluorescent Mycobacterium when avid IgG raised by this or by the other protocols examined in this work was tested.

It may be argued that as L.pneumophila itself was reported to be a good adjuvant (Wong and Feeley, 1984) that the use of Freund Complete adjuvant was not necessary. However, it was important not to depart from the published protocols' conditions when these were being compared and evaluated. The only variation introduced was the use of a different breed of rabbit.

4.1.1 Using non-NZW rabbits to raise antisera

When a Half Lop rabbit, described in section 2.5.1, was used instead of a NZW, differences in the avidity of the antibody produced by the same immunisation protocol were noted. Also, the antibody titre as determined by IFAT, of R7/88 (table 6) appeared to be less predictable than those produced in NZW rabbits inoculated according to the McKinney protocol. No Half Lop rabbits produced any working antiserum for the ELISA by either of the two

immunisation schedules in which they were used (6 rabbits in the McKinney and 1 in the Kohler). In fact, it is notable that when only NZW rabbits are considered, the success rate calculated from :

$$\frac{\text{No. of rabbits producing avid IgG which worked in the ELISA}}{\text{Total No. of rabbits inoculated}}$$

for the McKinney protocol was 6/8 rabbits (75%), a figure remarkably different from 6/14 (43%) when the Half Lop rabbits were included.

4.2 Antigen/ IgG reactions

Further work was performed to investigate the antigen with which these antibodies were reacting. Ouchterlony double diffusion tests were done. They showed that IgG from antisera Nos. 43 and 59 (purified by DEAE 52 and effective in the ELISA) gave a single precipitin line with either the boiled or non-boiled preparations of sonicated L.pneumophila (figure 14). Both antisera reacted with the same antigen as shown by the reaction line of identity. However whole serum (No. 42) which was ineffective in the ELISA, produced no precipitin line with either of the sonicates. This lack of reaction may have been due to the IgG being too dilute after purification by protein-A. A line of identity was observed when greater quantities of the same serum (No.42) were purified for IgG by either the DEAE 52 or the Protein-A method and reacted in an identical Ouchterlony test. When

this antigen/antibody system was investigated by CIE it was confirmed that the IgG fractions from Nos. 43 and 59 (figures 15 & 16) reacted with only a single antigen and that this was the same antigen in each case. The peaks produced from these antigen/antibody reactions closely matched peak No. 61 of the CIE reference system which was identified as a heat-stable antigen, giving a positive LAL reaction. (Collins et al., 1983).

It was notable that when IgG (purified by DEAE 52) from two samples of antiserum taken only 7 days apart were compared at the same protein concentration by CIE, quite different patterns of precipitin arcs were obtained. Antiserum No. 59 (figure 16) produced only one peak whereas No. 67 (collected 7 days later from the same rabbit, R6/88) produced eight peaks on staining. Similar such differences in counter-immunoelectrophoresis results have been observed (personal communication, Brown,W.) also, when antisera raised in rabbits to Neisseria meningitidis and collected only days apart were reacted with meningococcal polysaccharides. These results indicate the possible changes in serum/ antibody composition which can take place over a short time which may account, at least in part, for the difficulty encountered in producing avid IgG. The single peaks observed in the CIE experiments were excised and tested in the LAL assay, which has a high affinity for endotoxins including that of L.pneumophila LPS (Wong et al.,

1979, Fumarola, 1979). Such preparations were strongly reactive, indicating the presence of LPS. Although peak No.61 was LAL positive (Collins et al., 1983) it did not stain by lipid or polysaccharide stains although in mobility and morphology, it resembled the LPS of other Gram-negative bacteria (Esperson et al., 1980, Schoitz et al., 1979). Studies by other investigators suggest that the lipopolysaccharide of L.pneumophila contains the serogroup-specific antigen (Conlan and Ashworth, 1986, Joly and Kenny, 1982). The use of a purified antigen for immunising rabbits is discussed later. Interestingly, CIE gels incorporating IgG from test bleeds (e.g. No. 68) whose IgG was not effective in the ELISA system also showed precipitin arcs to LPS (figure 19). LPS, is a complex and heterogeneous molecule which has several epitopes. It is conceivable that antibodies can be raised to other epitopes of this immunodominant antigen which are not those found in urine accounting for IgG reacting with LPS in the CIE and not urinary antigen.

The method of raising antibodies described by Kohler et al. (1981) used boiled or autoclaved whole cells. Avid IgG was present in only one test bleed when this heated antigen was used as the immunogen. Although only two rabbits were immunised according to this protocol, they were rested from immunisations for a few months, then re-immunised and effective Cab (test bleed No. 11) from the

NZW rabbit (R1/86) was only then obtained, 19 months after the first immunisation. The reasons for these results are unclear but IgG class switching may be involved.

The method of Tang and Toma (1986) used formolised whole cells as in the McKinney et al., (1979) protocol but antigen was administered in the first inoculation as 12 intradermal injections (1ml total) followed 30 days later by intramuscular and dorsal subcutaneous injections, after which each monthly boost was followed two weeks later by bleeding. As this protocol lasted for 8 months it was much longer than the protocol of McKinney et al. (1979). It used a different strain of L.pneumophila (Philadelphia-1), but in the one rabbit which was immunised in this way, initially very low IFAT titres (up to 1K) were obtained and it was only from the 5th month onwards that the IFAT titre rose to 32K. No avid IgG suitable for the ELISA was detected. The protocol was extended for a further 11 months but again no avid IgG was detectable. Although R7/87 did not produce avid IgG in our hands, the authors claim that antibody raised by this method, when used as capture antibody and as conjugate bound to alkaline phosphatase produced an ELISA which detected not only soluble antigens from culture extracts of L.pneumophila SG's 1-8 as well as L.micdadei and nine other Legionella species (Tang and Toma, 1986) but also urinary antigen from a patient with L.pneumophila SG 12 (Tang et al., 1989). The reason for this ELISA having such a

broad spectrum of reactivity may be due to the size of the epitope to which IgG has successfully been raised. If it was a large epitope spanning several sugars and some (though not necessarily all) of these are constituents common to LPS repeat units of other Legionella strains then this could account for the detection of urinary antigen from a patient with L.pneumophila SG12. No other urinary antigen diagnostic system reports antigenuria detectable in patients who have Legionella infections other than than those caused by L.pneumophila SG 1. Furthermore, effective capture antibody (Nos. 43 and 59) has been used in the ELISA to test for antigenuria in two patients with serum IFA titres > 128 to L.jordanis. Both were negative.

4.3 Soluble antigens for raising antisera

The ideal situation for raising avid antisera is to use a purified and characterised antigen as the immunogen, and in an effort to reduce the chances of raising IgG to cellular antigens not found in the urine, the method of Conlan and Ashworth (1986) was adopted. The preparation of serogroup antigen (Conlan and Ashworth, 1986) differed in its preparation from other more conventional methods of LPS preparation, e.g. a phenol-water extraction where Legionella LPS was reported to partition into the phenol phase making it more difficult to separate from other cellular proteins. When Conlan and Ashworth compared the phenol extract with a saline extract of the Corby strain of L.pneumophila SG1,

similar patterns of banding typical of smooth LPS were observed by SDS-PAGE. The staining method used did not, however, reveal the levels of contaminating protein in each preparation. The band with highest mobility is thought to represent the rough LPS and then the next band the LPS plus one repeat unit, the third band with two repeat units etc. An identical, smooth-type LPS banding pattern was obtained (figure 21) with a proteinase-K digest of whole cells of the Knoxville-1 strain and when this digest was electroblotted and probed with 4 different IgG preparations, all effective as capture antibody in the ELISA, immunostaining of the proteinase-K digest corresponding to the LPS pattern (figure 22) was observed with three of the antisera. In addition to this, two intensely stained bands at 60 and 80 kD were revealed in two of these blots (figure 22). These bands were observed on the blots probed with IgG fractions from No.43 and the Williams antiserum, yet these differed in their characteristics as CAb in the ELISA where No. 43 reacted more strongly with "positive" urine compared with IgG from Williams antiserum. When the IgG fraction of No. 43 was reacted in the CIE only one precipitin arc was observed. The IgG fraction from the McKinney antiserum produced the highest $O.D._{492}$ values with the same "positive" test urines. Unfortunately, no McKinney antisera was available for testing in the CIE. One reason which may explain why two bands stained more intensely with IgG fractions from test

bleed No.43 and the Williams antiserum, may be that these bands transferred more efficiently or bound more strongly under the conditions used.

None of the 4 IgG fractions reacted with positive urine even when this was concentrated 100 fold. It may be that there are technical problems with the urinary antigen not sticking to the transfer membrane or dissassociating from it after binding. Radiolabelling experiments could be useful to determine the fate of the urinary antigen. However, it seems most probable that there simply is not enough urinary antigen in urine concentrated even 100 fold for a reaction to be observed either as a precipitin line in the CIE or as a band on immunoblotting with avid IgG.

The three saline extracts produced were each separated into fractions (A, B, [A+B]) and used as immunising antigens in a total of nine rabbits produced avid antiserum (test bleed No.139) three weeks after the priming immunisation in only one of these, R6/89. As before, with the McKinney protocol, the avid IgG was not isolated from subsequent test bleeds. When peaks A and B (figure 2) were used individually as immunogens, peak B which contained no protein by A_{280} measurement was non-immunogenic at 16ug [$C(H_2O)n$] concentration and failed to produce antibody detectable by IFAT. Peak A at the same concentration was mildly immunogenic with IFA titres up to 4K detected by IFAT

(e.g. R1/90). A higher titre of 16K by IFAT was produced when the concentration of antigen was doubled. This may have been due to protein, which co-eluted with peak A of the serogroup antigen, providing an adjuvant-like activity. Fractions from peaks A and B combined at 32ug [$C(H_2O))n$] concentration, produced one test bleed which contained avid IgG from R6/89 where the IFA titre was 32K. No other bleeds from this rabbit produced as high an antibody level. Another rabbit, R7/89, inoculated with the same dosage and the same combined pools of antigen (A+B) produced only low titres and no avid IgG was isolated as would be expected when titres as low as 2K were found. Therefore, only high titre antisera produced when the immunising dose of carbohydrate was 32ug may contain enough avid IgG effective in the ELISA.

When a portion of the saline extract described by Conlan and Ashworth (1986) was provided for use in this project and used as an immunogen for two rabbits (R5/90 and R10/90), titres by IFAT of 32K were obtained from each rabbit and both of these produced avid IgG, in each case on two occasions when test bleeds were taken 7 days apart.

In conclusion, particulate antigens were the easier to prepare and in general produced higher antibody titres, due in part presumably to the adjuvanticity of L.pneumophila itself. The soluble antigen prepared according to the Conlan and Ashworth (1986) protocol was less immunogenic and where the antigens were made in this laboratory, less successful

than results obtained with the McKinney et al. (1979) protocol. The two rabbits immunised with donated saline extract of the Corby-1 strain of L.pneumophila provided effective antisera. However, the IgG obtained from these test bleeds produced a higher background $O.D._{492}$ with negative control urines than that obtained with any of the IgG raised by the McKinney protocol, perhaps as a result of presenting the rabbit with a purer form of LPS where antibodies might be produced to sugars found in the LPS of other Gram-negative bacteria.

The protocol which yielded the best results from this work to produce avid IgG of diagnostic potential was that described by McKinney et al. (1979) where IgG could be made in 75% of NZW rabbits and collected on the 35th day after the priming immunisation. Protein-A was also the method of choice to purify IgG for use as capture antibody. A combination of these two methods produced avid IgG where the background $O.D._{492}$ was low (generally less than 0.05) which increased the differential between positive and negative antigen-containing urine and so provided unequivocal results even without the aid of an ELISA plate spectrophotometer. This ELISA was as sensitive as the commercial RIA (Dupont), detecting antigen in the urine at a 16 fold dilution from a patient (G.L.) with cultural and serological evidence of L.pneumophila SG1 infection. The latex agglutination test (Meridian diagnostics) was found to

be unreliable in our hands and is not recommended for diagnostic purposes.

Another ELISA has been developed in the U.K. since the start of this project (Birtles et al., 1990). It utilises a finite supply of capture antibody donated by Fehrenbach and uses a conjugate consisting of a monoclonal antibody (LCK-2b) coupled to fluorescin isothyocyanate (FITC). This is amplified by another monoclonal directed to FITC and conjugated to HRP for development in the normal way. Preliminary experiments (results not shown) using capture antibody (No. 43) and the above described monoclonal amplification system works to differentiate positive from negative control urines. The same monoclonal antibody system to detect L.pneumophila SG 1 urinary antigen but modified by using luminol instead of O-phenylenediamine in the presence of H_2O_2 in a chemiluminescence ELISA has also been described (Samuel et al., 1990). Light emission produced by the catalysed oxidation of luminol was recorded as a photographic print. Advantages claimed by the authors over the conventional ELISA are that it takes less time to perform than the colorimetric assays without any loss in sensitivity. However, quantitative results are not obtained with the chemiluminescence ELISA.

4.4 ELISA optimisation

Two variables in the ELISA assay were investigated, the binding properties of different microtitre plates, and the

effectiveness of different BSA preparations in blocking non-specific interactions.

Demands of this test on plate-type were low as the test appeared to be satisfactory on all eight plates tested, although three plates from different manufacturers were marginally superior in producing greater differential $O.D._{492}$ results between "positive" and "negative" control urines. These were Costar plate No. 3590, made from polyvinylchloride (PVC). The other two (Virion F and Nunc) were made from rigid polystyrene. As the other plates which were slightly less satisfactory plates were made from either of these polymers, it appeared that the small differences in affinity observed did not originate from polymer type, but probably reflected differences in manufacturing methods or in sterilisation methods. For example, gamma-irradiation is known to affect binding properties.

BSA from different sources may be produced by different methods such as alcohol precipitation, heat shock, salt fractionation or a combination of these methods. Two types were used, BSA from Sigma (No. 7906) and BSA fraction V (Miles Scientific). Each was tested at different dilutions and the BSA from Sigma performed satisfactorily and was adopted for routine use whereas BSA Fraction V (Miles Scientific) was not effective. No blocking activity was evident at concentrations which were effective with the BSA from Sigma. Fraction V preparations are not 100% albumin and

impurities may have been the reason for the lack of binding activity (personal communication, Sigma).

4.5 Sensitivity and specificity testing of capture antibody

The final evaluation of capture antibody (table 11) involved the use of 77 clinical specimens of urine. Only one of the negative controls (No. 39, table 10) was obtained from a patient whose clinical diagnosis of pneumonia may have been due to L.pneumophila infection. Circumstantial evidence came from IFAT testing where a titre of 128 to L.pneumophila SG 1 was observed in one serum sample only. The significance of this is uncertain since there was no conclusive evidence that this antibody titre was raised against L.pneumophila. However, no false positives were obtained by ELISA.

Cross-reactions were reported by Collins et al. (1983) between sonic extracts of 18 non-Legionella bacterial species and L.pneumophila SG 1 when examined by CIE. Antigen No.66 of the reference system (not LPS) of L.pneumophila SG 1 was similar to antigens found in many Gram-negative bacteria e.g. Pseudomonas aeruginosa, Bordetella pertussis and Heamophilus influenzae). The causative organisms isolated from patients diagnosed as having pneumonia included Klebsiella and H.influenzae. No raised $O.D._{492}$ values using urine from patients infected with these organisms were detected in the ELISA. The pathogens isolated from the urines of patients with urinary tract infection

included Escherichia coli, P.aeruginosa and Acinetobacter but none of these produced "false positives" in the ELISA.

Some antisera yielded IgG which was avid as CAb in the ELISA yet the whole serum was not effective. This was probably due to interference in the ELISA by other serum proteins (including IgM, albumin, transferrin etc.). Some of the sera raised using each of the four protocols yielded IgG which, when used in the ELISA reacted with both positive and negative urines. The reason for this is not clear but it could be due to IgG of low avidity for Legionella-antigen cross-reacting with other antigens in urine from indigenous flora.

4.6 Concentration and analysis of urinary antigen

The investigative methods, were all standard and established systems with little or no modification from those originally reported to obtain satisfactory results, with three notable exceptions. These were:

1. Concentration of urine produced inconsistent results when the concentrate was analysed by SDS-PAGE. The use of macrosolute concentrators may have been a factor in not being able to isolate LPS consistently. The incorporation of 3% (w/v) SDS into the rinsing solution to extract LPS from the macrosolute chamber would also have solubilised any adsorbed protein. However, the 29kD heat-stable protein described by Gosting et al. (1984) was not observed in any gel stained by either silver staining method for protein or

LPS. If Legionella urinary antigen is LPS, then a better method for its concentration may be by precipitation using cold ethanol (Zanen-Lim and Zanen, 1988) since this method has been used to isolate LPS from L.pneumophila SG 12 cells, though not from urine. Also immobilised lectin in an affinity column or Detoxi-Gel (Pierce) might be used to isolate LPS for further experiments.

2. It is possible that the failure to reproduce the detection of LPS in SDS-PAGE gels may have been due to an inadequacy of the staining procedure. The sensitivity may not have been sufficient to detect LPS or other antigens in urine after 100 fold concentration. Fomsgaard et al. (1990) only recently described a modified silver staining technique for certain LPS types which improved their detection. The presence of bound fatty acids in some LPS preparations accounts for their poor chemical detection by conventional silver staining, as much of the LPS is washed out of the gel at the fixing stage. As the whole cell FA profile of legionellae is so complex (and some of these FA's will have been cleaved from the lipid part of LPS) this may be a reason for not detecting LPS on more than one occasion.

It is possible that the detection of Legionella-LPS in gels of concentrated urine could be improved by omitting the fixing stage and increasing the oxidation time from 5 to 20min as recommended by Fomsgaard et al. (1990).

3. The other experimental procedure where problems were

encountered was that of immunoblotting where after the transfer of one "positive" urine (Co.) no reaction on immunostaining with avid antisera was seen. This suggested that Ag was not efficiently transferring or binding to the membrane. When different membrane types were used and where these had different binding properties (although the binding capability of LPS was unknown), immunostaining experiments still produced disappointing results. It would be worthwhile experimenting with other methods of LPS transfer (e.g. semidry electroblotting, Fomsgaard et al., 1990) to establish the best results for staining with specific antiserum.

4.6.1 The nature of urinary antigen

Different methods were employed in attempts to concentrate urinary antigen for the purpose of its identification, mainly by SDS-PAGE. SDS-PAGE was the first choice for analysing urine as highly sensitive stains for protein (Hitchcock and Brown, 1983) and for LPS (Tsai and Frasch, 1982) have been described will detect ng quantities of these components. Methods for the concentration of urine (section 2.13) included the use of a macrosolute concentrator with a molecular weight exclusion limit of 15kD. The advantage of such a system was that during concentration, salts were removed which interfere with the distribution of electric field across the polyacrylamide gel. This was the method chosen to concentrate a positive

urine (Co.) which although sterile, produced an LPS ladder-like pattern (figure 21) after electrophoresis and LPS staining. Silver staining for protein revealed little or no detectable protein (Gel A). This type of pattern from the analysis of concentrated urine has not been reported in the literature before and unfortunately could not be repeated. Confirmation of the LPS pattern to be that of L.pneumophila SG 1 by immunoblotting using avid IgG was not achieved even when two different membrane types were used. This was due to one or a combination of three factors:

1. The methods for the concentration of urine were not suitable. Even after modification of the method for concentrating urine by including 3% (w/v) SDS in the rinsing solution to wash the chamber of the concentrator in case LPS or other components were adsorbed to the plastic chamber, no bands were observed on the SDS-Page gel in lanes loaded with "positive" urines which were not also seen in concentrated urine from healthy people. However, similar bands were seen in concentrated urine obtained from patients with UTI infections. These bands may have been globulin (perhaps from locally-secreted IgA, albumin or even bacterial components. Immunoblotting against avid anti-Legionella antiserum produced no staining.
2. The method of transfer of the electrophoresed urinary antigen components was not suitable.
3. The urinary antigen may be a modified cellular component

where the affinity of the antibody is important and this may be difficult to show by immunoblotting or indeed by CIE.

One urine (Co.) produced an LPS pattern by SDS-Page which differed from the proteinase-K digest of L.pneumophila when reacted with avid antisera after immunoblotting (figure 22). This supports the idea that any Legionella LPS components found in urine are modified from the bacterial form of LPS. No staining was observed on immunoblotting with avid antisera. On repeating this experiment with other positive urines (figures 23 and 24) a pattern similar to that in figure 21 was not observed.

Finally, affinity chromatography was adopted to try to overcome the difficulties in transfer of urinary antigen onto nitrocellulose for immunostaining. Avid IgG antibody was bound to Sepharose 4B and this was exposed to both antigen and non-antigen containing urines. Eluted urine, tested by ELISA showed that the affinity columns were binding the urinary antigen. This was further confirmed with increasing $O.D._{492}$ values on the ELISA plate (figure 25) as the IgG in the columns became saturated with antigen with an increasing quantity of urine.

However, although a unique band (80 kD) was observed on an SDS-Page gel silver stained for LPS (figure 27) in lanes loaded with Sepharose, IgG and bound antigen from "positive" urine, neither this nor the other band (60kD) common to both positive and negative urines were

immunostained with avid IgG (No. 43) after electrophoretic transfer onto nitrocellulose or PVDF. This was probably due to no or poor binding of the urinary antigen to either of the two membranes (figure 26), although a reason for this is not obvious. However, it may indicate that the antigen is hydrophobic in nature. When SDS-PAGE of Sepharose/IgG bound urinary antigen were probed with sheep anti-rabbit IgG, only two bands in the control rabbit IgG lane were stained. These were 50 and 55kD in molecular weight and therefore not of the same molecular weight as the two bands previously reported (80 and 60 kD). So it remains possible that the unique band observed on the SDS-PAGE gel was indeed a component only found in "positive" urines.

4.7 Urinary antigen excretion in a patient with L.D.

The details of a case history of one patient (J.McM) diagnosed as having Legionnaires' disease highlighted the benefit of a working and reliable diagnostic test which provided evidence of a Legionella infection before either cultural or serological methods.

It can be seen from this and other published information (Kohler et al., 1984) that the test is best suited to the early diagnosis of L.D. where urine is collected in the early stages of infection and tested in the ELISA to detect urinary antigen before either cultural or even serological evidence is obtained. The sharp decline in the amount of antigen after 57h from the time of admission

(day to day experimental ELISA controls produced similar values and these results were confirmed when retested on one ELISA plate) is probably more marked as a result of the patient responding to intravenous (and later oral) antibiotics. Antigen excretion may be prolonged after infection in some cases where this is not related to the clinical condition of the patient. The possible recurrence of infection in such cases cannot be determined by this test.

SPECIES IDENTIFICATION

4.8 Fatty acid (FA) profiles of the Legionellaceae

Total cellular FA composition has been shown to be useful in the identification and classification of bacteria (Moss and Dees, 1975). This is particularly so for the *Legionellaceae*, and as a result, an increase of interest in this area is evident with more comprehensive information on FA profiles of many legionellae being published. Usually, as in the most recent publication (Lambert and Moss, 1989) it is information on the nonhydroxy FA profiles which is available. However, studies on hydroxy FA's of a limited number of *Legionella* strains, representing only 10 species have also been published (Mayberry, 1981, 1984). Hydroxy FA analysis can differentiate between some species of *Legionella* (e.g. *L.pneumophila* and *L.micdadei*, Mayberry, 1984). However, these two species also have easily distinguishable hydroxy FA profiles. Most new species

have their nonhydroxy FA composition described (e.g. L.moravica and L.brunensis in Wilkinson et al., 1988) but in a few cases the hydroxy FA composition is also described (e.g.L.tucsonensis in Thacker et al., 1989). As more information on nonhydroxy FA profiles is available for comparative purposes, these were studied in detail in this thesis.

It was found that the FA profile within a Legionella species varied with the period of incubation. This study of growth rates in different species varied, even on the same medium (BCYE). Most species showed confluent growth on BCYE agar after 48-72 days, but a few species grew only to form distinct colonies which ceased to increase in diameter after 72-96 days. The length of incubation for reproducible results was therefore judged to be 96h. Table 19 shows mainly quantitative differences in FA between identical strains after 24 and 96h. However, some qualitative differences were also noticed. Similar observations were made when strains were grown on two different media. Fortunately, growth under "standard" conditions (96h at 37°C on BCYE) on different batches of the same medium (BCYE) resulted in little or no differences in the nonhydroxy FA profiles, making these analyses useful and reproducible. Unlike the ubiquinone profiles, it was decided not to group species of the Legionellaceae on the basis of their predominant FA as they were extremely complex and minor

variations of strains representing the same species were found. Species which could be confused with one another on the basis of their GLC profiles include L.dumoffi, L.maceachernii, L.micdadei, and L.wadsworthii, but a closer examination of quantitative differences in their nonhydroxy FA's aids in their differentiation. It is recommended that where differences are small, the unidentified Legionella is grown along with the known species which it resembles, and the FA profiles are assessed together with table 21. This is especially important because quantitative differences in some FA are evident between some strains identified to be of the same species (e.g. L.micdadei Bari 2/158, Moss and Lambert, 1989 and L.anisa isolated from a patient). Members of the three DNA groups of L.pneumophila as proposed by Brenner et al. (1988) could not be distinguished on the basis of FA data (table 24). Type strains representing four serogroups of L.pneumophila (4, 5, 10 and Lansing-3) produced some variation in FA from other L.pneumophila strains, particularly in a15:0 and n16:1. These differences were not substantiated when the type strains were compared with serologically identified environmental strains of the same serogroup.

The use of mass spectrometry to confirm the structure of FA found in the legionellae is a widely used technique and all FA's reported in this study in the Legionellaceae were found within the four strains analysed (including

86/35784). Only one or two very small peaks (< 0.5%) present in FA profiles were identified as contaminating hydrocarbons. These were not significant since they were not included in calculations. Retention times of standard FA's in a commercially available mixture were determined along with each batch of analyses to allow identification.

Differences in the FA composition for the same species have been reported by different laboratories. These may reflect differences in growth conditions, methods used to prepare FAMES, or conditions for analysis.

It is apparent that the analysis of FA profiles will be of value to a laboratory with a large throughput of Legionella isolates, most of which will be identified by this and by RPTLC without the need for DNA homology or RFLP analysis.

However, if new species continue to be identified at the current rate, it is likely that the family will become very large. Comparing FA analyses will prove to be difficult. This is more likely as the number of isolates from cooling towers will increase as a result of safety guidelines being enforced.

4.9 Ubiquinone profiles of the Legionellaceae

Ubiquinones were extracted from all the Legionella strains shown in table 1 (Materials and Methods) representing L.pneumophila SG 1-14 (and one proposed new serogroup) and other strains representing 29 species, 7

proposed new species and 6 possible new species. These were analysed by the method of reversed-phase thin-layer chromatography. Ubiquinone profiles as determined by this method (Mitchell and Fallon, 1990) were reproducible, both qualitatively and semi-quantitatively, and results were compiled to produce a useful reference "library" (table 27).

As minor quantitative differences between laboratories were noted in ubiquinone profiles of the same species (even those who used HPLC, table 28) it is recommended that unless identical conditions and equipment are used, a laboratory should produce its own standard reference library of ubiquinone profiles.

RPTLC is a cheap and simple method of obtaining reliable results and therefore a realistic alternative to HPLC, which requires expensive and sophisticated equipment.

New information is presented (table 27) on the ubiquinone profiles of seven Legionella species: L.birminghamensis, L.brunensis, L.cincinnatiensis, L.moravica, L.quinlivanii, L.tucsonensis and proposed new species No. 1347, "L.worsleiensis".

As with the FA profiles of the autofluorescent species, ubiquinone profiles are also useful as a differential character in their identification. Other species, whose FA profiles are similar e.g. L.longbeachae and L.pneumophila (figures 50 and 58 respectively) can also be differentiated

by their ubiquinone profiles.

However, some species e.g. L.anisa and L.bozemanii are impossible to differentiate either by their GLC or RPTLC profiles. Serologically, L.bozemanii SG 2 and L.anisa are not easily separated. They can however be distinguished by using absorbed antisera or by other methods such as DNA homology.

Mass spectrometry does not provide an accurate method for the quantitative determination of ubiquinone mixtures. The intensity of peaks attributable to molecular ions may vary considerably, and does not always accurately reflect the relative proportions of ubiquinones in a mixture (Collins and Gilbart, 1983). This problem becomes particularly acute when complex mixtures are analysed and this may explain why the peak heights of each of the ubiquinones in figure 93 do not correspond to the semi-quantitative result as determined by RPTLC (table 27). Other possible reasons for the loss of ubiquinone material before mass spectrometry may be that the recovery of ubiquinones from the TLC plate (purification step) < 100% due to incomplete elution or more simply by photodegradation.

4.10 Patient and environmental isolates: GLC and RPTLC data compared with data from type strains

Many strains from both patient and environmental sources have now been analysed for their FA and ubiquinone

content. L.pneumophila strains, which represent the most commonly isolated species of this family, have consistent profiles whether isolated from the environment or from patients. These closely resemble profiles compiled in table 21. Other species e.g. L.longbeachae and L.quateriensis (table 29) showed minor differences in FA from their respective type strains. Such minor variations may also be observed in legionellae isolated from patients when these are not L.pneumophila. Strain-88/24485 (table 31), serologically identified as L.anisa produced, on FA analysis, some minor quantitative differences from the type strain (up to 4 mol%). In addition to this, variation of hydroxy FA within the type strain was also recorded (up to 5%). The L.micdadei strain (88/1058), which caused the Lochgoilhead outbreak, also showed some differences in FA from those of the type strain. Variation in some FA's (up to 10 mol%) of isolated L.micdadei strains and the type strain (Tatlock) has also been reported (Moss and Lambert, 1989).

It may be that any variation observed in FA composition analysed under "standard" conditions between an isolate whether from a patient or a water sample and its corresponding type strain is determined by the species itself and is governed at a genetic level.

Where this FA and ubiquinone data may also be of use is in the identification of a new species, strain or sub-species. An example where one isolate (86/35784), was

deduced to be a new sub-species of L.quinlivanii (section 3.8.3) has already been detailed.

4.11 Future work

The production of diagnostic antisera for the purpose of detecting Legionella antigen in urine has obvious commercial value. Experiments to investigate the McKinney protocol (the most successful immunisation protocol from this work) further, might include varying some of the conditions within the protocol. Modifications to the protocol could include using boiled whole cells as the inoculum, increasing or decreasing the dose used for immunisation and investigating the influence of different strains of L.pneumophila SG 1 (e.g. Pontiac-1).

The relative proportions of different subclasses of IgG in test bleeds obtained from the same rabbits only days apart, which differed in their ability to capture urinary antigen should be investigated further. In addition, the ability of DEAE 52 to bind both IgG subclasses could be tested.

The eventual aim should be to use a purified and well characterised antigen to raise the specific capture antibody for ELISA testing of positive urines samples. This problem may be approached by using various fragments of LPS (after acid or alkali hydrolysis. It may be necessary to look for other carbohydrate-containing antigens in the outer membrane

of L.pneumophila, which may be comparable to the enterobacterial common antigen in structure.

If such fragments are haptenic rather than antigenic, then effective immunisation may be achieved by coupling to diptheria or tetanus toxoid via an amide bond under acidic conditions.

The concentration of urinary antigen by cold ethanol precipitation used in conjunction with a modified silver stain may be the key to consistently identify Legionella antigen in urine and the blotting of this may afford detection after immunoblotting. However, the electroblotting process also merits further investigation. Standard preparations of LPS and its fragments should be used to monitor transfer efficiency, probably by using radiolabelled LPS in order to determine whether failure to detect urinary antigen by immunoblotting was due to poor transfer or poor fixation to the membrane.

Finally, the FA and the ubiquinone libraries should be assembled onto a computer database where a software package can compare these with data from new Legionella isolates. This is likely to make the process of comparing results less difficult and the methods of identification described in this thesis more widely available.

REFERENCES

Abe, K., Ishibashi, M., Ohmae, M., Kawabe, K. and Katsui, G. (1978) "Determination of ubiquinone in serum and liver by high speed liquid chromatography". J.Nutritional Science and Vitaminology, 24, 555-567.

Aguero-Rosenfeld, M.E. and Edelstein, P.H. (1988) "Retrospective evaluation of the Dupont radio-immunoassay kit for the detection of Legionella pneumophila serogroup 1 antigenuria in humans". J.Clin.Micro., 1775-1778.

Bang, F.B. and Levin, J. (1968) "Clottable protein in Limulus : It's localisation and kinetics of it's coagulation by endotoxin". Thromb.Diath.Haemmorrh., 19, 186.

Bartlett,C.L.R., Macrae, A.D., Macfarlane, J.T.(Eds.) 1986 "Legionella Infections". (Arnold Press).

Benson, R.F., Thacker, W.L., Waters, R.P., Quinlivan, P.A., Mayberry, R.W., Brenner, D.J. and Wilkinson, H.W. (1989) "Legionella quinlivanii sp. nov. isolated from water". Current Micro.Vol., 18, 195-197.

Berdal B.P., Farshy, Carol E., and Feeley, J.C. (1979) "Detection of Legionella pneumophila antigens in urine by enzyme-linked immunospecific assay". J.Clin.Micro., 9, No.5, 575-578.

Bibb, W.F., Arnow,P.M., Thacker, L. and McKinney, R.M. (1984) "Detection of soluble antigens in serum and urine specimens by enzyme-linked immunosorbent assay with monoclonal and polyclonal antibodies". J.Clin.Micro., 20, 478-482.

Birtles, R.J., Harrison, T.G., Samuel, D. and Taylor, A.G. (1990) "Evaluation of urinary antigen ELISA for diagnosing Legionella pneumophila SG 1 infection". J.Clin.Path., 43, 685-690.

Brenner, D.J., Steigerwalt, A.G., McDade, J.E.(1979) "Classification of Legionnaires' disease bacterium: Legionella pneumophila, genus novum, species nova of the family Legionellaceae familia nova". Ann.Intern.Med., 90, 656-8.

Brenner, D.J. (1984) "Classification of Legionellae". In Legionella: Proceedings of the 2nd International Symposium: Washington DC, 55-60.

Brenner, D.J., Steigerwalt, A.G., Epple, A., Bibb, W.F., McKinney, R.M., Starnes, R.W., Colville, R.K., Selander, R.K., Edelstein, P.H., and Moss, C.W. (1988) "Legionella pneumophila serogroup Lansing-3 isolated from a patient with fatal pneumonia, and descriptions of L.pneumophila subsp. pneumophila nov., L.pneumophila subsp. fraseri subsp. nov., and L.pneumophila subsp. pascullei subsp. nov." J.Clin.Micro., 26, No.9, 1695-1703.

Brenner, D.J. Steigerwalt, A.G., Gorman, G.W. (1985) "Ten new species of Legionella". Int.J.Syst.Bact. 35, 50-59.

Chandler, F.W., Blackman, J.A., Hicklin, M.D., Cole, R.M. and Callaway, C.S. (1979) "Ultra structure of the agent of Legionnaires' disease in the human lung". Am.J.Pathol., 71, 43-50.

Chart, H. and Trust, T.J. (1984) "Characterisation of the surface antigens of the marine fish pathogens Vibrio anguillarium and Vibrio ordalii". Can.J.Micro., 30, 703-710.

Cherry, W.B. Pittman, B., Harris, P.P., Hebert, G.A., Thomason, B.M., Thacker, L. and Weaver, R.E. (1978) "Detection of Legionnaires' disease bacteria by direct immunofluorescent staining". J.Clin.Micro., 8, 329-338.

Cherry, W.B. and McKinney, R.M. "Detection of Legionnaires' disease bacteria in clinical specimens by direct immunofluorescence". In Jones and Hebert (Eds.) "Legionnaires", the disease, the bacterium and methodology. (1979) Center for Disease Control, Atlanta. 92-103.

Coonrod, J.D. (1983) "Urine as an antigen reservoir for diagnosis of infectious diseases". The American Journal of Medicine, 85-92.

a. Collins, M.T., Sang-Nae Cho, Neils Hoiby, Esperson, F. Leif Baek and J.S. Reif. (1983) "Crossed immuno-electrophoretic analysis of Legionella pneumophila serogroup 1 antigens". Infect. and Immun., 39, No.3, 1428-1440.

b. Collins, M.T.,Frank Esperson, Neils Hoiby, Sang-Nae Cho, Alice Friis-Moller, and John S. Reif.(1983) "Cross-reactions between Legionella pneumophila (serogroup 1) and twenty eight other bacterial species , including other members of the family Legionellaceae". Infect. and Immun., 39, No.3, 1441-1456.

Collins, M.D. and Gilbart, J. (1983) "New members of the coenzyme Q series from the Legionellaceae". FEMS Microbiology Letters, 16, 251-255.

Conlan, J.W. and Ashworth, L.A.E. (1986) "The relationship the serogroup antigen and lipopolysaccharide of Legionella pneumophila". J.Hyg., Camb., 96, 36-48.

Darveau, R.P. and Hancock, R.E.W.(1983) "Procedure for isolation of bacterial lipopolysaccharides from both rough and smooth Pseudomonas aeruginosa and Salmonella typhimurium strains". J. Bacteriol., 155, No.2,831-838.

Domardzski, M.D., Havsser, J.L. and Gosselin, F. (1984) "Coexistence of Legionnaires' disease and Q fever in a single patient". Can.Med.Assoc.J., 130, 1022-1023.

Dondero, T.J., Rendtorff, R.C., Mallison, G.F. (1980) "An outbreak of Legionnaires' disease associated with a contaminated air-conditioning cooling tower". N.Engl.J.Med., 90, 694-696.

Dournon, E. (1988) "Isolation of Legionellae from clinical specimens". In "A Laboratory Manual For Legionella." Harrison, T.G. and Taylor, A.G. (Eds.), 13-30.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) "Colorimetric method for determination of sugars and related substances". Anal.Chem., 28, No.3, 350-356.

Dumoff, M. (1979) "Direct *in-vitro* isolation of the Legionnaires' disease bacterium in two fatal cases". Ann.Int.Med., 90, 694-696.

Edelstein, P.H. (1986) "Evaluation of the Gen-probe DNA for the detection of Legionellae in culture". J.Clin.Micro., 23, No.23, 481-484.

Edelstein, P.H., Meyer, R.D. and Finegold, S.M. (1979) "Isolation of Legionella from blood". Lancet, 1, 750-751.

Edelstein, P.H., Meyer, R.D., Finegold, S.M. (1980) "Laboratory diagnosis of Legionnaires' disease". Am.Rev.Resp.Dis., 121, 317-327.

Edelstein, P.H. (1984) "Laboratory diagnosis of Legionnaires' disease". In Legionella: Proceedings of the 2nd International Symposium:Washington, DC., 3-5.

Edelstein, P.H. (1987) "Laboratory diagnosis of infections caused by Legionellae". Eur.J.Clin.Micro., 6, No.1, 4-10.

Edwards, R. and Feltham, R.K.A. (1983) "Taxonomic implications of the cellular fatty acid content of the Legionellaceae and possibly related species". FEMS Micro. Lett., 17, 251-255.

Esperson, F., Hertz, J.B., Hoiby, N. and Mogensen, H.H. (1980) "Ultrastructural localisation and protective activity of a high molecular-weight antigen isolated from L.pneumophila". Infect. and Immun., 31, 822-824.

Fallon, R.J. and Abraham, W.H. (1982) "Polyvalent heat-killed antigen for the diagnosis of infection with Legionella pneumophila". J.Clin.Path., 35, 434-438.

Fallon, R.J. and Rowbotham, T.J. (1990) "Microbiological investigations into an outbreak of Pontiac fever due to Legionella micdadei associated with use of a whirlpool". J.Clin. Path., 43, 479-483.

Farshy, C.E., Klein, G.C. and Feeley, G.C. (1978) "Detection of antibodies to Legionnaires' disease organism by microagglutination and micro enzyme-linked immunosorbent assay tests". J.Clin.Micro., 7, No.4, 327-331.

Feeley, G.C., Gorman, G.W., Weaver, R.E., Mackel, D.C. and Smith, H.W. (1978) "Primary isolation media for the Legionnaires' disease bacterium". J.Clin. Micro., 8, 320-325.

Fehrenbach, F.J., Horbach, I., Ruf, B., Sharman, D. and Pohle, H.D. (1986) "Rapid detecting of Legionella antigen in tissues and body fluids". Israel Journal of Medical Sciences, 22, 706-710.

Fomsgaard, A., Fruedenberg, M.A., and Galanos, C. (1990) "Modification of the silver staining technique to detect lipopolysaccharide in polyacrylamide gels." J.Clin.Micro., 28, No.12, 2627-2631.

Fox, A., Rogers, J.C., Fox, K. Schnitzer, G., Morgan, S.L., Brown, A. and Aono, R. (1990) "Chemotaxonomic differentiation of Legionellae by detection and characterisation of aminodideoxyhexoses and other unique sugars using gas chromatography-mass spectrometry". J.Clin.Micro., 28, No.3, 546-552.

Flesher, A.R., Susumu, I., Mansheim, B.J. and Kasper, D.L. (1979) "The cell envelope of the Legionnaires' disease bacterium". Ann.Intern.Med., 90, 628-630.

Fliermans, C.B. Cherry, W.B., Orrison, L.H., Smith, S.J., Tison, D.L., and Pope, D.H. (1981) "Ecological distribution of Legionella pneumophila". Appl.Envir.Micro., 41, 9-16.

Friedman, H., Newton, C., Blanchard, D.K., Klein, T., Widen, R. and Wong, K.H. (1987) "Immunogenicity and adjuvanticity of lipopolysaccharide from Legionella pneumophila". Proceedings of the society for experimental biology and medicine, 184, 191-196.

Friedman, R.L., Iglewski, B.H. and Miller, R.D. (1980) "Identification of cytotoxin produced by Legionella pneumophila". Infect. and Immun., 29, No.1, 271-274.

Fumarola, D. (1979) "Legionnaires' disease agent and limulus endotoxin assay". IRCS Medical Sciences:Parasitology and Infectious diseases, 7, 9.

Galanos, C., Luderitz, O. and Westphal, O. (1969) "A new method for the extraction of R. lipopolysaccharides". Eur.J.Biochem., 9, 245-249.

Gilbart, J. and Collins, M.D. (1985) "High-performance liquid chromatographic analysis of ubiquinones from new Legionella species". FEMS Micro. Lett., 26, 77-82.

Glick, T.H., Gregg, M.B., Berman, B. (1978) "An epidemic of unknown aetiology in a Health Department". Am.J.Epidemiol., 167, 149-160.

Goldberg, D.J., Collier, P.W., Fallon, R.J., McKay, T.M., Markwick, T.A., Wrench, J.G., Emslie, J.A., Forbes, G.I., Macpherson, A.C. and Reid, D. (1989) "Lochgoilhead Fever: Outbreak of non-pneumonic Legionellosis due to L.micdadei". The Lancet:Communicable Disease,316-318.

Gorman, G.W., Feeley, J.C., Steigerwaltii, A., Edelstein, P.H., Moss, C.W. and Brenner, D.J. (1985) "Legionella anisa : a new species of Legionella isolated from potable waters and a cooling tower". Appl. and Enviro.Micro., 49, 3305-3309.

Gosting, L.H., Cabrian, K., Sturge, J.C. and Goldstein, L.C. (1984) "Identification of a species-specific antigen in Legionella pneumophila by a monoclonal antibody". J.Clin.Micro., 20, 1031-1035.

Gress, F.M., Myerowitz, R.L., Pasculle, A.W., Rinaldo Jr. C.R., and Dowling, J.N. (1980) "The ultrastructural morphological features of Pittsburgh Pneumonia agent" Am.J.Pathol., 101, 63-69.

Grimont, P.A.D., Popoff, M.Y., Grimont, F., Coynault, N. and Lemelin, M. (1980) "Reproducibility and correlation study of three deoxyribonucleic acid procedures". Curr. Micro., 4, 325-330.

Harrison, T.G. and Taylor, A.G. (1982) "A rapid micro-agglutination test for the diagnosis of L.pneumophila (serogroup 1) infection". J.Clin.Pathol., 35, 1028-1031.

Harrison, T.G. and Taylor, A.G. (Eds.) "A laboratory manual for Legionella". (1988), John Wiley and sons. (Publ.).

Harrison, T.G., Saunders, N.A., Doshi, N. and Wait, R. (1988) "Serological diversity within the species Legionella spiritensis". J. of Appl.Bact., 65, 425-431.

Harvey, D.J. (1982) "Picolinyl esters as derivatives for the structural determination of long chain branched and unbranched fatty acids". Biomed.Mass Spectrom., 9, 33-38.

Hebert, G.A., Moss, C.W., McDougal, D.K., Bozeman, F.M., McKinney, R.M. and Brenner, D.J. (1980) "The rickettsia-like organisms TATLOCK (1943) and HEBA (1959): bacteria phenotypically similar to but generally distinct from L.pneumophila and the WIGA bacterium". Ann.Int.Med., 92, 45-52.

Helms, C.M., Viner, J.P., Weisenburger, D.D., (1984) "Sporadic Legionnaires' disease. Clinical observations on 87 nosocomial and community acquired diseases". Am.J.Med.Science, 285, 2-12.

Herwaldt, L.A., Gorman, G.W., McGrath, T., Toma, S., Brake, B., Hightower, A.W., Jones, J., Reingold, A.L., Boxer, P.A., Tang, P.W., Moss, C.W., Wilkinson, H., Brenner, D.J., Steigerwalt, A.G. and Broome, C.V. (1984) "A new Legionella species, Legionella feeleii species nova, causes Pontiac Fever in an automobile plant"., 100, No.3, 333-338.

Hitchcock, P.J. and Brown, T.M. (1983) "Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver-stained polyacrylamide gels". J. of Bact., 154, No.1, 269-277.

Huebner, R.E., Reeser, P.W.,and Smith, D.W. (1984) "Comparison of the virulence of the Philadelphia and Pontiac isolates of Legionella pneumophila". In Legionella; Proceedings of the 2nd International Symposium, Washington DC., 123-124. Editors of the A.M.Soc. for Microbiol., Washington.

Jantzen, E., Bryn, K., Hagen, N., Bergan, T. and Bovre, K. (1978) "Fatty acids and monosaccharides of Neisseriaceae in relation to established taxonomy." NIPH Ann., 2, 59-71.

Joly, J.R. and Kenny, G.E. (1982) "Antigenic analysis of Legionella pneumophila and Tatlockia micdadei (Legionella micdadei) by two-dimensional (crossed) immunoelectrophoresis". Infect.and Immun., 721-729.

Karr, D.E., Bibb, W.F. and Moss, C.W. "Isoprenoid quinones of the genus Legionella". (1982) J.Clin.Micro., 15, 1044-1048.

Kirby, B.D., Snyder, K.M., Meyer, R.D. and Finegold, S.M. (1980) "Legionnaires' disease:Report of 65 nosocomial acquired cases and review of the literature" Medicine, 59, 188-205.

Kawai, Y and Moribayashi, A. (1982) "Characteristic Lipids of Bordetella pertussis : Simple fatty acid composition, hydroxy fatty acids, and an ornithine containing lipid". J. of Bact., 151, No.2, 996-1005.

Kjeldahl. (1982) "Determination of protein nitrogen". Chapt. 7, 300-304. In Fundamentals of Clinical Chemistry (Eds.) by Tietz, N.W., Caraway, W.T., Freier, E.F., Kachmar, J.F. and Rawnsley, H.M. W.B.Saunders (Publ.).

Kohler, R.B., Allen, S.D. and Wilson, E.R. (1984) "Cross-reacting bacterial antigens in a radiometric assay for urinary Legionella pneumophila antigen"., 40-41, In Thornsberry, C., Balows, A., Feeley, J.C. and Jakubowski, W. (Eds.), Legionella. Proceedings of the IInd International Symposium. Am. Soc. for Micro.,Washington, D.C.

Kohler, R.B., Winn, C.W. Jr., Girod, J.C. and Wheat, J.L. (1982) "Rapid diagnosis of pneumonia due to Legionella pneumophila SG1". The Journal of Infectious Diseases, 146, 444-446.

Kohler, R.B. and Sathapatayavongs, B. (1983) "Recent advances in the diagnosis of serogroup 1 Legionella pneumophila by detection of urinary antigen". Zbl.Bakt.Hyg.,I.Abt.Orig.A, 225, 102-107.

Kohler, R.B., Winn, W.C. Jr. and Wheat, J.L. (1984) "Onset and duration of urinary antigen excretion in Legionnaires' disease" J.Clin.Micro., 20, 605-607.

Kohler, R.B., Wheat, L.J., French, M.L.V., Meenhorst, P.L., Winn, W.C. and Edelstein, P.H.(1985) "Cross-reactive urinary antigens among patients infected with Legionella pneumophila serogroups 1 and 4 and the Leiden 1 strain (SG 10)". The Journal of Infectious Diseases, 152,1007-1012.

Kohler, R.B., Zimmerman, S.E., Wilson, E., Allen, S.D., Edelstein, P.H., Joseph Wheat, L. and White, A. (1981) "Rapid radioimmunoassay diagnosis of Legionnaires' disease-detection and partial characterisation". Ann.Int.Med., 94, No.5, 601-605.

Kaufmann, A.F., McDade, J.E., Patton, C.M. (1981) "Pontiac fever isolation of the aetiologic agent (Legionella pneumophila) and the demonstration of it's mode of transmission". Am.J. Epidemiol., 114, 337-347.

Laemmli, U.K. (1970) "Cleavage of Structural proteins during the assembly of the head of Bacteriophage T4". Nature, 227, 680-685.

Lambert, M.A., and Moss, C.W. (1983) "Comparisons on the effects of acid and base hydrolyses on hydroxy and cyclopropane fatty acids in bacteria". J.Clin.Micro., 18, 1370-1377.

Lambert, M.A., and Moss, C.W. (1989) "Cellular fatty acid compositions and isoprenoid contents of 23 Legionella species". J.Clin.Micro., Vol. 27, No. 3., 465-473.

Lindquist, D.S., Nygaard, G., Thacker, W.L., Benson, R.F., Brenner, D.J. and Wilkinson, H.W. (1988) "Thirteenth serogroup of Legionella pneumophila isolated from patients with pneumonia". J.Clin.Micro., 26, 586-587.

Macfarlane, J.T., Ward, M.J. and Mayhew, J. (1982) "Creatine kinase and confusion pneumonia" Lancet, 2, 1221.

Mackie and McCartney (Eds., 1989) "Medical Microbiology". Cruickshank (Publ.)

Mangiafico, J.A., Hedlund, K.W. and Knott, A.R.(1981) "Rapid and sensitive method for quantitation of Legionella pneumophila serogroup 1 antigen from human urine". J.Clin.Micro., 843-845.

Mayberry, W.R. (1981) "Monohydroxy and dihydroxy fatty acids in Legionella species". J. of Bact., 14, 313-318.

Mayberry, W.R. (1984) "Monohydroxy and dihydroxy fatty acid composition in Legionella species". Int.J.of Syst.Bact., 34, 321-326.

McDade, J.E., Sheppard, C.C. and fraser, W. (1977) "Legionnaires' disease. Isolation of a bacterium and its role in other respiratory disease". New Engl.J.Med., 297, 1197-1203.

McDade, J.E., Brenner, D.J. and Bozeman, J. (1979) "Legionnaires' disease isolated in 1947" Ann.Int.Med., 90, 659-661.

McKinney, R.M., Thomason, B.M., Harris, P.P., Thacker, L., Lewallon, K.R., Wilkinson, H.W., Hebert, G.A. and Moss, C.W. (1979) "Recognition of a new serogroup of Legionnaires' disease bacterium". J.Clin.Micro., 19, 103-107.

Milder, J.E. and Rough, R.R. (1982) "Concurrent Legionnaires' disease and active pulmonary tuberculosis". Am.Rev.Resp.Dis., 125, 759-761.

Miller,T.J. and Stone H.O. (1978) "The rapid isolation of ribonuclease-free immunoglobulins by protein-A sepharose affinity chromatography". J.of Immunol.Methods, 24, 111-125.

Mitchell, K. and Fallon, R.J. (1990) "The determination of ubiquinone profiles by reversed-phase high-performance thin-layer chromatography as an aid to the speciation of Legionellaceae". J.Gen.Micro., 136, 2035-2041.

Morrill, W.E., Barbaree, J.M., Fields, B.S., Sanden, G.N. and Martin, W.T. (1990) "Increased recovery of Legionella micdadei and Legionella bozemanii on buffered charcoal yeast extract agar supplemented with albumin". J.Clin.Micro., 28, No.3, 616-618.

Moss, C.W. and Dees, S.B. (1975) "Identification of microorganisms by gas-liquid chromatographic-mass spectrometric analyses of cellular fatty acids". J.Chromatography, 596-604.

Moss, C.W., Weaver, R.E., Dees, S.B. and Cherry, W.B. (1977) "Cellular fatty acid composition of isolates from Legionnaires' disease". J.Clin.Micro., 6, 140-143.

Moss, C.W. and Dees, S.B. (1979) "Further studies of the cellular fatty acid composition of Legionnaires' disease bacterium". J.Clin.Micro., 9, 648-649.

Moss, C.W., Bibb, W.F., Karr, D.E., Guerrant, G.O. and Lambert, M.A. (1983) "Cellular fatty acid composition and ubiquinone content of Legionella feelii sp. nov". J.Clin.Micro., 18, No.4, 917-919.

Moss, C.W. and Lambert, M.A. (1990) "Re-evaluation of the cellular fatty acid composition of Legionella micdadei Bari 2/158". J.Clin.Micro., 28, No.2, 389-390.

Moyer, N.P., Lange, A.E., Hall, N.H. and Housler, W.J. Jr. (1984) "Application of capillary gas-liquid chromatography for sub-typing Legionella pneumophila epidemiology". In Legionella; Proceedings of the 2nd International Symposium, Washington DC. (Eds. from the Am.Soc.Microbiol.), 274-276.

Muder, R.R., Yu, V.L. and Kroboth, F.J. (1983) "Pneumonia due to the Pittsburgh pneumonia agent; new clinical perspective with a review of the literature". Medicine (Baltimore), 62, 120-128.

Muder, R.R., Reddy, S.C., Yu, V.L. and Kroboth, F.J. (1984) "Pneumonia due to the Pittsburgh pneumonia agent; radiological manifestations". Radiology, 150, 630-637.

Nakane, P.K. and Kawaoi, A. (1974) "Peroxidase-labelled antibody ; A new method of conjugation". The J. of Histochemistry and Cytochemistry (1974), 22, No.12, 1084-1091.

Nakane, P.K. and Pierce, G.B. (1966) "Enzyme-labelled antibodies:preparation and application for localisation of antigens". J.Histochem.Cytochem., 14, 790.

Orrison, L.H., Bibb, W.F., Cherry, W.B. and Thacker, L. (1983) Determination of antigenic relationships among legionellae and non-legionellae by direct fluorescent antibody and immunodiffusion test". J.Clin.Micro., 17, 332-337.

Parsons, N.J., Andrade, J.R.C., Patel, P.V., Cole, J.A. and Smith, H. (1989) "Sialylation of lipopolysaccharide and loss of absorbtion of bactericidal antibody during conversion of gonococci to serum resistance by cytidine 5'-monophospho-N-acetyl neuraminic acid". Microbial pathogenesis, 7, 63-72.

Pasculle, J.W., Myerowitz, R.L. and Rinaldo, C.W.Jr. (1979) "New bacterial agent of pneumonia isolate from renal-transplant patients". Lancet, II, 58-61.

Pasculle, A.W., Feeley, J.C., Gibson, R.J., Cordes, L.J., Myerowitz, R.L., Pattonn, C.M., Gorman, G.W., Cormack, C.L., Ezzell, J.W. and Dowling, J.W. (1980) "Pittsburgh pneumonia agent; direct isolation from human lung tissue". J. of Inf.Dis., 141, 727-732.

Pasculle, A.W., Veto, G.E., Krystofiak, S., McKelvey, K. and Vrsalovic, K. (1989) "Laboratory and clinical evaluation of a commercial DNA probe for the detection of Legionella spp.". J.Clin.Micro., 27, No.10, 2350-2358.

Pharmacia, LKB Biotechnology. (1988) "Affinity Chromatography, principles and methods". Pharmacia (Sweden).

Reitschel, E.T., Gottert, H., Luderitz, O. and Westphal, O. (1972) "Nature and linkages of the fatty acids present in the Lipid-A component of Salmonella lipopolysaccharide." Eur.J.Biochem., 28, 166-173.

Pine, L., Hoffman, P.S., Malcolm, G.B., Benson, R.F. and Franzus, M.J. (1986) "Role of keto acids and reduced-oxygen-scavenging enzymes in the growth of Legionella species". J.Clin.Micro., 23, No.1, 33-42.

Ristuccia, P.A., Cunha, B.A. and Ristuccia, A.M. (1984) "*In-vitro* activity of antimicrobial agents against Legionella pneumonia and type strains of four other Legionella species". In Legionella; Proceedings of the 2nd International Symposium, Washington DC. (Eds. from the Am.Soc.Microbiol.), 103-105.

Rodgers, F.G., Greaves, P.W., Macrae, A.D. and Lewis, M.J. (1980) "Electron microscopic evidence of flagella and pili on Legionella pneumophila". J.Clin.Pathol., 33, 103-105.

Salton, M.R.J. (1987) "Bacterial membrane proteins". Microbiological Sciences, 4, No.4, 100-105.

Samuel, D., Harrison, T.G. and Taylor,A.G. (1990) "Detection of Legionella pneumophila serogroup 1 urinary antigen using an enhanced chemiluminescence ELISA" J. of Bioluminescence and Chemiluminescence, 5, 183-185.

Sande, M.A., Hudson, L.D. and Root, R.K. (1986) (Eds.) "Respiratory infections". Churchill Livingstone (Publ.).

Sathapatayavongs, B., Kohler, R.B., Wheat, L.J., White, A., Winn, W.C. Jr., Girod, J.C. and Edelstein, P.H. (1982) "Rapid diagnosis of Legionnaires' disease by urinary antigen detection; comparison of ELISA and RIA". Am.J.Med., 72, 576-582.

Sathapatayavongs, B., Kohler, R.B., Wheat, L.J., White, A. and Winn, W.C. Jr. (1983) "Rapid diagnosis of Legionnaires' disease by latex agglutination". Am.Rev.Respir.Dis., 127, 559-562.

Saunders, N.A., Harrison, T.G., Haththotuwa, A., Kachwalla, N. and Taylor, A.G. (1990) "A method for typing strains of L.pneumophila serogroup 1 by analysis of restriction fragment length polymorphisms". J.Med.Micro., 31, 45-55.

Schoitz, P.O., Hoiby, N. and Hertz, J.B. (1979) "Cross reactions between Haemophilus influenzae and nineteen other bacterial species". Acta Pathol. Microbiol. Scand. Sect. B, 87, 337-344.

Selander, R.K., McKinney, R.B., Whittam, T.S., Bibb, W.F., Brenner, D.J., Nolte, F.S. and Pattison, P.E. (1985) "Genetic structure of populations of Legionella pneumonia". J.Bact., 1021-1037.

Sonesson, A., Jantzen, E., Bryn, K., Larsson, L., and Eng, J. (1989) "Chemical composition of a lipopolysaccharide from L.pneumophila". Arch. Micro., 153, 72-78.

Tang, P.W., Savigny, D. and Toma, S.(1982) "Detection of Legionella antigenuria by reverse passive agglutination". J.Clin.Micro., 998-1000.

Tang, P.W. and Toma, P.S. (1986) "Broad-spectrum enzyme-linked immunosorbent assay for detection of Legionella soluble antigens". J.Clin.Micro., 24, 556-558.

Tang, P.W., Toma, S. and Rajkumar, W.D. (1989) "Detection of urinary antigens of Legionella pneumophila serogroup 12 by broad-spectrum enzyme-linked immunosorbent assay". J.Clin.Micro., 27, No.4, 783-784.

Tayler, A.G., Harrison, T.G., Dighero, M.W. and Bradstreet, C.M.P. (1979) "False positive reactions in the indirect fluorescent antibody test for Legionnaires' disease eliminated by use of formolised yolk-sac antigen". Ann.Int.Med., 90, 686-689.

Thacker, W.L., Benson, R.F., Schifman, R.B., Pugh, E., Steigerwalt, A.G., Mayberry, W.R., Brenner, D.J. and Wilkinson, H.W. (1989) "Legionella tucsonensis sp. nov. isolated from a renal transplant recipient". J.Clin.Micro., 27, No. 8, 1831-1834.

Thacker, W.L., Plikaytis, B.B. and Wilkinson, H.W. (1985) "Identification of 22 Legionella species and 33 serogroups with the slide agglutination test". J.Clin.Micro., 21, 779-782.

Thacker, W.L., Wilkinson, H.W., Benson, R.F., Edberg, S.C. and Brenner, D.J. (1988) "Legionella jordanis isolated from a patient with fatal pneumonia". J.Clin.Micro., 26, No. 7, 1400-1401.

Thomason, B.M. and Bibb, W.F. (1984) "Use of absorbed antisera for demonstration of antigenic variation among strains of Legionella pneumophila serogroup 1". J.Clin.Micro., 19, No.6, 794-797.

Thomason, B.M., Chandler, F.W., and Hollis, D.G.(1979) "Flagella on Legionnaires' disease bacteria; an interim report". Ann.Int.Med., 91, 224-226.

Thornsberry, C.L., Baker, C.N. and Kirven, L.A. (1978) "*In-vitro* activity of antimicrobial agents on Legionnaires' disease bacterium". Antimicro.Agents Chemother., 78-80.

Timbury, M.C., Donaldson, J.R., McCartney, C., Winter, J.H. and Fallon, R.J. (1988) "How to deal with a hospital outbreak of Legionnaires' disease". J.Hosp.Infect., 11, 189-195.

Tsai, C.M. and Frasch, C.E. (1982) "A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels". Anal.Biochem., 119, 115-119.

Voller, A., Bidwell, D.E. and Bartlett, A. (1976) "Enzyme immunoassays in diagnostic medicine". Bulletin W.H.O., 53, 55-65.

Wait, R. (1988) "Confirmation of the identity of Legionellae by whole cell fatty-acid and isoprenoid profiles". In "A Laboratory Manual for Legionella". (Eds. Harrison, T.G. and Taylor, A.G. Chapter 7, pp. 69-101.

Weaver, R.E. and Feeley, J.C. "Cultural and biochemical characterisation of the Legionnaires' disease bacterium". Jones and Hebert (Eds), In "Legionnaires' disease" the bacterium and methodology. CDC, Atlanta. 20-25.

Westphal, O. and Jann, K. (1965) "Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure". In R.L. Whistler (Ed.), Methods in carbohydrate chemistry, 5, Academic Press, Inc., New York.

Whang, H.W., Mayer, H., Schmidt, G. and Neter, E. (1972) "Immunogenicity of the common Enterobacterial antigen produced by smooth and rough strains". Infect. and Immun., 533-539.

Wilkinson, H.W., Sampson, J.S. and Plikaytis, B.B. (1986) "Evaluation of a commercial gene probe for identification of Legionella cultures". J.Clin.Micro., 23, No.2, 217-220.

Wilkinson, H.W., Fikes, B.J. and Cruce, D.D. (1979) "Indirect immunofluorescence test for serodiagnosis of Legionnaires' disease; evidence for serogroup diversity of Legionnaires' disease bacterial antigen and for multiple specificity of human antibodies". J.Clin.Micro., 19, 379-373.

Wilkinson, H.W. and Fikes, B.J. (1980) "Slide agglutination test for serogrouping Legionella pneumophila and atypical Legionella-like organisms". J.Clin.Micro., 11, 99-101.

Wilkinson, H.W., Draser, V., Thacker, W.L., Benson, R.F., Schindler, J., Potuznikova, B., Mayberry, W.R. and Brenner, D.J. (1988) "Legionella moravica sp. nov. and Legionella brunensis sp. nov. isolated from cooling tower water". Ann.Inst.Pasteur/Micro., 139, 393-402.

Wilkinson, I.J., Sangster, N., Ratcliff, R.M., Mugg, P.A., Davos, D.E. and Lanser, J.A.(1990) "Problems associated with identification of Legionella species from the environment and isolation of six possible new species" Appl. and Enviro.Micro., 56, No.3, 796-802.

Williams, A. and Featherstone, A.S.R. (1988) "The early diagnosis of Legionnaires' disease in a Legionella pneumophila aerosol-infected guinea pig model; comparison of a method developed for detecting Legionella pneumophila antigens in urine and the demonstration of circulating antibody by enzyme-linked immunosorbent assay". J. of Inf., 16, 47-54.

Wills, J.E., Thornton, S., and Gardiner, D.J. (1987) "Factors affecting the choice of immunising schedule". Animal Technology, 38, No.4, 183-190.

Winter, J.H., McCartney, A.C., Fallon, R.J., Telfer, A.B.M., Drury, J.K., Reece,I.J. and Timbury, M.C. (1987) "Rapid diagnosis of an outbreak of Legionnaires' disease at Glasgow Royal Infirmary". Thorax, 42, 596-599.

Wong, K.H., Moss, C.W., Hochstein, D.P.H., Arko, D.V.M. and Schalla, W.O. (1979) "Endotoxicity of the Legionnaires' disease bacterium". Ann.Inter.Med., 90, 624-627.

Wong, K.H. and Feeley, J.C. (1983) "Antigens and toxic components of Legionella in pathogenesis and immunity". Zbl.Bakt.Hyg.,I.Abt.Orig.A, 255, 132-137.

Wong, K.H. and Feeley, J.C. (1984) "Lipopolysaccharide of Legionella as adjuvant for intrinsic and extrinsic antigens". Proc.Soc.Exper.Biol.Med., 177, 475-481.

Wreghitt, T.G., Naginton, J. and Gray, J. (1982) "An ELISA test for the detction of antibodies to L.pneumophila". J.Clin.Micro., 35, 657-660.

Yonke, C.A., Hartan, E.S., Wilson, D.L. and Wentworth, B.B. (1981) "Evaluation of an indirect heamaglutination test for Legionella pneumophila serogroups 1-4". J.Clin.Micro., 13, 1040-1045.

Zanen-lim, O.G. and Zanen, H.C. (1988) "Differences in lipopolysaccharide profiles of serologically identical Legionella pneumophila serogroup 6 strains". J.Clin.Path., 41, 826-829.

Zanen-lim, O.G. and Zanen, H.C. (1988) "Lipopolysaccharides as a marker system in the epidemiology of Legionella pneumophila serogroup 12". FEMS Micro.Lett., 50, 85-88.

Zuravleff, J.J., Yu, V.L., Shannard, J.W. (1983) "Legionella pneumophila contamination of a hospital humidifier". Am.Rev.Respir.Dis., 128, 657-651.

APPENDICES

APPENDIX I

Buffers used for IgG purification

IgG purification on DEAE 52-cellulose

0.3 M Phosphate buffer :

Part A

NaH_2PO_4 (0.3M)		46.77g
dH_2O	ad	1.0 L

Part B

Na_2HPO_4 (0.3M)		42.58g
dH_2O	ad	1.0 L

0.1 M Phosphate buffer pH 7.5 :

160ml Part A + 840ml Part B + 9 L dH_2O

IgG purification on Protein-A Fast-Flow

Buffer A - pH 8.0 (with HCl)

Boric acid	(0.016M)	4.95g
NaCl	(0.012M)	3.5g
NaOH	(0.025M)	5.0g
Phenylmethyl sulfonyl fluoride (PMSF, Sigma)	(0.1mM)	0.085g
NaN_3	(0.02%)	1.0g
dH_2O	ad	5 L

Buffer B - pH 3.0(with HCl)

Glycine	(0.1M)	37.5g
PMSF	(0.1mM)	0.085g
NaN_3		1.0g
dH_2O	ad	5 L

Buffer A (dialysis)

As Buffer A excluding:

PMSF and NaN_3.

APPENDIX II

Buffers for ELISA

0.01M PBS pH 7.4	Per 4 L
Part A	
$NaH_2PO_4:2H_2O$	31.2g/L
Part B	
Na_2HPO_4	28.39g/L

19ml Part A + 81ml Part B + 0.1 L dH_2O

+ 3.8 L 0.85% (w/v) NaCl

Substrate

Mix together in order just before use.

dH_2O	8.5ml
4% o-Phenylenediamine (in dH_2O)	0.5ml
Substrate buffer	1.0ml
3% H_2O_2	50ul

Substrate Buffer

Citric acid	(170mM)		3.57g
NaH_2PO_4	(650mM)		9.23g
Thiomersal	(0.1%)		0.1g
dH_2O		ad	0.1 L

APPENDIX III

Buffers for conjugation

PBS pH 7.2

Na_2HPO_4		10.96g
NaH_2PO_4		3.15g
NaCl		85.00g
dH_2O	ad	10.0 L

1% BSA Tris-HCl, 0.05M pH 8.0

Trizma base (Sigma)		30.27g
Bovine albumin (Sigma A-7906)		5.00g
NaN_3 (0.02%)		0.1.g
conc. HCl		
dH_2O	ad	5.0 L

PBS

NaCl		40g
KH_2PO_4		1g
$Na_2HPO_4.12H_2O$		14.4g
KCl		1g
dH_2O	ad	5L

Tris-HCl, 0.05M pH 8.0

Trizma base (Sigma)		30.27g
conc. HCl		
dH_2O	ad	5.0 L

APPENDIX IV

Freezing medium for legionellae

Peptone water (Oxoid, Code CM9)		15.0g
(Formula; Peptone L37 [10g] NaCl [5g])		
dH_2O	ad	1L

(Autoclaved at 121°C for
15 min., pH 7.2 approx.)

APPENDIX V

Barbital buffer for electrophoresis

0.05M, pH 8.4, ionic strength = 0.1

Sodium barbitone		10.3g
Barbitone		1.84g
dH_2O	ad	1L

(Dissolve using a magnetic stirrer)

May crystallise on storage, especially at 4°C.