CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Legionnaires' disease was first described after a pneumonia outbreak at an American Legion Convention held in Philadelphia during 1976. In total, 182 delegates were affected and 29 died before workers at the Centres for Disease Control and Prevention (CDC) in Atlanta, GA, isolated the causative organism during January 1977. The organism was placed in the family *Legionellaceae*, genus *Legionella* (Brenner *et al.* 1979), to commemorate the first victims of the disease and the first species was named *pneumophila*, Greek for 'lung loving'. It soon became clear that these organisms were not new: retrospective studies showed that an organism isolated by Tatlock in 1944 and called *Tatlockia micdadei*, actually belonged to the genus Legionella. The organism was subsequently renamed *Legionella micdadei* (strain TATLOCK) (Pasculle 1992). The first strain of *L pneumophila*, (OLDA), was isolated in 1947 from a guinea pig that had previously been inoculated with blood from a patient with 'unknown febrile disease' (Bozeman 1968). Another historical isolate, WIGA, was isolated in 1980 by Bozeman and coworkers and named *L bozemanii*.

The two decades following the discovery of the family Legionellaceae was marked by rapid developments in Legionella detection and identification of numerous new species. In fact, some workers dispute the creation of a single genus containing such a large number of species (Pasculle 1992). Twenty-eight new Legionella species and two Legionella-like amoebal pathogens (LLAPs) (LLAP-1 and LLAP-6) were isolated during the 1980s, mostly from sources in the USA. The 1990s were marked by an increase in Legionella isolation from countries in Europe and Australia with fifteen new Legionella species and LLAP-10 were described. LLAP-1, LLAP-6 and LLAP-10 are now known as *L drozanskii*, *L rowbothamii* and *L fallonii* respectively (Adeleke *et al.* 2001). The addition of these three organisms to the genus now brings the total number of species to 48. *L lytica* was previously known as *Sarcobium lyticum* while the TATLOCK strain of *L micdadei* is referred to as *L pittsburghensis* in some of the older literature. The currently known Legionella species are listed in Table 1.1 and LLAPs in Table 1.2.

Table 1.1 Currently known Legionella species

ORGANISM	SGs	AUTHOR	YEAR	SOURCE	PATHOGEN
L adelaidensis	1	Benson et al.	1991	Cooling water (Adelaide Australia)	Unknown
L anisa	1	Gorman et al.	1985	Faucet (Chicago), tap water (LA)	Yes
L beliardensis	1	*	2002	Water, France	Unknown
L birminghamensis	1	Wilkinson et al.	1987	Lung biopsy (Alabama)	Yes
L bozemanii	2	Brenner et al.	1980	Lung aspirate (Toronto)	Yes
L brunensis	1	Wilkinson et al.	1989	Cooling tower water (Czechoslovakia)	No
L cherrii	1	Brenner et al.	1985	Thermally altered water (Minnesota)	Yes
L cincinnatiensis	1	Thacker et al.	1988	Lung tissue (Cincinnatti)	Yes
L donaldsonii*	*	*	*	*	*
L drozanskii (LLAP-1)	1	Adeleke et al.	2001	Tank of well water (Leeds 1981)	Yes
L dumoffii	1	Brenner et al.	1980	Lung tissue	Yes
L erythra	2	Brenner et al.	1985	Cooling tower water (Seattle)	No
L fairfieldensis	1	Thacker et al.	1991	Cooling tower water (Fairfield Australia)	Unknown
L fallonii (LLAP-10)	1	Adeleke et al.	2001	Ship airconditioner (1994)	Yes
L feeleii	2	Herwaldt et al.	1984	Grinding machine coolant fluid	Yes
L geestiana	1	Dennis et al.	1993	Hot water tap, office building (London)	Unknown
L gormanii	1	Morris et al.	1980	Bronchial wash of pneumonia patient	Yes
L gratiana	1	Bornstein et al.	1991	Thermal spa water (France)	No
L gresilensis	1	*	2002	Water, France	Unknown
L hackeliae	2	Brenner et al.	1985	Bronchial biopsy (Ann Arbour)	Yes
L israelensis	1	Bercovier et al.	1985	Water (Israel)	No
L jamestowniensis	1	Brenner et al.	1985	Wet soil (New York)	No
L jordanis	1	Cherry et al.	1982	Water and sewage (Israel)	Yes
L lansingensis	1	Thacker et al.	1994	Bronchial washing, leukemia patient	Yes
L londiniensis	1	Dennis et al.	1993	Office building cooling tower (London)	Unknown
L longbeacheae	2	McKinney et al.	1982	Human lung (Longbeach Australia)	Yes
L lytica	1	Hookey et al.	1996	Previously Sarcobium lyticum	Yes
L maceachernii	1	Brenner et al.	1985	Water (Phoenix)	Yes
L micdadei	1	Hebert et al.	1980	Human blood via yolk sac	Yes
L moravica	1	Wilkinson et al.	1989	Cooling tower water (Czechoslovakia)	No
L nautarum	1	Dennis et al.	1993	Hot water tap (London)	Unknown
L oakridgensis	1	Orrison et al.	1983	Cooling tower water (Pennsylvania)	Yes
L parisiensis	1	Brenner et al.	1985	Cooling tower water (Paris)	Yes
L pittsburghensis	1	Pasculle et al.	1980	Synonym for L micdadei, strain TATLOCK	Yes
L pneumophila	15	Brenner et al.	1979	Water (Pennsylvania)	Yes
L quateirensis	1	Dennis et al.	1993	Shower in hotel bathroom (Portugal)	Unknown
L quinlivanii	2	Benson et al.	1990	Water in bus airconditioner (Australia)	No
L rowbothamii (LLAP-6)	1	Adeleke et al.	2001	Water and sludge, industrial liquefier	Yes
L rubrilucens	1	Brenner et al.	1985	Tap water (Los Angeles)	Yes
L sainthelensi	2	Campbell et al.	1984	Spring water (Washington)	Yes
L santicrucis	1	Brenner et al.	1985	Tap water (Virgin Islands)	No
L shakespeari	1	Verma et al.	1992	Cooling tower water (England)	Unknown
L spiritensis	1	Brenner et al.	1985	Lake water (Washington)	No
L steigerwaltii	1	Brenner et al.	1985	Tap water (Virgin Islands)	No
L taurinensis	1	Lo Presti et al.	1999	Water, hospital humidifier (Italy)	Unknown
L tusconensis	1	Thacker et al.	1990	Pleural fluid, transplant patient (Arizona)	Yes
L wadsworthii	1	Edelstein et al.	1983	Sputum	Yes
L waltersii	1	Benson et al.	1996	Potable water system (Australia)	Unknown
L worsleiensis	1	Dennis et al.	1993	Industrial cooling water (England)	Unknown

*Sources: ATCC website, NCTC website, L donaldsonii mentioned once only in the literature (Adeleke 1996). Information regarding pathogenesis: De Gheldre et al. (2001); * no information available at time of print.*

The first Legionella-like amoebal pathogen (LLAP) was isolated from soil in Poland in 1954 and was named *Sarcobium lyticum* (Adeleke 1996). The next isolation of an LLAP was in England more than 20 years later. Since then, LLAPs have been isolated from various sources and found to infect a variety of amoebae (Table 1.2). Except for *S lyticum*, LLAP-1, LLAP-3 and LLAP-5, all LLAPs were originally isolated

Table 1.2 Currently known Legionella-like amoebal pathogens (LLAPs)

STRAIN	HOSTS	YEAR	ORIGINAL SOURCE	PATHOGENIC
Sarcobium lyticum	A polyphaga H vermiformis	1954	Soil	Yes
LLAP-1	A polyphaga	1981	Tank of portable water well	Yes
LLAP-2	A polyphaga H vermiformis	1986	Garage steam cleaning pit	Yes
LLAP-3	A polyphaga	1986	Sputum from pneumonia patient	Yes
LLAP-4	A polyphaga	1986	Hospital whirlpool bath	Yes
LLAP-5	A polyphaga	1988	Nursing home plant spray	Yes
LLAP-6	A polyphaga H vermiformis	1988	Factory liquefier tower	Yes
LLAP-7	A polyphaga H vermiformis	1991	Hotel whirlpool spa	Yes
LLAP-8	H vermiformis	1990	Hospital shower	Yes
LLAP-9	A polyphaga H vermiformis	1992	Factory cooling tower	Yes
LLAP-10	A polyphaga	1994	Ship air-conditioning system	Yes
LLAP-11	A polyphaga	1993	Furnace cooling system	Yes
LLAP-12	A polyphaga	1994	Furnace cooling system	Yes

Adapted from Adeleke 1996

from sources associated with confirmed cases or outbreaks of Legionnaires' disease (Adeleke 1996).

Legionnaires' disease outbreaks have been documented from as early as 1957 in Philadelphia (Terranova *et al.* 1978), Wadsworth (Haley *et al.* 1979), Washington DC (England *et al.* 1981) and Minnesota (Osterholm *et al.* 1983). The first outbreak of Pontiac fever was reported in Pontiac, Michigan, in 1968 (Glick *et al.* 1978). Today serious Legionnaires' disease outbreaks are still regularly reported (Table 1.3).

Table 1.3 Recent Legionnaires' disease outbreaks (1999-2001)

DATE	SITE AND SOURCE	CASES	DEATHS
March 1999	Flower show, Netherlands: spa bath	242	28
June 1999	Hotel, Belgium: hot water system	4	1
February 2000	Hotel, South Wales: food display humidifier	5	3
March 2000	Office building, Melbourne: cooling tower	5	0
April 2000	Aquarium, Melbourne: cooling towers	110	2
June 2000	Public bath, Japan: hot water system	14	1
June 2000	Hospital, Maryland: hot water system	5	3
March 2001	Office building, Melbourne: cooling tower	5	2
March-April 2001	Ford plant, Ohio: burst pipe	6	2
March-June 2001	Hospital, Melbourne: source unknown	7	2
May-June 2001	Central London building: hot water system	3	0
February-June 2001	Las Vegas: source unknown	3	0
July 2001	Hospital, Paris: hot water system	12	6
July 2001	Murcia, Spain: cooling towers	315	2
August 2001	Hospital, Spain: hot water system	18	3
October 2001	Murcia Spain	U	U
October 2001	Melbourne Australia	U	U

Sources: http://www.q-net.net.au/~legion/Legionnaires_Disease_2001.html,
http://www.hcinfo.com/outbreaks-news.htm#0107Spain

1.2 CLASSIFICATION

The family Legionellaceae contains one genus (Legionella) and 46 species, together consisting of 58 serogroups (SGs) (Table 1.1). *L pneumophila* contains 15, and *L bozemanii, L feeleii, L hackeliae, L sainthelensi* and *L longbeacheae* each contain two serogroups. Legionellae are distantly related to the purple sulfur bacteria and their non-photosynthetic relatives. Hybridisation studies demonstrated that the legionellae were closely related to common members of the Enterobacteriaceae and to Pseudomonas, all of which are also related at the ribosomal level to the purple photosynthetic bacteria. The guanine plus cytosine (G+C) content of the DNA of Legionella species ranges between 38 and 52 mol% (Brenner *et al.* 1985). The different species in the genus Legionella were created on the basis of their DNA relatedness. The various Legionella species share up to 67% DNA relatedness at optimal hybridization temperature while strains within a given species have 70% or greater relatedness and <5% deviation in related sequences.

1.3 MORPHOLOGY

Legionella species are thin, obligatory aerobic, faintly staining gram-negative bacilli that are non-spore forming and contain no capsules. Although legionellae are generally non acid-fast, partial acid-fastness has been described in *L micdadei* and one strain of *L pneumophila* SG 6. All legionellae except *L oakridgensis* are motile (Brenner 1986, Winn 1984).

Legionellae are typically between 0.3-0.9 μm wide and 2-20 μm long. However, coccobacillary forms measuring 1-2 μm may be observed in clinical specimens and under conditions of iron deprivation (Ristroph *et al.* 1981) whereas long, filamentous organisms may result from growth on certain culture media (Yu 1990). The organisms often appear tapered at one or both ends but without the needle-shaped appearance of Fusobacterium species (Winn *et al.* 1984).

Although physically hardy and heat tolerant in nature, legionellae require special laboratory media and techniques for isolation (Brenner *et al.* 1984). Under laboratory conditions, they can survive in distilled water for long periods of time (up to 139 days) and in tap water for more than a year when kept at room temperature. Although they grow and multiply in temperatures between 25-42°C, with an optimum of 35-37°C, they cannot survive for long periods at temperatures above 50°C.

Legionella-like amoebal pathogens are nonculturable, protozoonotic, gram-negative bacilli. They were named LLAPs because of their ability to infect amoebae and replicate

intracellularly, in the same way that legionellae infect protozoa and human macrophages. They differ from legionellae in that they cannot be cultured on laboratory media (Adeleke 1996).

1.4 CELL ENVELOPE

The typical gram-negative cell envelope is a multi-layered structure composed of two trilaminar membranes, the outer membrane (cell wall) situated outside a thin peptidoglycan layer which is separated from the cytoplasmic (inner) membrane by a periplasmic space. The cell envelope contains approximately 10-20% peptidoglycan and polysaccharides, proteins and a glycolipid known as lipopolysaccharide (LPS). Toxins and enzymes are located in the periplasmic space and are present in concentrations sufficient to assist in the destruction of substances that may harm the organism (Craeger *et al.* 1990).

The cell envelope of Legionella species is similar to that of other gram-negative bacteria consisting of two trilaminar membranes, each 75Å wide (Chandler *et al.* 1979, Neblett *et al.* 1979). Between the two membranes a fine peptidoglycan layer is visible by electron microscopy after plasmolysis or papain treatment. The two layers of the cell envelope can be separated by sucrose density gradient centrifugation but not by treatment with detergents (Gabay *et al.* 1985).

The outer and cytoplasmic membranes of legonellae have very distinct protein profiles, but their enzymatic activity and 2-keto-3-deoxyoctonate (KDO) content is similar (Winn 1984). *L micdadei* contains an unusual, electron-dense layer between the trilaminar membranes, visible after cultivation in eggs or cell culture, but not after culture on agar media. The significance of this layer is unknown, but may be related to the partial acid fastness of the organism. Complex surface structures, resembling capsules and containing mainly polysaccharides, have been demonstrated in *L pneumophila*. However, the superficial antigens of these structures are different from those normally found in capsules; they migrate in the opposite direction from most polysaccharides when subjected to counter-current electrophoresis. In addition, membrane-bound granules that resemble beta-3-hydroxylbutarate granules have been demonstrated by freeze-fracture methods (Winn 1984).

1.4.1 Cell membrane

The main function of the cell membrane is to regulate the movement of materials into and out of the cell by various transport mechanisms. The membrane also synthesises cell wall (outer membrane) components, assists with DNA replication, secretes proteins, carries on respiration and captures energy in ATP. Furthermore, it contains the bases of flagella and responds to chemical substances in the environment. The typical cell membrane consists of about 70% protein and 30% phospholipid by weight and contains small amounts of carbohydrate. Two forms of cell membrane (CM-1 and CM-2) were detected in *L pneumophila* (Hindahl and Iglewski 1984). The CM-1 fraction was the purest form of cell membrane while CM-2 was consisted mainly of cell membrane associated with small amounts of peptidoglycan.

The phospholipids in the membrane form a bilayer and are in a fluid state with protein molecules scattered among, and embedded in, the lipid molecules in the membrane, forming a mosaic pattern. Some of the protein molecules extend through the entire membrane and act as carriers or form pores or channels through which materials enter and leave the cell. Others are embedded in or loosely attached to the inner or outer surface of the membrane. Proteins on the inner surface are usually enzymes; those on the outside include proteins that make the cell identifiable as a particular organism (Craeger *et al.* 1992).The phospholipid composition of legionellae species is different from that of other gram-negative bacteria in that they possess an unusually high content of phosphatidylcholine. However, there are no marked differences in the phospholipid composition of the different Legionella species.

1.4.2 Peptidoglycan

Peptidoglycan, a macromolecule unique to prokaryotes, is synthesised in the cell cytoplasm and comprises 10-20% of the cell envelope. It surrounds the cell membrane, providing rigidity, shape and mechanical protection to the cell. Peptidoglycan consists of a glycan (polysaccharide) backbone containing mainly N-acetyl-muramic acid (murNAc) and N-acetyl-glucosamine (gluNAc), which can be cleaved by the bacteriolytic enzyme lysozyme. Peptide side-chains containing amino acids and in some instances diaminopimelic acid are attached to the backbone and are cross-linked by peptide bridges that vary in structure among bacterial species. The peptidoglycan layer is attached to the outer membrane by a small, 75 kilodalton (kDa) lipoprotein, which assists in anchoring the

peptidoglycan to the outer membrane and in maintaining the structure and organisation of the cell. The peptidoglycan layer of a gram-negative cell differs from that of a gram-positive cell in that it is thinner and more flexible although sufficiently strong to protect the cell against osmotic lysis.

The *L pneumophila* peptidoglycan layer was demonstrated for the first time in 1979 by electron microscopy, following plasmolysis or papain treatment (Chandler *et al.* 1979, Neblett *et al.* 1979). It is similar in composition to that found in most gram-negative bacteria, except for the absence of diaminopimelic acid in most species. Diaminopimelic acid has been demonstrated in some isolates of *L pneumophila* SG1. The peptidoglycan of *L pneumophila* is composed of muramic acid, glucosamine, glutamic acid, alanine and meso-diaminopimelic acid with a molar ratio of 0.8:0.8:1.1:1.7:1, sensitive to lysozyme hydrolysis and insensitive to alkali dissolution (Amano and Williams 1983).

1.4.3 Cell wall (outer membrane)

The cell wall, situated outside the peptidoglycan layer, is a porous structure containing mainly proteins and lipopolysaccharide that performs two main functions. It maintains the shape of the cell and prevents it from bursting when fluids flow into it by osmosis. The phospholipids in the cell wall are similar to those in the cell membrane, being primarily phosphatidyl ethanolamine and phosphatidyl glycerol. The remainder of the cell wall comprises proteins.

Lipopolysaccharide (LPS) or endotoxin is a complex glycolipid present in the outer membrane of all gram-negative bacteria. LPS is essential for bacterial growth and viability. It contributes to the low membrane permeability and participates in physiological functions of the membrane, increases the resistance of the cell to hydrophobic agents and is the primary target for the attack of antibacterial drugs and proteins such as components of the host's immune response (Wiese 1999). LPS consists of three distinct, covalently bound regions: lipid A, core polysaccharide and O-specific side chains.

Lipid A, the biologically active part of the LPS molecule in which the toxicity of the LPS resides, is embedded in the outer part of the cell wall. It consists of a carbohydrate backbone of alternating pyrophosphate units and glucosamine and resembles a glycerol-based phospholipid but is larger and more rigid. Long fatty acid side chains, usually containing 10-18 carbons, are attached to the backbone. The core polysaccharide chain contains mainly two sugars, 2-keto-3-deoxyoctulosonic acid (KDO) and heptose and is attached to the inner portion of the carbohydrate backbone via the KDO. Other sugars usually present in the core polysaccharide are glucose, glucosamine, galactose, rhamnose

and mannose (Sonesson *et al.* 1989, Petitjean *et al.* 1990). O-specific side chains are attached to the terminal sugar of the core polysaccharide and extend outward into the medium surrounding the cell. They consist of variable numbers (usually 10-50) of repeating oligosaccharide units of 3-5, often very unusual, sugars each. The O-specific side chains enhance the toxicity of the cell by keeping the hydrophobic lipid A portion water-soluble (Rietschel *et al.* 1985, Wiese *et al.* 1999). The major serologic determinants of most gram-negative bacteria reside in the O-specific side chains; organisms lacking these chains (rough or 'R' type) are less virulent than their smooth ('O' type) counterparts.

The LPS of Legionella species has a complex, unusual structure and chemical features that may account for its importance as a bacterial virulence factor (Gabay *et al.* 1985, Otten *et al.* 1986, Sonesson *et al.* 1989, Zähringer *et al.* 1995, Yu 1990). All the legionellae tested to date, except *L longbeacheae* and *L dumoffii*, produce a smooth form of LPS (Sonesson *et al.* 1994). Carbohydrates represent 10-20% of the total weight of the *L pneumophila* LPS; 1-13% of the carbohydrate backbone consists of KDO (Ciecielski *et al.* 1986).

The carbohydrate backbone of the Legionella lipid A is antigenically related to that of Enterobacteriaceae but possesses some unique structural features (Ciecielski *et al.* 1986). It consists of glucosamine, quinovosamine and glycerol, in the molar ratio 3.9:1.0:3.4 with glycerol as a phosphorylated moiety and contains phosphatidylcholine (Lambert *et al.* 1989). The major constituent of *L longbeacheae, L bozemanii, L israelensis, L maceachernii* and *L micdadei* lipid A is 2,3-dideoxy-2,3-diamino-d-glucose. *L israelensis* contains a substantial amount of d-glucosamine while *L bozemanii* and *L longbeacheae* SG 1 lipid A contains d-glucosamine and glycerol as minor constituents of the backbone structure (Sonesson *et al.* 1989)

The core polysaccharide of *L pneumophila* contains rhamnose, mannose, glucose, quinovosamine, glucosamine and KDO in the molar proportion 1.6:1.8:1.0:1.5:4.1:2.7. Heptose is absent and glucose is mainly phosphorylated (Sonesson *et al.* 1989). The outer core polysaccharide of *L pneumophila* exhibits hydrophobic properties due to the presence of N- and O-acetyl groups and 6-deoxy sugars (Zähringer *et al.* 1995). The inner core contains KDO but lacks heptose and phosphate groups. Sonesson's group (1994) demonstrated the presence of d-glucosamine, d-mannose, d-glucose, l-rhamnose, d-glycero-d-manno-heptose, l-glycero-d-manno-heptose, KDO and glycerol in the core polysaccharides of *L bozemanii* and *L longbeacheae*. In their study, the core polysaccharide from *L longbeacheae* appeared shorter, but composed of the same sugars as *L bozemanii*, except for the absence of l-fucosamine. Glycerol- and glucosamine

phosphate was present in both organisms and *L longbeacheae* contained glucose phosphate as well. The d-quinovosamine and l-fucosamine constituted 80mol% of the *L bozemanii* core polysaccharide.

The core polysaccharides of *L maceachernii and L micdadei* both contain mainly l-rhamnose, l-fucose, d-mannose, d-glucose, l-fucosamine, d-glucosamine, KDO and the rare octose yeersiniose A. The composition of the *L israelensis* core is simpler and consists of mainly l-rhamnose and 3-amino-3,6-dideoxy-D-mannose. In *L israelensis* and *L micdadei* the 2-keto-octonic acid is linked to the KDO (Sonesson *et al.* 1994). Phosphorylated sugars have been demonstrated in *L israelensis, L micdadei* and *L maceachernii*, whereas ethanolamine is only present in *L maceachernii*. This group also demonstrated that *L micdadei* contains a higher proportion of the low molecular weight constituents than *L maceachernii* and *L israelensis*.

The O-chain of *L pneumophila* SG 1 constitutes a homopolymer of an unusual sugar, legionaminic acid, of which approximately 10-75 residues are present (Zähringer *et al.* 1995, Knirel *et al.* 1996). The lack of free hydroxyl groups of this homopolymer renders the cell surface highly hydrophobic, thereby supporting adherence to the membranes of target cells. Epidemiologic studies have shown an epitope situated in the environment of the *L pneumophila* SG 1 legionaminic acid to be associated with virulence (Zähringer *et al.* 1995). The O-chain is linked to position 3 of the terminal rhamnosyl group that, together with the occurrence of the reducing KDO residue in multiple forms, contributes to the heterogeneity of the core oligosaccharide. This highly O-acetylated core oligosaccharide is unique to legionellae (Knirel *et al.* 1996).

1.5 CAPSULAR-LIKE ENVELOPE MATERIAL

Legionella species do not contain capsules but complex surface structures, resembling capsules and containing mainly polysaccharides, have been demonstrated in *L pneumophila, L micdadei* and *L dumoffii* (Hébert *et al.* 1984). The capsular-like envelope contains approximately 35% carbohydrate, 2.6% protein, 1.8% phospholipid and 1% KDO and has been a variable finding in more recent studies (Yu 1990).

1.6 FLAGELLA AND FIMBRIAE

Flagella (H-antigens) are long, hollow, helical filaments, usually several times the length of the cell and 10-20nm in diameter that are involved in cell movement. The number and location of flagella are distinctive for each genus. Fimbriae (pili) are straight filaments

composed of protein subunits called pilin, are thinner and shorter than flagella, extend out from the surface of the cell and are not associated with cell movement. They are present only on gram-negative cells and are an example of a class of surface structures termed adhesins that allow attachment of bacterial cells to other cell surfaces. As such they are extremely important for bacterial survival in an animal host. Most fimbriae are lectins, able to bind to specific sugar residues on glycoproteins and other surface components on host cells.

Most Legionella species and serogroups possess single polar flagellae and fimbriae on primary isolation (Hébert 1981, Yu 1990). The *L pneumophila* flagellum is composed of one major subunit, the FlaA protein (Dietrich *et al.* 2001). Recent studies have indicated that expression of this flagellum is temperature regulated and influenced by the growth phase of the organism, the viscosity and the osmolarity of the growth medium and by amino acids (Heuner *et al.* 1999). Flagellated legionellae are usually short forms (Chandler *et al.* 1980) whereas dividing organisms are seldom flagellated.

Fimbriae, approximately 10-11 nm in diameter and coiled and bent at irregular intervals, similar to the fimbriae found on *Pseudomonas aeroginosa*, have been demonstrated on *L pneumophila.* The presence and expression of a type IV pilin gene may provide an advantage for colonisation of lung tissues during infections and invasion of amoebae in the environment (Stone and Abu Kwaik 1998).

1.7 CHEMICAL COMPOSITION

1.7.1 Enzymes and toxins

Legionellae produce a number of enzymes and extracellular products (potential toxins). These products are usually linked to virulence although their exact role in pathogenesis and tissue damage is still not clear, especially since some of these products are produced by avirulent strains. The products produced by legionellae include hemolysins, proteases, esterases, phosphatases, aminopeptidases and endonucleases (Yu 1990). Cianciotto (2000) reported the presence of acid phosphatase, zinc-metalloprotease, monoacylglycerol lipase, phospholipase A, lysophospholipase A, RNAse and pNPCC hydrolase in the type II *pilD*-dependent exoproteins of legionellae.

Under natural conditions aminopeptidases are necessary for the degradation of algal extracellular products in aquatic environments and are known to split the amino acids used as carbon and energy sources off from appropriate compounds. In legionellae

they may be involved in virulence together with esterases through their ability to degrade peptides and proteins of the infected host.

Phosphatases are important bacterial virulence factors. They are believed to be involved in pathogenesis of several intracellular bacteria, including *L micdadei*. Although *L pneumophila* possesses phosphatase activity, its exact role in pathogenesis is still unclear. However, *L pneumophila* produces an organic pyrophosphatase during intracellular survival but acid phosphatase is apparently not essential for intracellular infection (Aragon *et al.* 2000, Inglis *et al.* 2000). *L pneumophila* has been reported to secrete phospholipase A but not phospholipase C (Cianciotto *et al.* 2000).

Low catalase activity has been reported in *L pneumophila, L wadsworthii, L oakridgensis* and *L gormanii* whereas *L jordanis, L longbeacheae, L micdadei* and *L bozemanii* possess high catalase activity. Peroxidase activity has been demonstrated in *L pneumophila, L gormanii* and *L dumoffii* (Pine *et al.* 1984). *L pneumophila* contains two enzymes (KatA and KatB) with catalytic and peroxidatic activity (Bandyopadhyay and Steinman 2000). KatA is induced during exponential growth and is the predominant peroxidase in the stationary phase. When KatA is inactivated, the sensitivity of *L pneumophila* to exogenous hydrogen peroxidase is increased and its virulence to macrophages of the THP-1 cell line is decreased. KatB has similar characteristics, except that its inactivation had no effect on the stationary phase in this study.

Superoxide dismutase (SOD) is present in *L pneumophila, L wadsworthii, L oakridgensis, L gormanii, L jordanis, L longbeacheae, L micdadei* and *L bozemanii* at levels ranging between 8.2 and 30.5 U/mg protein. *L pneumophila* contains three types of SOD: iron-containing (Fe-SOD), iron,zinc-containing (Fe,Zn-SOD) and copper,zinc-containing (Cu,Zn-SOD). Cu,Zn-SOD is not often present in bacteria but is widespread in eukaryotes. St. John and Steinman (1996) reported an increase in the activity of Cu,Zn-SOD during the transition from the expoenetial to the stationary phase of *L pneumophila* growth, suggesting that this product probably contributes to the pathogenecity of the organism but is not essential for intracellular survival or macrophage killing.

1.7.2 Fatty acids

Bacterial cells usually contain fatty acids with 16-18 carbons that exist in saturated and monounsaturated forms. Small amounts of cyclopropane and branched fatty acids, the latter containing large amounts of ubiquinones with more than 10 isoprenes in the side chain, are often encountered. These fatty acids are relatively uncommon in gram-negative bacteria except in *Thermus aquaticus* and a thermophilic

flavobacterium but are common in gram-positive bacteria like Listeria, Propionibacterium, Bacillus and Staphylococcus species (Brenner 1986, Ehret *et al.* 1986, Moss *et al.* 1977).

The fatty acid composition of Legionella species has been studied extensively. The cell walls of Legionella species contain distinctive fatty acids, 80-90% of which are branched-chain, containing 14-17 carbons. Lambert and coworkers (1989) found only one type strain of *L erythra* that did not contain such a high percentage of branched-chain fatty acids. Trace amounts (<0.5-5%) of ester-linked hydroxy fatty acids are present in about 50% of Legionella species. The predominant fatty acids found in legionellae are C^{i}16:0, C^{a}15:0 and C^{a}17:0 (Ehret *et al.* 1986, Lambert *et al.* 1989). Ubiquinones Q11 and Q12 are major components in *L pneumophila* and Q13 is present in small quantities (Brenner *et al.* 1986, Ehret *et al.* 1986). The most recent findings regarding the fatty acid composition of Legionella species are summarised in Tables 1.4 and 1.5.

Table 1.4 Major fatty acids of the genus Legionella

MET-B	(%)	UNSAT	(%)	SAT	(%)	SAT-OH	(%)	BC-OH	(%)	CYCLO	(%)	UNSAT-OH	(%)
a11:0	(4)	a15:1A	(4)	14:0	(86)	10:0 3OH	(2)	i14:0 3OH	(58)	$_{cyc}$17:0	(77)	16:0 2OH	(5)
i12:0	(2)	a17:1A	(1)	15:0	(99)	11:0 3OH	(1)	i15:0 3OH	(1)				
i13:0	(4)	16:1H	(57)	16:0	(100)	12:0 3OH	(1)	i16:0 3OH	(24)				
a13:0	(10)	15:1$^{(n-6)}$	(100)	17:0	(93)	15:0 2OH	(16)						
i14:0	(100)	16:1$^{(n-5)}$	(51)	18:0	(79)	15:0 3OH	(8)						
i15:0	(84)	16:1$^{(n-7)}$	(100)	19:0	(54)								
a15:0	(100)	16:1$^{(n-11)}$	(3)	20:0	(74)								
i16:0	(100)	17:1$^{(n-8)}$	(4)										
16:0$^{10\text{-MET}}$	(1)	17:1$^{(n-9)}$	(16)										
i17:0	(73)	a17:1$^{(n-9)}$	(42)										
a17:0	(100)												
i18:0	(53)												
i19:0	(13)												
a19:0	(24)												
i20:0	(8)												

MET-B: methyl branched; UNSAT: unsaturated; SAT: saturated; SAT-OH: saturated hydroxy; BC-OH: branched chain hydroxy; CYCLO: cyclopropoane; UNSAT-OH: unsaturated hydroxy; (%): percentage of Legionella species that contain this fatty acid; Information adapted from Diogo et al. 1999.

1.4.4 Outer membrane proteins (OMPs)

Associated with the peptidoglycan layer, *L pneumophila* contains a major outer membrane protein (MOMP) that acts like a porin by forming ion-permeable channels in contact with lipid membranes (Gabay *et al.* 1985, Hindahl *et al.* 1986, Yu 1990). This MOMP is similar to the *E coli* K-12 OmpF, OmpD and LamB porins and can be partially dissociated from the peptidoglycan layer in the presence of high salt concentrations (Hindahl *et al.* 1986). The MOMP is similar in all Legionella species. The major protein in the *L pneumophila* MOMP has a molecular weight of 28kDa and is exposed on the cell surface (Kniros *et al.* 1999).

It has been suggested that the Legionella MOMP is composed of 28kDa and 31kDa subunits, crosslinked by interchain disulfide bonds. It is covalently bound to the underlying peptidoglycan via the 31kDa subunit (Butler *et al.* 1990, Hoffman *et al.* 1992a). Further studies however revealed that the 31kDa subunit is in fact a 28kDa subunit containing a bound fragment of peptidoglycan (Hoffman *et al.* 1992a). Butler et al (1990) demonstrated that the banding patterns ranging between 31-45kDa and between 55-60kDa resolved as a single 31kDa protein when the material was treated with N-acetylmuramidase.

Table 1.5 Major fatty acids in individual Legionella species

UNKNOWN	METHYL BRANCHED				UNSATURATED	
	15:0 (%)	i16:0 (%)	a17:0 (%)	i14:0 (%)	$16:1^{(n-7)}$(%)	$16:1^{(n-11)}$(%)
cincinnatiensis	anisa (24)	cherrii (30)	lansingensis(34)	waltersii (U)	feeleii* (24)	adelaidensis (27)
fallonii	birminghamensis(32)	erythra (30)	londiniensis (22)		geestiana (33)	
lytica	bozemanii (26)	fairfieldensis (38)			moravica (32)	
rowbothamii	brunensis (37)	gratiana (26)			quateirensis (32)	
taurinensis	dumoffii (37)	longbeacheae(23)			sainthelensii (29)	
	feeleii* (25)	oakridgensis (25)			worsleiensis (33)	
	gormanii (25)	pneumophila (33)				
	hackeliae (36)	rubrilucens (30)				
	israelensis (38)	santicrucis (27)				
	jamestowniensis(39)	shakespearii (37)				
	jordanis (54)	spiritensis (30)				
	maceachernii (32)					
	micdadei (39)					
	nautarum (38)					
	parisiensis (28)					
	pittsburghensis(39)					
	quinlivanii (26)					
	steigerwaltii (27)					
	tusconensis (35)					
	wadsworthii (48)					

*Adeleke et al. 2001, Diogo et al. 2001, Lo Presti et al. 1999, Woods et al. 1988. *Diogo et al. reported differences in fatty acid contents of L feeleii species tested. (%):percentage of total fatty acids of the particular species*

1.5 ANTIGENIC COMPOSITION

Legionella antigens are high molecular weight complexes with the chemical properties of a lipopolysaccharide (endotoxin) on the cell surface. Serogroup-spcecific antigens are high molecular weight, protein-carbohydrate-lipid complexes containing the major fatty acids and enzymes like hemolysins, proteases, esterases, phosphatases, amino-peptidases and endonucleases. The majority of these antigens are situated in the O-side chain of the LPS, contain small amounts of KDO, consist of lipid A and resemble the endotoxin of other gram negative bacteria like *S minnesota, C burnetti* and *C psittaci* (Ciecielski *et al.* 1986).

Table 1.6 Antigenic composition of Legionella species

MOL WT	CHARACTERISTICS	REFERENCES
11kDa	Species-specific for *L pneumophila*	Sampson et al. 1986
19kDa	Similar to the PplA in *E coli*. SG-specific for *L pneumophila* SGs 1-8 but not identical in all SGs. Exhibits sequences homologous to *E coli* and *H influenza* LPS. *L pneumophila* SG 1 LPS is tightly bound to this protein. Sensitive to formalin.	Ott et al. 1991 Hindahl et al. 1987 Boissinot et al. 1987 Sampson et al. 1986
24kDa	Mip (macrophage infectivity potentiator), surface antigen in *L pneumophila*. Prokaryotic homolog of FK506 protein needed for optimal infection of macrophages. Required for optimal infection of human macrophages but not for extracellular growth. Other legionellae express a 24-30kDa Mip-like protein. Mip-like analogs have also been described in other prokaryotes for example Chlamydia, Neisseria and Pseudomonas species. It is believed that Mip-related proteins may constitute a family of prokaryotic proteins.	Engleberg et al. 1993 Dumais-Pope et al. 1993 Hurley et al. 1993 Cianciotto et al. 1992 Hacker et al. 1993
25kDa	Adhesive molecule for host cells and plays a major role in Legionella virulence. Species-specific.	Kniros et al. 1999 Sampson et al. 1986
28kDa	Some workers regard this protein as the Legionella MOMP. It is covalently bound to the underlying peptidoglycan via a 31kDa subunit and is exosed on the cell surface.	
29kDa	Generally regarded as the Legionella MOMP. All legionellae have the same MOMP except for a few species with a 46-48kDa band. Present *in L pneumophila* SGs 1-8 but not identical among serogroups. Major component of the *L pneumophila* serogroup-specific antigen and is heat-stable. Only partly exposed on the cell surface; exposure can be increased by treatment with the detergent EDTA.	Boissinot et al. 1987 Gosting et al. 1984 Sampson et al. 1986 Ehret et al. 1985 Hindahl et al. 1986 Yu 1990 Ciecielski et al. 1986
30kDa	The molecular weight of the MOMP falls between 24-30kDa. Legionella species other than *L pneumophila* possess a Mip-like protein of 24-30kDa.	
31kDa	Part of MOMP, binding the 28kDa subunit to the peptidoglycan.	Butler et al. 1990
43kDa	Genus-specific.	
44kDa	Present in some legionellae. Most prominent 2-4 hours after infection of various cell lines. Synthesis not dependent on protein synthesis. Also known as 'Legionella intracellular growth antigen' (LIGA) since it is found exclusively in intracellularly grown *L pneumophila*.	Susa et al. 1996
45kDa	Present in *L pneumophila* SGs 1-8 but not identical in all serogroups.	Boissinot et al. 1987
46kDa	Present in some legionellae.	
47kDa	Present in some legionellae.	
48kDa	Present in some legionellae.	
55kDa	The banding pattern between 55-60kDa proteins resolves as a single 31kDa protein when treated with N-acetylmuramidase.	Butler et al. 1990.
58kDa	Genus-specific.	
61kDa	Genus-specific.	
65kDa	Single major proteint in the cell membrane of legionellae.	Gabay et al. 1985
66kDa	Identity unknown.	
68kDa	Genus-specific.	
88kDa	Species-specific for *L pneumophila*.	Sampson et al. 1986
97kDa	Present in some species.	
135kDa	Species-specific for L pneumophila; surface exposed; not peptidoglycan-associated	Lever 1993
154kDa	Genus-specific.	

The antigenic composition of Legionella species has been studied quite extensively since the late 1970s, when the presence of serogroup-specific antigens was first reported (Johnson *et al.* 1979). By the early 1980s, 85 different family-, genus-, species- and serogroup-specific antigens had been described (Collins *et al.* 1983). Soon thereafter, in 1984, *L pneumophila* alone was shown to possess at least 23 different antigens of which 11 were heat stable and presumably non-protein in composition (Winn 1984). The complete chemical structure of the species- and serogroup-specific antigens and the role of individual antigens in pathogenesis were unknown at the time. It was suggested that they play a role in attachment to mucosal surfaces, antibody binding and the coiling phagocytosis characteristic of legionellae (Pearlman *et al.* 1985, Ciecielski *et al.* 1986). The different antigens and their functions are summarised in Table 1.6.

1.6 GENETIC COMPOSITION

The Legionella genome is currently being sequenced in its entirety; to date approximately 80% of the genome has been sequenced. The guanine plus cytosine (GC) content ranges between 38 and 52mol% (Brenner *et al.* 1988). Legionella species share up to 67% DNA relatedness at optimal hybridisation temperature. Strains within one species share ≥70% DNA relatedness with <5% deviation in related sequences. Although not closely related the other groups of organisms, their closest relatives are the Enterobacteriaceae and Pseudomonas species. They are also distantly related to the purple sulfur bacteria. Plasmids of 85, 84, 80, 60 and 43Mdal (megadaltons) have been described in some Legionella species in the early 1980s. (including *L pneumophila* SGs 2, 3 and 4, *L bozemanii, L dumoffii, L micdadei* and *L gormanii* but not *L pneumophila* SGs 1, 5 and 6) (Aye *et al.*1981, Brown *et al.* 1982, Maher *et al.* 1982). Nolte *et al.* (1984) detected a 21-Mdal plasmid in clinical and environmental strains of *L pneumophila* SG 1. The genetic composition of legionellae is summarised in Table 1.7.

1.6 CLINICAL SIGNIFICANCE

Infections caused by legionellae are collectively known as legionellosis and include Legionnaires' disease and Pontiac fever. Subclinical infections have been reported. Legionella infections occur worldwide, in people of all ages and race groups with no evidence of person-to-person spread of infection (Kurtz 1988). The mode of transmission, inoculum size, particle size and host susceptibility appear to influence the severity of the

Table 1.7 Genetic composition of Legionella species

COMPONENT	CHARACTERISTICS AND FUNCTIONS	REFERENCES
enh	enh1, enh2 and enh3 loci involved host cell entry of *L pneumohila*.	Cirillo et al. 2001
icm	Icm locus prevents phagosome-lysosome fusion for intracellular multiplication and macrophage killing. Contains 10 genes: icmW, icmX, icmY, icmZ, icmT, icmS, icmR, icmQ, icmP and icmO. IcmQ-icmR-complex regulates formation of translocation channel that delivers proteins to host cells. IcmS-icmW-complex important in virulence. Icm system in *L pneumophila* transfers effector molecules to host cells.	Sadowski et al. 1993 Brand et al. 1994 Coers et al. 2000 Segal and Shuman 1997
dot	Dot virulence genes encode a large complex that functions as a secretion system and can transfer plasmid DNA from one cell to another. DotA is highly variable among *L pneumophila* strains, involved in macrophage killing and the lysis of red blood cells and engaged in organelle traficking during intracellular stage of life cycle. DotH, dotO and dotI apparently play a role in intracellular growth.	Kirby et al. 1998 Brand et al. 1998 Coers et al. 2000 Andrews et al. 1998
dot/icm	Encode a type IV-related transport apparatus. Subsets of genes are necessary for pore formation and modulation of phagosome trafficking. Most of the icm and dotA gene products act during the early stages of phagosome establishment.	Coers et al. 2000 Wiater et al. 1998
gsp	Encoding for the global stress proteins of *L pneumophila*, gspA, is induced upon intracellular infection in macrophages and exposure to in vitro stress stimuli.	Abu Kwaik et al. 1997
pts	Necessary for in vitro virulence in *L pneumophila* and apparently also for normal growth within macrophages.	Higa et al. 2000
nmv	Non-macrophage virulence gene. Nmv negative bacteria are complement resistant. Although the mechanisms of nmv virulence is still unknown, it may be related to the production of a toxin or to resistance of the organism to host factors important in early pathogenesis of disease, before macrophage ingestion.	Edelstein et al. 2000
hel	Three loci, helA, helB and helC, have been described. They are similar to the cnz and cnr loci of Alcaligenes xylooxidans, but their functions remain to be determined.	McClain et al. 1996
eml	This locus probably expressed in response to non-stress stimuli during the early stages of intracellular infection.	McClain et al. 1996
lly	The lly-locus (legiolysin) produces browning of culture medium during the late stationary growth phase. Lly negative strains have decreased resistance to ordinary light. This locus encodes for a 39kDa protein that is involved in hemolysis, pigment production and fluorescence in L pneumophila.	Steinert et al. 1995 Wintermeyer et al. 1994
mip	The gene coding for Mip, the macrophage infectivity potentiator protein. It is essential for optimal intracellular survival of L pneumophila. Mip-like genes have been described in other Legionella species.	Helbig et al. 2001 Cianciotto et al. 1990
msp	A set of genes in L pneumophila with homology to the protein of type II general secretion pathway of gram-negative bacteria. They are not necessary for growth in amoebae but other proteins that are important for growth in amoeba are likely to be secreted by this pathway.	Hales and Shuman 1999
lcy	This locus is also known as Legionella cyclophilin. It shows similarity to the cytoplasmic and perplasmic cyclophilins of E coli.	
fla	The flaA gene encodes for the flagellum subunit of L pneumophila SG1. Two strains of L pneumophila, L bozemanii and L feeleii SG 1 were shown to possess flaA-specific sequences but were not able to produce flagella. It has high sequence homology to the flil family of genes, responsible for the formation of flagellae.	Heuner et al. 1995
pil	The genes in the pil locus (pilB, pilC, pilD) are homologous to type IV pilin genes and may enhance macrophage colonization during legionella infections and amoeba invasion in the environment. PilD is involved in type IV pilus biogenesis and type II protein secretion.	Stone and Abu Kwaik 1998

infection (Girod *et al.* 1982, Yu 1990). A seasonal distribution of legionellosis has been reported, with the majority of cases being identified during summer to autumn months in most studies (Roig *et al.* 1991, Tobiansky *et al.* 1986). Approximately 50% of the currently known Legionella species have been implicated in human infections. However, all the legionellae should be regarded as potentially pathogenic under appropriate conditions.

1.6.1 Legionnaires' disease

Legionnaires' disease is a severe multisystem disease with pneumonia as the most predominant clinical finding. Symptoms may range from a mild cough and slight fever to a coma with widespread pulmonary infiltrates and multisystem failure. Clinical features of the disease are similar to those of other pneumonias and may lead to the formation of lung abscesses. Asymptomatic infections have been reported; they occur more frequently in *L micdadei* infections (also referred to as Pittsburgh pneumonia) than in those caused by *L pneumophila* (Finch 1988, Roig *et al.* 1991). Survivors usually recover completely although lung fibrosis and some neurological abnormalities may persist in some cases. Legionnaires' disease has a low attack rate with a ratio of 10-20% in sporadic cases. A mortality rate of 12% in Britain and 15% in South Africa has been reported but this figure may be as high as 50% in the absence of appropriate treatment (Zumla 1988). Legionnaires' disease may have a sudden or gradual onset and usually an incubation period of 2-10 days.

Erythromycin was historically the drug of choice for the treatment of Legionnaires' disease, but the newer macrolides, especially azithromycin, have superior in vitro activity and greater intracellular and lung-tissue penetration. Quinolones have even greater in vitro activity and better intracellular penetration than the macrolides and numerous successes have been reported with these drugs, especially with ciprofloxacin. Rifampin is highly active both n vitro and in vivo against Legionella and is recommended as part of combination therapy (with a macrolide or a quinolone) for patients who are severely ill. The total duration of therapy is usually 10-14 days; however a 21-day course may be needed for immunocompromised patients or those with extensive evidence of disease on chest radiographs.

1.6.2 Pontiac fever

Pontiac fever is an acute, self-limiting, flu-like illness without symptoms of pneumonia. It is characterised by high fever, chills, myalgias and malaise but without the pneumonia or cough typical of Legionnaires' disease. Some authors suggest that it is a hypersensitivity pneumonitis caused either by infection with Acanthamoeba filled with legionellae (Vandenesch 1990) or as a result of a toxic reaction to the organism (Muder *et al.* 1986). The incubation period is short, ranging from 3-5 days, and the attack rate high, exceeding 90% in some cases. The fatality rate is low. Pontiac fever symptoms usually resolve spontaneously within one week, only symptomatic treatment is needed and the chest radiograph is clear. There is no evidence of secondary spread of the infection in Pontiac fever. Diagnosis can only be made by seroconversion, which may be delayed for up to 6 weeks after onset of symptoms.

1.6.3 Incidence of legionellosis

The incidence of legionellosis varies from country to country and from region to region. In the United States, Legionnaires' disease is considered fairly common and serious and legionellae are among the top three causes of sporadic, community-acquired pneumonia. However, many cases are still not reported, as Legionnaires' disease is difficult to distinguish from other forms of pneumonia. Although only approximately 1,000 cases are reported to the Centers for Disease Control and Prevention (CDC), it is estimated that over 25,000 cases occur every year, causing more than 4,000 deaths.

The epidemiology of legionellosis in Australia is significantly different from that in other parts of the world, in that *L pneumophila* SG 1 infections are rare and usually associated with international travel, especially during winter months. The majority of infections in this region occurs during spring and summer, is caused by *L longbeacheae* and is usually associated with the use of contaminated potting soil mixes and other garden products.

Only one outbreak of Legionnaires disease has been reported in South Africa to date but the prevalence of antibodies in the general population is high. Ratshikhopha (1990) reported antibodies to *L pneumophila* SGs 1-6 in 65% of healthy blood donors from Gauteng. Furthermore, antibodies to *L pneumophila* SGs 1-4 were reported in 36% of health mineworkers, compared to 10% of healthy factory workers and 16% of pneumonia patients (Bartie and Klugman 1997). Maartens *et al.* (1994) reported

seroconverions to *L pneumophila* SGs 1-4 in 9% of patients hospitalised between 1987 and 1988 with symptoms of community-acquired pneumonia.

1.7 ECOLOGY

Legionellae are natural inhabitants of water, found in a wide range of habitats. They are ubiquitous in natural sources like streams, rivers and lakes, thermal ponds and thermally polluted waters (Dennis 1993). *L longbeacheae* is the only species that is more often isolated from potting soil than from water to such an extent that soil has been suggested as the natural habitat of the species (Steele *et al.* 1990).

Legionellae from these natural environments can enter man-made water systems by various means. The organisms can be transmitted from raw water collected directly from rivers and reservoirs, during water treatment, as part of post-treatment aftergrowths within water distribution systems or during building or plumbing repair and construction activities (Colbourne and Dennis 1985). Once established, they can persist in the water supply for long periods of time and are difficult to eradicate. Therefore, as it is impossible to remove legionellae from natural waters and to control their entrance into water distribution systems completely, their presence must be considered in the design, operation and maintenance of buildings. This makes collaboration between engineers, occupational health practitioners and microbiologists essential.

Water distribution systems of hotels, hospitals, ships, retail establishments, office blocks and public buildings, industrial premises, power stations and domestic premises are often colonised with legionellae. Misting machines in supermarkets, fountains, spas, whirlpools, ice machines and dental water lines (Williams *et al.* 1996) have also been implicated in legionellosis. Eyewash stations are often colonised by legionellae and amoebae (Paszko-Kolva *et al.* 1991).

Legionellae are usually present in low numbers in natural sources but certain factors present in man-made reservoirs may amplify their numbers significantly:

- Temperature Environmental legionellae can survive over a wide temperature range (20-43°C) with an optimum of 30°C (Hoffman 1984, Kusnetsov *et al.* 1996). Kusnetsov and coworkers (1996) showed that legionellae can survive at 50°C for up to one hour and at 55°C for up to 40 minutes. The temperature tolerance of legionellae is illustrated in Figure 1.2
- pH Legionellae can survive and multiply at a pH of 5.0-8.5.
- Stagnant water The presence of stagnant water in distribution systems may increase the risk of Legionella contamination.

- ⇨ Water treatment The type of water treatment used in the system may affect legionellae numbers
- ⇨ Disinfectants The concentration and persistence of residual disinfectant in the system affect
- ⇨ The chemical environment Turbidity and the presence of organic carbon and certain metals like zinc and copper have been implicated in an increased risk of contamination (Brundett *et al.* 1989, Marrie *et al.* 1992, States *et al.* 1987). Legionellae can store carbon and energy in the form of polyhydroxybutarate granules, allowing them to survive without substrates for long periods of time (http://www.e-tiology.com/legion.htm).
- ⇨ High relative humidity (RH) Legionellae survive best at 65% RH, they are least stable at ≤55% RH (Tiefenbrunner *et al.* 1993).
- ⇨ A build up of algae, slime and protozoa Amoebae, cyanobacteria and flavobacteria have been associated with the growth of *L pneumophila* (Colbourne and Dennis 1985). The organism was shown to adhere to the slime coat of Fisherella species (Bohach *et al.* 1983).
- ⇨ The presence of biofilm, scale and sediment In hot water systems, concentrations of the organism are usually highest in biofilms within the system and at the openings of water outlets.
- ⇨ The presence of corrosion products
- ⇨ Construction Major construction has been associated with numerous cases of legionellosis. It is believed that legionellae are released from soil during excavations. The organisms can then enter the cooling tower of the building, air intakes or water pipes, or may even be inhaled directly. Dust and dirt created during construction may also provide nutrients to legionellae already present in a cooling tower or potable water system (Freije 1996). New buildings may present legionellae problems if the plumbing is not flushed to remove dirt before occupation of the building (Frieje 1996). The water systems of renovated, idle buildings may contain stagnant water, a known source of legionellae.
- ⇨ Water pressure shock An increase in water pressure may send dirt into a system, providing a food source for the bacteria, or may dislodge scale and sediment, containing legionellae from pipes (Freije 1996).

1.7.1 Non-potable water sources

Heat rejection devices like cooling towers, evaporative condensers and HVAC systems are often implicated as sources of Legionella infections. They contain reservoirs filled with warm, recirculating water that makes them ideal for the growth, amplification and dissemination of microorganisms like legionellae. In a typical water-cooled condenser air is induced through, or blown over, packing of the cooling tower down which water, circulating from a pond beneath the packing, is allowed to fall by gravity, producing a large wetted surface that cools the descending water. The constant fall of water through the tower, the large area of the basin, fill, pipes and heat exchanger, the warm temperature of the water, the high relative humidity and high organic content within these devices provide conditions that favour contamination by legionellae, algae, protozoa, fungi and other bacteria. This risk is increased further by the open nature of the systems, excessive aeration and the constant addition of fresh water to make up that which is lost by evaporation increase the degree of biofouling in these systems.

Water is continually added to the ponds of cooling towers to make up for that lost by evaporation. Particles from the air drawn into the tower are captured in the water pumped down the structure and added to the pond. Algae and other organic substances released from construction, protective and plumbing materials can serve as nutrients (Fields et al. 1989, Muraca *et al.* 1988, Yamamoto *et al.* 1992). In towers that are not regularly cleaned, sludge accumulates in the reservoir and the slime adheres to the water covered surfaces, resulting in the presence of large concentrations of organisms including legionellae on these surfaces. In addition, water temperatures below 60°C, high relative humidity (Freije 1996), the age and configuration of the system, the pH of the water and the presence of certain metals may increase the risk of biofouling further (Yamamoto *et al.* 1992).

Water derived from municipal supplies but subsequently stored in cisterns, or conditioned prior to heating, is not considered potable, because of the deterioration in chemical and bacteriological quality during storage (Colbourne and Dennis 1985). The authors suggest that colonisation of such non-potable water sources inside large buildings, such as hotels, factories or hospitals, may be a major cause of legionellosis outbreaks.

1.7.2 Potable water systems

Legionellae are often present potable water supplies, especially in the hot water sections of these systems. The organisms may enter potable water supplies from the main water source, even from municipal water. Because most municipal water systems are not routinely screened for the presence of legionellae and the organisms are chlorine tolerant, they may survive many of the standard water treatment protocols. Once inside the system, they find a suitable environment to multiply and are usually very difficult to eradicate.

Legionella counts can rise from very low to very high within a short period of time but the factors that give rise to these changes in counts are not well understood and often very hard to determine (Joly 1993). The age and condition of the pipes, the degree of scaling and sediment and the potential for biofilm formation within the system increase the risk of contamination. Furthermore, water temperatures of 25-42°C, stagnation and the presence of certain free-living amoebae that are capable of supporting the intracellular growth of legionellae are amplifying factors often mentioned in published reports. Biofilm and scale that form in valves and fittings and on pipe walls not only feed legionellae, but also protect them from hot water and chemical disinfectants. Some materials used in the construction of plumbing systems, for example neoprene washers, are more readily colonised than others (Marrie *et al.* 1992). The location of the building may also play a role in legionellae contamination of potable water systems.

Hot-water tanks are often colonised with legionellae, especially at the bottom where a warm zone often develops and scale and sediment accumulate (Alary and Joly 1992, Freije 1996). However, hot water piping presents an even higher risk of contamination than tanks. Biofilm and scale that form in valves and fittings and on pipe walls feed legionellae and protect them from disinfectants. Rubber washers and gaskets in fittings also support and protect the organisms. Unused piping (dead-legs) creates additional problems because legionellae grow well in stagnant water.

1.7.3 Biofilms

Survival of microorganisms in low-nutrient environments such as man-made water systems regularly treated with biocides usually involves attachment to solid surfaces with subsequent formation of biofilms that are rich in nutrients and consist of a mixed population of organisms. Bacteria in industrial water systems therefore tend to grow in

biofilms where they have access to more nutrients, are in close proximity to other cells and are protected from various antimicrobial mechanisms like biocides, antibiotics and protozoan predators (Wireman *et al.* 1993, LeChevalier *et al.* 1998). It has been suggested that the slow growth rates within biofilms and the particular nutrient deficiencies under biofilm conditions may cause the expression of distinct phenotypes that are often resistant to chemical and antibiotic agents (Barker *et al.* 1992).

In these systems, legionellae may exist as planktonic cells, as free-living components of the biofilm ecosystem or in association with other organisms that colonise surfaces, like flavobacteria, cyanobacteria and amoebae that they may infect (Barker *et al.* 1993, Rogers *et al.* 1993, Wright *et al.* 1989). Wireman *et al.* (1993) suggested that legionellae are primarily biofilm organisms. Although their exact ecology in biofilm conditions is not yet known it is influenced by factors like contact time, Legionella concentration and surface type, but not by flow rate, temperature, water hardness or the frequency of contact with the surface. Recent studies have continued to demonstrate the importance of biofilms in Legionella persistence and growth in both natural and man-made water systems, even in the absence of eukaryotes like protozoa (Keevil 2000).

1.7.4 Intracellular replication

Legionellae are slow-growing organisms that require a combination of nutrients for growth. Due to their fastidious nature and lack of antibiotic activity, they may be replaced by faster growing organisms if they do not have an alternative means of survival in aquatic environments. The fact that legionellae are ubiquitous in these environments suggests that protozoa, especially amoebae, play a supportive role in their survival and multiplication and that they may in fact act as natural hosts and amplifiers for legionellae.

Rowbotham (1980) was the first to demonstrate interactions between legionellae and protozoa. To date, protozoa of the genera Acanthamoeba, Tetrahymena, Naegleria, Echinamoeba and Vanella species have been implicated in these interactions. Although little is known about the metabolic and physiological status of legionellae after passage through protozoa, in vitro studies have shown alterations in their physiological status resulting in iron deprivation, possibly changing the susceptibility of the released bacteria to chemical inactivation.

Amoeba trophozoites feed and multiply in water and biofilm. When conditions become unfavourable, these trophozoites are transformed into cysts with hard, impermeable outer walls that provides protection for ingested Legionella organisms. When conditions

become more favourable, the cysts change to trophozoites again and the bacteria are set free. Legionellae have been recovered from cysts treated with 50 ppm chlorine suggesting a high level of protection by the cysts (Kilvington and Price 1990). This high resistance of amoebal cysts to biocides may be the mechanism for the apparent reseeding of water systems by legionellae often experienced in the water treatment industry. However, recontamination may also occur via transmission of airborne cysts acting as carriers for the legionellae.

The intracellular replication of legionellae within free-living amoebae has recently been studied in vitro in detail using in vitro models/studies. McNealy *et al.* (2001) reported that a multiplication ratio as small as 0.001:1 (*L pneumophila* : amoebae) may establish a strong intracellular replication cycle in amoebae suspended in spring water. Their results indicated a 7,000-fold increase in *L pneumophila* colony forming units and lysis of all amoebae trophozoites. Furthermore, the addition of non-Legionella species did not decrease the replication cycle in amoebae but the addition of a ciliate (Tetrahymena) to amoebae and *L pneumophila* suspended in cooling tower water resulted in packaging of the legionellae into vesicles. This process was dependent on the presence of amoebae, which was necessary to initially amplify the low number of legionellae. Their results suggested that different protozoa probably function sequentially to increase the number and influence the distribution of legionellae in aquatic environments.

On the other hand, studies by Keevil *et al.* (2000) and Lüneberg (2000) suggested that intracellular growth is not essential for the proliferation of *L pneumohila* within mixed bacterial populations. They reported that, even when mixed populations were treated with cycloheximide to eradicate all eukaryotes, the numbers of *L pneumophila* actually increased >50-fold and represented a significant fraction of the total biofilm flora (Keevil 2000). It is therefore unsafe to assume that the absence of protozoal hosts within water systems precludes the survival and growth of legionellae; as long as there are other bacterial species present appropriate measures should be taken to prevent Legionella proliferation.

Legionellae can tolerate a broad range of physical parameters, since pH, temperature and conductivity have no significant influence on the frequency of detection in a natural aquatic environment (Grimm *et al.* 2000).

1.8 TRANSMISSION

The mere presence of legionellae in a water distriubtion system does not necessarily imply a human health risk. For human infection to occur certain conditions are necessary

(Colbourne and Dennis 1989, Breiman 1993). Interfering with any link in this 'chain of infection' will decrease the occurrence of disease significantly. The links in the chain of infection are as follows (Figure 1.3):

- ⇨ Reservoir Water is the major environmental reservoir for legionellae but other sources have been recognised.
- ⇨ Amplification A better understanding of the amplification process may lead to the development of methods to limit the multiplication of legionellae and to interrupt the chain of events leading to organism transmission with subsequent infection (Breiman 1993).
- ⇨ Dissemination The primary mechanism of Legionella dissemination is inhalation of droplets of respirable size. However, other routes have been described. In most cases of Legionnaires' disease, airborne spread [from cooling towers] has been implicated.
- ⇨ Virulence The species disseminated must be virulent enough to cause infection. In nature legionellae can survive and possibly replicate in free suspension and grow in biofilms and protozoa, thus leading to diverse phenotypes, potentially increasing their virulence and susceptibility properties (Barker and Brown 1995).
- ⇨ Susceptibility The organism must be disseminated to a susceptible person; this is a key factor in the chain of causation.
- ⇨ Inoculation site The organism must be inoculated at a site on the person where it can cause infection.

1.8.1 Aerosolisation

Aerosolisation is generally recognised as the major mechanism of Legionella transmission. The survival and viability of legionellae in aerosols are influenced by a number of factors (Colbourne and Dennis 1989, Tiefenbrunner 1993).

- ⇨ Relative humidity (RH) The survival time of legionellae in aerosols increases from 3 minutes at RH 30% to 15 minutes at RH 80% under laboratory conditions. Some authors suggest that the organisms will survive best at RH 65%.
- ⇨ Metabolic state Water is a low nutrient growth medium, therefore legionellae in an aerosol will be in a low metabolic state and relatively stable when compared to the same organism in a high metabolic state when grown in a nutrient-rich medium.
- ⇨ Aerosol age affects the viability of legionellae by allowing longer action of degrading factors such as high temperatures, biocides and ultraviolet light.
- ⇨ Aerosol composition influences the survival of aquatic bacteria. This applies particularly to legionellae as it is suggested that other aquatic organisms may

secrete protective substances which, when coating *L pneumophila*, may enhance its survival in an aerosol.

Apart from affecting persons outside a building, aerosols can be disseminated to people inside a building via contaminated ventilation and dehumidification systems. The distance legionellae can drift from a cooling tower and still be viable depends on humidity, sunlight and wind conditions. In most cases the distance will be less than 200 metres but some workers suggest that viable legionellae can travel up to 1-3 km in an aerosol. It is therefore no surprise that aerosols have been implicated in numerous outbreaks and sporadic cases (Freije 1996, Joly 1993). Aerosols may also transmit legionellae via showerheads and tap faucets as well as respiratory care equipment, whirlpool baths, humidifiers, decorative fountains, mist machines as these devices can cause droplets of respirable size (Breiman 1993). The rate of particle deposition from an aerosol depends on the particle size. Small particles (<5 μm in diameter) will remain in suspension thereby increasing their potential to cause infections. However larger particles may also cause infection in conditions of low relative humidity when they may evaporate before deposition, thereby becoming smaller and more respirable and thus capable of reaching the smaller airways of the lung.

1.8.2 Direct installation

Respiratory therapy devices have the potential to disseminate pathogenic microorganisms by delivering gases at high pressures and volumes and by generating intensive aerosols. These devices may thus be able to bypass the normal defence mechanisms of the respiratory tree. Numerous cases of nosocomial Legionnaires' disease have been traced to the use of respiratory therapy devices (Muder *et al.* 1986).

1.8.3 Aspiration

Aspiration as a mode of transmission has been largely overlooked because of the popularity of the aerosolization theory, but warrants further investigation. Pneumonia can develop in humans via two mechanisms. Those associated with aerosolization (for example tuberculosis) typically have a high attack rate while pneumonias caused by of aspiration typically have a low attack rate as infection depends mainly on the susceptibility of the individual (Muder *et al.* 1986). There are a number of reasons for suggesting aspiration as a possible mode of transmission. Legionnaires' disease has a low attack rate; the attack rate in Pontiac fever is high. Also, bacteria normally associated with aspiration pneumonia

tend to be normal flora possessing pili to mediate adherence to pharyngeal epithelial cells; legionellae also possess pili. Symbiosis between selected oropharyngeal flora and legionellae has been reported (Muder *et al.* 1986, Roig *et al.* 1991). Muder *et al.* (1986) also suggested that, given previously overlooked information, the only parameter that could be linked statistically to acquisition of the disease during the first outbreak was the ingestion of water and not as previously thought, the inhalation of contaminated aerosols.

1.8.4 Ingestion

A potential mechanism by which legionellae might be transmitted via ingestion is that of bacteremic spread after penetration of the intestinal tract. It is well known that *L pneumophila* can disseminate to extrathoracic organs (Muder *et al.* 1986). The fact that diarrhoea is one of the symptoms of Legionnaires' disease, further suggests the possibility that the gastrointestinal tract may be the portal of entry in some cases. However there is not much evidence to support this theory.

1.8.5 Other potential routes of entry

There have been reports of pericarditis followed by seroconversion to L pneumophila in the absence of pneumonia. In addition, one case of culture-proven *L pneumophila* prosthetic valve endocarditis has been reported (Muder *et al.* 1986).

1.9 PREVENTION

1.9.1 Non-potable water systems

The key factors to reduce the risk of Legionella contamination of non-potable water sources are to keep the Legionella counts as low as possible and to prevent transmission from the tower to people. In order to prevent Legionella contamination in these sources, the following are recommended (Freije1996):

- ⇨ Physical cleaning Physical cleaning alone has little effect on the risk of Legionella contamination, but it makes water treatment more effective by reducing any build-up of slime, dirt, biofilm, algae, fungi and amoebae, placing a greater demand on the biocide by preventing proper contact with microorganisms.
- ⇨ Water treatment Non-potable water sources should be treated regularly to control scale, corrosion and the build-up of microorganisms. Because biocides are designed

only to control slime and algae for optimum system efficiency and not to eliminate all bacteria in a system, water treatment programs should be evaluated by checking the total bacteria count (TBC) to indicate the general cleanliness of the system. A high TBC count in a cooling tower generally indicates the presence of a food source for legionellae, although there is no reliable correlation between TBC counts and Legionella counts (Freije 1996).

- ⇨ Periodic disinfection A regular water treatment routine is believed to be more effective than periodic disinfection. Broadbent (1987) suggested that hyperchlorination with cleaning usually had only a short-term effect on legionellae counts. The counts usually rise again within one month after treatment. However, periodic disinfection may be useful for some institutions in some instances.
- ⇨ Maintenance Overall good maintenance is widely recommended for the control of legionellae in cooling towers. Maintenance plans should be based on manufacturers' instructions and regulations published in formal guidelines. Cooling towers should be inspected regularly for leaks, corrosion and blockages and fans, motors and pumps should be operating properly. All operating and maintenance procedures should be documented and inspection reports kept for future reference. Cold water humidifiers should be provided with a domestic water source rather than using recirculated water. However, replace cold water humidifiers wherever possible with steam humidifiers because steam units operate at ≥70°C. Humidifiers should be kept clean and leak free at all times.

1.9.2 Potable water sources

Freije (1996) recommends the following preventive measures for potable water systems:

- ⇨ Water lines and fixtures Removing deadlegs to reduce the risk of stagnant water is critical to legionellae control. Natural rubber in plumbing fixtures provides nutrients for Legionella growth and protects the organisms from disinfectants. Washers and gaskets should preferably be made of neoprene or other synthetics. It is believed that copper is the best material to use in plumbing fixtures because it resists scale and biofilm formation. Steel is reasonably good while plastics are not recommended. Heavily scaled faucets and showerheads should be replaced. Faucet aerators and shock absorbers should be avoided as legionellae grow well in these fixtures. Water should be treated to avoid the formation of scale. Recirculation lines should be extended to the point furthest from the supply, lines should be run at a slight fall to simplify draining of the system and hot water piping should be run above cold water

piping to prevent warming of cold water. Freije also recommends the reaming of pipe ends to remove parts that can trap sediment and cause corrosion.

- ⇨ Hot water generators and cold water storage tanks The typical hot water tank keeps approximately 65% of the tank at the set temperature; the other 35% will be cooler. Some water heaters develop a warm zone at the bottom of the tank where scale and sediment can accumulate and provide a suitable habitat for legionellae. This problem can be overcome by installing thermometers at various locations of the tank. If possible, high-temperature semi-instantaneous heaters should be installed, as they are not conducive to legionellae growth. It is also important to keep in mind that horizontal tanks are less prone to Legionella growth than vertical tanks. When new tanks are purchased, it is recommended to consider a tank that recirculates the water to keep the temperature constant. The tank should also be capable of producing water at 70°C in case high temperatures are needed for disinfection.
- ⇨ Regular operation and maintenance Water temperatures at or above 60°C in tanks and 50°C at taps will control legionellae while cold water should be stored and distributed at ≤ 20°C. The optimum temperature will depend on the physical and mental conditions of the users and may therefore vary among institutions. In addition, hot water tanks should be drained at least once a year to remove scale and sludge and flushed with chlorine, and should be operated so that water is stored in the tank for one day or less. Coldwater storage tanks should be cleaned approximately once a year and preferably also flushed with chlorine. New systems should be flushed with chlorine prior to start-up, while idle systems require periodical running of water to all outlets to avoid stagnation.

1.10 DISINFECTION AND CONTROL

In order to reduce or eliminate the risk of legionellosis, the concentration of legionellae in the affected system should be minimised and transmission of the organisms to susceptible persons should be prevented. This can be achieved in a number of ways.

- ⇨ Superheating is the most common way of decontaminating a water distribution system contaminated by legionellae. The water temperature is raised to ≥70°C for 24 hours and each outlet is flushed for 20 minutes. The water temperature at each outlet should reach 60°C. Prior to superheating, dead legs should be removed from the system to prevent reseeding of the system. Sediment and scale can act as insulators, thus protecting legionellae from the effects of superheating. These layers

can be removed by opening each outlet slightly for about 8 hours. This should allow enough time so that the entire scale layer can be heated to ≥60°C.

⇨ Periodic chlorination is another method of controlling legionellae in hot water systems. After removing scale and sediment, sodium hypochlorite is added until approximately 50 parts per million (ppm) of free residual chlorine is produced. The pH of the water should be maintained between 7.0 and 7.6 to maintain the biocidal activity of the chlorine. All taps are subsequently flushed until the odour of chlorine is evident and the water allowed to stand for one hour prior to draining and refilling the tank and flushing each outlet to remove the chlorine from the system (Springston 2000). There are some disadvantages to chlorine shock treatment as compared to superheating in hot water systems. Firstly, chlorine is very corrosive and will shorten the service life of metal plumbing materials. Secondly, chlorine will not penetrate layers of scale, resulting in legionellae contained in such layers will recontaminate the system soon after hyperchlorination. The best way of overcoming this problem is continuous chlorination with 2ppm free chlorine following superchlorination (Springston 2000).

⇨ Alternative methods to control legionellae growth include the use of metal ions (copper and silver), in-line ultraviolet systems and ozone. Cooling towers can also be treated with algaecides and bactericides (biocides) such as gluteraldehyde to assist in controlling the multiplication of bacteria, protozoa and algae. Biocides should be added continuously to maintain constant concentrations in the system. Cleaning of the system is also important in the control of legionellae. A buildup of protozoa, algae, fungi, dirt and biofilm can prevent a biocide from making contact with microorganisms. The correct type and concentration of biocide used is therefore very important.

For cooling towers that have been implicated in outbreaks or are highly contaminated, emergency treatment is necessary. Springston (2000) recommends chlorination of the system with 50ppm free residual chlorine, together with a dispersant. This is followed by 10ppm free residual chlorine for 24 hours, taking into account the correct pH for the chlorine to be active. The entire system is then drained and the chlorination process repeated. Some workers recommend the use of bromine (that is less affected by pH) instead of chlorine. In order to prevent transmission of legionellae to susceptible persons, by using drift eliminators and by locating cooling towers as far as possible away from windows, air intakes and outdoor areas frequented by people. In addition, prevailing wind directions should be taken into account with regard to cooling towers and air intakes. [http://www.esmagazine.com/es/cda/articleinformation/features/bnp_features_item/0,2503,3615,00.html]

1.11 NUTRITIONAL REQUIREMENTS

Legionellae are chemo-organotrophic and use amino acids, catabolized through the Krebs cycle, as their main source of carbon and energy. *L pneumophila* can synthesise all the other chemical components *de novo* and apparently has no vitamin requirements. However, the exact nutritional requirements for legionellae are not clear as they appear to be influenced by variations in culture conditions. It has been suggested that the addition or deletion of certain nutrients from culture media stimulate the induction of enzymes that bypass certain metabolic steps in order to use only the materials available in the medium.

Early studies suggested that the amino acids needed for growth include arginine, isoleucine, methionine, threonine, valine, phenylalanine, tyrosine. alanine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, tryptophan and tyrosine. Threonine and tyrosine stimulate oxygen uptake of legionellae, depending on the medium used for culture. Most strains require serine that can be used as the sole source of carbon and energy. Some strains also require proline (Rowbotham 1980, Muller 1981, Tesh *et al.* 1983). However, Wadowski *et al.* (1985) suggested that legionellae can meet all their carbon and energy needs with nine amino acids. This need for amino acids implies that legionellae can use these products from other organisms that produce them in excess or from decaying matter.

Extensive differences in the sensitivity of legionellae to chelators have been reported. In a study by Reeves *et al.* (1981) Legionella growth was completely inhibited by ethylene-diamine-tetra-acetic acid (EDTA), citrate, acetate and malate and partly by lactate. He also reported that the strains that were the least sensitive to the chelators appeared to need the smallest amount of metals for optimal growth and survived longer. In addition, the strains of *L pneumophila* tested by this group needed a greater variety of metals and were more sensitive to the loss of metals by chelation than *P aeroginosa*. It is however important to bear in mind that the salt content or the pH of a medium can also alter the specificity of chelators (Reeves *et al.* 1981). L-cysteine is one of the most commonly used chelating agents. Although normally added to culture media to act as a chelating agent, it acts as a required amino acid and not as a chelating or reducing agent in media used for Legionella culture.

The addition of trace metals to culture media stimulates heme proteins in aerobic organisms and non-heme enzymes. Most organisms need boron, calcium, chromium, cobalt, copper, iron, magnesium, manganese, molybdenum, nickel, vanadium and zinc for

growth. Calcium and magnesium are cofactors in many bacterial enzymes and are important components in cell walls and membranes. Iron, manganese and zinc are important cofactors for some enzymes and are important in oxidation-reduction systems. Molybdenum plays and essential role in nitrogen metabolism. Although cobalt, copper and nickel are less frequently required, they are essential cofactors in certain enzymes. Vanadium is not a common bacterial nutrient but stimulates growth in some strains of *L pneumophila* and can substitute for molybdenum in some bacterial strains and for iron in some strains of lactic acid streptococci (Reeves *et al.* 1981, Hutner 1972). Iron is present in ferredoxin and cytochrome and is an important cofactor for growth. It also plays a regulatory role in some fermentation processes. Iron salts are essential for the growth of all legionellae, although *L pneumophila* can grow over a wide range of iron concentrations. Under conditions of iron deprivation the lag phase of the organism is increased and cellular morphology is affected, giving rise to short, coccobacillary forms. Long slender rods are formed when the iron concentration is high (Ristroph *et al.* 1981). Legionellae will not grow on media deficient in all trace metals (Winn 1984).

It has been suggested that the difficulties in the isolation of legionellae from the environment may be explained by their sensitivity to relatively low levels of hydrogen peroxide and superoxide that are generated in culture media exposed to light. Although legionellae grow in the absence of charcoal in filter sterilised broth media, no growth occurs in broth media when sterilised by autoclaving. It is believed that toxic products might be released into the broth medium during autoclaving thereby inhibiting growth. The addition of charcoal to culture media can enhance Legionella growth by preventing this photochemical oxidation reaction and by detoxifying media of reduced forms of oxygen.

Yeast extract is rich in nucleotides and provides vitamins and coenzymes to culture media. The addition of alpha-ketoglutarate to agar media increases the isolation rate and the quantity of viable bacteria detected but may inhibit growth in broth media. Alpha-ketoglutarate is added to the media used for Legionella culture to stimulate the production of oxygen-scavenging enzymes in order to promote growth (Pine *et al.* 1986, Winn 1984). Potassium, a cofactor for some enzymes, is required for carbohydrate metabolism. It is used mainly for ribosomal function: when the protein content of bacterial cells bacterial cells is lowered, protein synthesis stops while glycolysis continues.

When exposed to light, yeast extract contains substantial levels of hydrogen peroxide and superoxide radicals. Cysteine is also known to form hydrogen peroxide upon auto-oxidation and when present in solutions of amino acids, increases the production of peroxide. Photo-oxidation or free radical-mediated oxidation of membrane lipids produces

toxic lipid peroxides and hydroperoxides. Hydrogen peroxide may therefore form in the medium used for Legionella culture.

The charcoal added to buffered charcoal yeast extract (BCYE) medium generally used for Legionella culture consumes oxygen but this effect decreases with time. Activated charcoal in suspension decomposes hydrogen peroxide and superoxide radicals, decrease their accumulation in complex media exposed to light and prevents yeast extract medium from light-accelerated oxidation of cysteine. According to Hoffman *et al.* 1983) this can be overcome by adding peroxide and superoxide radical scavengers (for example catalase and superoxide dismutase) to culture media that do not contain charcoal.

The presence of even low levels of hydrogen peroxide has a substantial effect on *L pneumophila* growth but apparently not on *L micdadei* and *L bozemanii* (Hoffman *et al.* 1983). This can probably be explained by the fact that, although it contains considerable amounts of superoxide dismutase (SOD), *L pneumophila* contains no catalase. Of the legionellae tested, *L micdadei* contains the highest amount of SOD. Autooxidation and/or photochemical oxidation of complex culture media, solutions of amino acids and vitamins may generate considerable levels of hydrogen.

1.12 DETECTION METHODS

Classical detection methods for Legionella species from the environment relied on the inoculation of susceptible guinea pig hosts (McDade *et al.* 1977). Although selective, these methods were expensive and time consuming and were soon replaced by isolation by culture on agar media. To improve the recovery of legionellae by culture, the use of certain selective media and pretreatment steps were introduced to minimise contamination by non-legionellae (Bopp *et al.* 1981, Groothuis *et al.* 1983). In attempts to simplify Legionella identification, radioimmunoassays (RIAs), enzyme linked immunosorbent assays (ELISAs), agglutination tests and nucleic acid probes and polymerase chain reaction (PCR)-based assays have since been developed and tested (Bej *et al.* 1991, Lye *et al.* 1997, Mahbubani *et al.* 1990). Although the majority of these new methods are fairly successful for the detection of environmental legionellae, culture remains the method of choice. However, no single culture method has so far proven to be ideal for all samples in all given circumstances and environments. Even in the absence of contaminating bacteria or other inhibitory substances, the detection of small numbers of legionellae from environmental samples remains difficult. This, together with the lack of standardisation of methods, complicates the interpretation of culture results and comparisons of results

from different institutions (Kusnetsov *et al.* 1995, Ta *et al.* 1995). Variations in bacterial numbers in different areas within a water distribution system and the sampling method used often complicate the interpretation of culture results even further.

1.12.1 Sampling

Numerous guidelines have been developed for environmental sampling for Legionella detection. The purposes of sampling for legionellae are firstly to evaluate the possibility of legionellae amplifying in building water systems that generate aerosols to which building occupants and passers-by can be exposed. Secondly, to determine the efficacy of water treatment programmes in preventing Legionella amplification and thirdly to determine whether legionellae may be causing disease in building occupants (Shelton *et al.* 2000). The appropriate sampling strategy should be based on the specific conditions of the building and the specific objectives of the investigation.

When deciding on the frequency of sampling for legionellae, one has to remember that Legionella testing will only be applicable to the area of the system sampled, at the time of sampling. The sampling frequency should therefore be based on factors such as

Table 1.8 Basic supplements in Legionella media

SUPPLEMENT	FUNCTION
Charcoal	Legionellae are sensitive to relatively low levels of peroxides and superoxides that are generated by media exposed to light. Activated charcoal prevents these reactions and detoxifies media from reduced forms of oxygen. It also prevents free radical-associated oxidation of cysteine, as essential growth factor for legionellae.
L-cysteine	L-cysteine is normally added to agar media as a chelating agent but in the case of legionellae it acts as a required amino acid and not a chelating or reducing agent.
Potassium hydroxide	Potassium hydroxide is necessary for protein synthesis. Because the addition of sodium, even in the small quantities used for pH adjustment can reduce growth of legionellae, KOH should also be used for this purpose.
Alpha-ketoglutarate	In general, alpha-ketoglutarate increases the isolation rate and viability of organisms on agar media but it may have an inhibitory effect in broth media.
Iron	A trace element that plays a regulatory role in various fermentation processes.
ACES buffer	Addition of a buffer with a pK at optimal pH for legionellae growth, ACES buffer, is normally added to culture media but any other suitable buffer can be used instead.

the potential for amplification of legionellae in the system, the suspected concentration and the need to verify the efficacy of treatment programmes (Shelton *et al.* 2000). The number of samples to collect will depend on the size of the facility and the number of systems in the facility that may be potential sources for legionellae (ASTM Guidelines D5952-96).

Legionellae can be isolated from a wide variety of environmental samples including water, biofilm, sediment and scale, as well as soil and dust. Water and biofilm samples are usually the first to be tested when contamination is suspected. However, aerosol samples may be useful in some investigations, for example to indicate how many legionellae organisms are passing from a water source into the air or the distance the legionellae are being transmitted. Samples should be collected from areas in the system where legionellae are most likely to be detected. Areas most likely to contain legionellae are (ASTM Guidelines D5952-96) incoming water supplies, water storage tanks and hot water heaters, hot and cold water faucets and shower heads, water-cooled heat-transfer equipment and humidifiers, spas, decorative fountains and other water systems suspected of containing legionellae.

The volume of water collected for legionella detection usually depends upon the nature of the system and the purpose of the examination. In general samples of one litre are collected in sterile glass, polyethylene or similar containers. Smaller sterile containers with screw caps are recommended for collecting biofilm, scale and sediment samples. Previously used sampling containers should be sterilised or pasteurised before use. Biocides can be inactivated by adding an inactivating agent to the sampling container before or during sampling. Chlorine and other oxidising biocides are inactivated by the addition of potassium thiosulphate or sodium thiosulphate at a concentration of 1 ml of a 30% solution per litre of sample. For other biocides adding a universal neutralising agent is not practicable.

In some instances swabs are more appropriate than water samples. Although experts agree that water rather than swab samples should preferably be collected from cooling towers, water tanks and sources like decorative fountains, some workers regard swab samples from biofilms critical for the evaluation of Legionella colonisation (Stout 1998, Ta 1995). For some sources, for example showerheads, some workers prefer swab samples, others prefer water samples and some prefer both. Water samples will provide a more accurate comparison of legionellae numbers in different water outlets in a building than swabs and will provide a total picture of the bacteria present in the system whereas swabs will only provide information on the particular area sampled. However swab samples

will provide more information on the presence of legionellae in a biofilm and are more convenient than water samples to collect. If both water and swab samples are collected, the water sample should be collected first and contain a minimum of 200ml (Stout 1998). Barbaree *et al.* (1987) recommended that swabs be dipped in sterile water before use.

Aerosol sampling for legionellae is usually done with Andersen air samplers or liquid impingers (Breiman *et al.* 1990, Ishimatsu *et al.* 2001). Barbaree *et al.* (1987) suggested sampling with a six-stage Andersen sampler containing a selective medium at a flow rate of 0.0283m^3/minute. Although selective media are not recommended for use in this type of sampling under normal circumstances it is suggested for legionellae to control the number of non-legionellae on the plates. Alternatively, an all-glass impinger containing 0.25% yeast extract can be used at a flow rate of 0.0108m^3/minute.

Despite the reasonable success in detecting legionellae from aerosols reported by some workers, water sampling has several advantages over aerosol sampling. Because legionellae live in water and on surfaces these samples check the actual source of contamination. Legionella counts will be more consistent in water than in air because the amount of contaminated water droplets entering the air will vary significantly over time; a water test will therefore be more accurate in indicating a potential health risk. Detection methods relying on culturing legionellae may fail to isolate legionellae from aerosol samples if the bacteria lose their culturability while being airborne, during the collection procedure, during sample storage or during the culturing process. However, methods not based on bacterial multiplication, may be useful for detection of legionellae from air samples that test negative by the culture method (ASTM Guidelines D5952-96)

A very important aspect of sampling for legionellae is personal protection during sampling. The ASTM Guidelines (D5952-96) recommends that equipment should be turned off while collecting samples, if possible. Disposable protective clothing should be worn as well as slip-proof footwear and eye protection, especially when working in wet, potentially contaminated areas and in areas recently treated with biocides, disinfectants, detergents or other chemicals. Half-mask respirators of at least 95% efficiency should be worn when working near potentially contaminated equipment that may generate aerosols.

1.12.2 Sample transport and storage

Samples should be allowed to reach ambient temperature and be protected from extreme temperatures and direct sunlight during transport and storage. In general, temperatures below 3°C and above 30°C should be avoided. The necks of sampling containers should be sealed with tape and the containers wrapped in absorbent paper and

placed in individual plastic bags if not delivered by hand to the laboratory. Samples should preferably reach the laboratory within 24 hours of collection and should be kept at room temperature until testing, which should be commenced within 24 hours of receipt. The longest period of time allowed between sample collection and culture of the concentrate is 14 days (Draft International Guidelines ISO/DIS 11731–1996).

The viable count of bacteria in a water sample can change during transport and storage because bacteria can become attached to the walls of the sample containers, may become non-culturable or die. For legionellae these losses are most apparent when stored between 0°C and 6°C. Thus the ISO method recommends that sample concentrates can be stored at temperatures between 6-18°C in the dark for no longer than14 days. However, some authors recommend refrigeration of the samples at 2-8°C before and after processing (Stout 1998).

1.12.3 Sample concentration

Environmental water samples may be inoculated directly onto agar media if the number of organisms is likely to exceed 10^5 per litre. However, as the number of legionellae in any given sample is usually not known, samples are usually concentrated by either membrane filtration or centrifugation, prior to culture. Both membrane filtration and centrifugation are widely accepted and used for this purpose, but there is no consensus among workers regarding the efficiency and accuracy of either of these methods (Boulanger *et al.* 1995, Brindle *et al.* 1987.

A number of studies were carried out in the late 1980s to early 1990s, comparing the different aspects of filtration and centrifugation. Smith and coworkers published a comprehensive comparison of different filter membranes in 1993. This group evalutated eight different filter types with pore sizes ranging from 0.2-0.8μm and found a polycarbonate filter with a pore size of 0.2μm most appropriate for sterile seeded samples. They reported a recovery rate of 66% after sonication and 59% after vortex resuspension. When using the same filter type with a pore size of 0.4μm, the recovery rate dropped to 49% after sonication and 35% after vortex. These recovery rates were significantly better than those reported with the other filter types they tested. Unfortunately this group did not study the efficiency of these filter types on non-sterile, environmental samples. Reinthaler *et al.* (1992) also preferred polycarbonate or cellulose nitrate filters with a pore size of 0.2μm. Other groups reported on the efficiency of black filters placed directly on agar (Szewzyk *et al.* 1991), sequential filtration through 3.0μm, followed by 1.0μm fibreglass cartridge filters (Payment *et al.* 1989) and nucleopore filters

examined directly by microscopy (Hobbie *et al.* 1977). Some workers reported an increase in recovery from environmental, but not seeded samples, when using flat black filters instead of white cast cellulose nitrate filters (Boulanger *et al.* 1995)

The Centres for Disease Control and Prevention (CDC) (Atlanta GA) recommends filtration of water samples through 0.2μm polycarbonate filters. The use of nylon or polycarbonate filters with pore sizes of 0.22μm or 0.45μm is recommended in the International Standard (ISO/DIS 11731) method. According to the Australian Standard (AS 3896-1991) filtration through 0.22μm and 0.45μm filters is appropriate; this method does not recommend a specific filter type. In South Africa, 0.45μm, type HA Millipore filters, optimised for microbiological analyses of potable water, are used.

For membrane filtration to be effective, several factors have to be taken into account when choosing the type of filter and the pore size to use. Smith *et al.* (1993) reported several defects in some of the filters they tested, including brittleness, wrinkles, non-wetting areas, and inhibitory compounds present on the surface of some filters. Furthermore, blocked pores, abnormalities and pore structure, the method of resuspension after filtration and lot-to-lot differences, even in the same filter type, may complicate sample concentration by filtration. Disadvantages of filtration as a means of concentration also include the slow filtration rate of dirty environmental samples and the presence of biocides in samples that may affect the filtration rate (Brindle *et al.* 1987).

Centrifugation is often used as an alternative for filtration but differences in opinion regarding the optimal centrifugation speed and time have been reported (Voss *et al.* 1984, Brindle *et al.* 1993, Boulanger *et al.* 1995). Some workers prefer centrifugation at 6000 *g* for 10 minutes while others consider 3000 *g* for 30 minutes more appropriate. Brindle *et al.* (1993) reported a good correlation between centrifugation at 6000 *g* for 10 minutes and filtration through 0.45μm pore size, nitro-cellulose filters, with centrifugation having the added advantage of saving on time and effort. The Australian Standard recommends centrifugation at 6000 *g* for 10 minutes whereas the International Standard regards either of the two methods of centrifugation as appropriate. Brindle and coworkers (1993) reported a loss of 57% of organisms after centrifugation at 6100 g for 10 minutes, with an even higher loss at lower centrifugation speeds whereas other authors reported losses of 80-90% with both centrifugation and filtration. Even from very large quantities of water they could only recover 14-55% by filtration, depending on the volume filtered.

Brindle *et al.* (1987) compared membrane filtration and centrifugation for Legionella recovery from seeded samples and found both methods equally successful. For filtration to be effective, several factors have to be taken into account when choosing the

type of filter and the pore size to use. It was also found that direct plating of filter membranes did not enhance recovery but rather experienced that their bacterial counts were inaccurate due to clustering of the colonies on the wet membranes.

1.12.4 Sample resuspension

The method of resuspension of the organisms after concentration may influence the accuracy of recovery from environmental samples. Organisms can be resuspended after filtration by either producing a vortex for a minimum of two minutes, or by placing the concentrate in an ultrasound tank until the membrane appears clean. There have been contradicting reports regarding the efficiency of both these resuspension methods; most authors believe that a combination of methods is necessary to optimise the recovery from different sample types. The general feeling is that each laboratory should find the method that works best for its samples and environment.

The ISO method recommends placing the filter in sterile diluent and shaking vigorously for not less than two minutes, or alternatively to place the container in and ultrasoud tank (sonicator) for 2-10 minutes, depending on the type of tank used. The Australian method recommends resuspension in water from the original sample followed by shaking the concentrate vigorously for 20-30 seconds, Sonication has been used successfully to release intra-amoebal legionellae from seeded samples (Boulanger *et al.* 1995), a method that may further increase the sensitivity of culture methods.

A scraping method to remove bacteria from membranes after filtration has been used in the past. Bacteria were aseptically scraped from filters using commercially available cell scrapers. This method is not routinely used any more.

1.12.5 Sample pretreatment

The presence of non-legionellae in environmental samples may decrease the recovery of legionellae due to overgrowth or inhibition by non-legionellae (Boulanger *et al.* 1995, Lye *et al.* 1997). Legionellae have thermophilic characteristics and are relatively stable in acidic conditions. To improve their isolation from highly contaminated samples, Bopp *et al.* (1981) and Groothuis *et al.* (1983) developed the pretreatment methods (acid treatment and heat treatment respectively) that are still widely used today. However, the efficacy of pretreatment varies among different sample types and institutions and if not carefully controlled, these pre-treatment procedures may inhibit or injure legionellae. Most authors believe that a combination of methods is necessary to optimise the

recovery from different sample types. The general feeling is that each laboratory should find the method that works best for their environment and sample types they process.

It is well known that laboratory-adapted legionellae react differently to pretreatment than environmental strains. Environmental strains from different sources or even from different areas in the same source may react differently to these selective procedures. A study by Roberts and coworkers (1987) indicated that environmental legionellae are more resistant to pretreatment than laboratory-adapted strains.

Acid treatment involves subjecting sample concentrates to a buffer at a pH of 2.2.for a period ranging from 3-15 minutes as preferred by different workers. As legionellae are not entirely acid fast, the length of exposure to this buffer must be carefully controlled. Stout (1998) recommended acid treatment of potable waters for 3 minutes and 15 minutes for non-potable water. Heat treatment relies on the thermophilic characteristics of legionellae and involves subjecting sample concentrates to a temperature of 50°C for 30 minutes.

1.12.6 Culture media

The primary isolation medium for Legionella species is buffered charcoal yeast extract (BCYE) agar supplemented with alpha-ketoglutarate, iron and cysteine. Legionellae do not grow in the absence of iron or cysteine. The presence of certain organisms may inhibit legionellae growth, but the selectivity of agar media can be improved by the addition of antimicrobial agents such as amphotericin B, colistin, cefamandole, polymyxin B, vancomycin and trimethoprim, antifungal agents like anisomycin and cycloheximide and inhibitors like glycine as summarised in Table 1.10. Glycine is an excellent inhibitor of environmental flora, but not effective against respiratory flora (Stout 1998).

The addition of 2.5-5% CO_2 to the atmosphere is essential for the culture of some Legionella species although concentrations above 5% will increase the pH of the medium, thereby inhibiting growth. An extracellular, water–soluble compound is produced when some legionellae are grown on BCYE agar, resulting in fluorescence when exposed to long-wave (365 nm) ultraviolet light. All the known strains of *L pneumophila* provide fluorescence yellow-green while *L bozemanii, L dumoffii, L gormani, L anisa, L cherri, L steigerwaltii, L gratiana, L tusconensis* and *L parisiensis* will produce blue-white and *L rubrilucens* and *L erythra,* red fluorescence. The colour of the fluorescence can assist in differentiation of different species when present in one sample (Figure 1.1). Dyes can be added to solid culture media to aid identification further. It is important to note that certain antibiotics, particularly cafamandole, may inhibit some Legionella species (Stout

1998). *L micdadei* is sensitive to cefamandole but can be recovered on PAV medium. In a study involving 28 species, 11 species failed to grow on a cefamandole-containing medium and 8 species grew only marginally (Lee 1993). The inclusion of a non-selective plate is therefore recommended, even in highly contaminated samples (Stout 1998).

Table 1.10 Supplements in Legionella selective media

Medium	Supplements									
	Aniso[1]	Cefam[1]	Cefal[1]	Colis[1]	Cyclo[1]	Glyc[2]	Pol-B[3]	B-p[1]	B-b[1]	Vanco[1]
BMPAα	80	4	0	0	0	0	80	0	0	0
CCVC	0	0	4	16	80	0	0	0	0	0.5
WY	0	0	0	0	0	3	100	0	0	5
MWY	80	0	0	0	0	3	50	10	10	1
GVPC	0	0	0	0	80	3	80	0	0	1
GVP	0	0	0	0	80	0	80	0	0	1
DGVP	0	0	0	0	0	3	50	10	10	1
PAV	80	0	0	0	0	0	100	0	0	5
GPAV	80	0	0	0	0	3	100	0	0	5

[1]µg/ml; [2]mg/ml; [3]IU/ml; aniso=anisomycin; Cefam=cefamandole; Cefal=cefalothin; Colis=colistin; Glyc=glycine; Pol-B=polymyxin B; B-p=bromocresol purple; B-b=bromothymol blue; Vanco=vancomycin

1.12.7 Standardised culture methods

There is no one generally accepted culture method for legionellae. However, a number of standards and guidelines have been developed in this regard and are widely accepted by different institutions. For example, in Australia the Australian Standard (AS 3896-1991) has been developed, in Europe the International Standard (ISO/DIS 11731-1996) and in the USA, the ASTM Standard Guide for Investigating Water Systems for Legionellae (D 595201996). A method published by Stout in 1998 is also often used in the USA. In South Africa, an adaptation of the Most Probable Number method is also used in addition to the European method.

The Draft International Standard (ISO/DIS 11731), accepted by most European countries as a standard method in 1996, is used to demonstrate the presence of confirmed Legionella species in the majority of environmental sample types, ranging from water to swabs, biofilm and sediment. Water samples are concentrated by centrifugation or membrane filtration and biofilm and scale samples are diluted prior to testing. To reduce the growth of unwanted, non-legionellae organisms, a portion of the sample is subjected to heat treatment and another to acid treatment before culture. Treated and untreated portions are then inoculated onto plates of agar medium and incubated aerobically at 37°C for up to 10 days. After incubation morphologically characteristic colonies (reported as *presumptive* legionellae) are tested for cysteine dependence. For

species identification direct immunofluorescence, latex agglutination or biochemical tests can be done. Results are reported as an estimated number of *confirmed* legionellae per millilitre of sample. Agar media used for this method are bufferened charcoal yeast extract (BCYEα) as a non-selective and GVPC (glycine/vancomycin/polymyxin/cycloheximide) agar as a selective medium.

Stout *et al.* (1998) published a method widely used in the USA. This method recommends slightly different procedures for potable and non-potable waters, biofilms and swab samples. In general, swabs are pretreated with acid for 3 minutes prior to inoculation onto BCYEα and DGVP agar plates. Samples from hot-water tanks are inoculated directly (without concentration or pretreatment) whereas samples collected from faucets and showerheads are concentrated and acid treated before inoculation. Sample concentrates are resuspended in a small volume of the original sample. Cooling water samples are not concentrated and are inoculated directly onto BCYEα. After an initial incubation period the concentrates are acid treated for 15 minutes if overgrowth by non-legionellae occurs and inoculated onto BCYEα, DGVP and CCVC agar. Confirmation is done as stipulated in the ISO method.

The Australian Standard (AS 3896-1991) is applicable to water samples only. Samples are concentrated by centrifugation or membrane filtration and inoculated onto a number of selective and non-selective agar media, either as untreated or as heat-treated portions. Acid treatment is recommended for highly contaminated samples only. Culture plates are incubated aerobically at 37°C for up to 10 days. Legionellae are confirmed by cysteine dependence and species identification by latex agglutination, direct or indirect immunofluorescence or any other suitable method. Results are reported as an estimated number of *confirmed* Legionella organisms in a millilitre of sample. Agar media used are BCYEα as a non-selective and MWY (modified Wadowski-Yee) and BMPAα as selective media.

The Most Probable Number (MPN) is a quantitative method, adapted for enumeration of legionellae in water samples by workers at the Centre for Scientific and Industrial Research (CSIR) (Grabow *et al.* 1991). This method does not make provision for the testing of biofilm, sediment or scale. After concentration by membrane filtration appropriate serial dilutions are made and inoculated in triplicate onto BCYEα agar. No selective media or sample pretreatment steps are included in the method. The plates are incubated aerobically at 37°C for three days where after a representative smear from each agar plate is stained by direct immunofluorescence (DFA). Plates are recorded as positive when they contain morphologically typical Legionella colonies and yield a positive

DFA test. MPN statistical tables are used to calculate the number of Legionella organisms in the original sample and are usually reported as *presumptive* legionellae.

1.12.8 Colony morphology

Colonies of legionellae are smooth with an entire edge and exhibit a characteristic ground-glass appearance (Figure 1.5). The type of agar media used for isolation will influence the colour of the colonies, ranging from white to grey and blue, brown, pink or even green or deep red. When viewed under a plate microscope, some colonies may display pale blue or pink edges. Typical Legionella colones on BCYEα agar are illustrated in Figure 1.1.

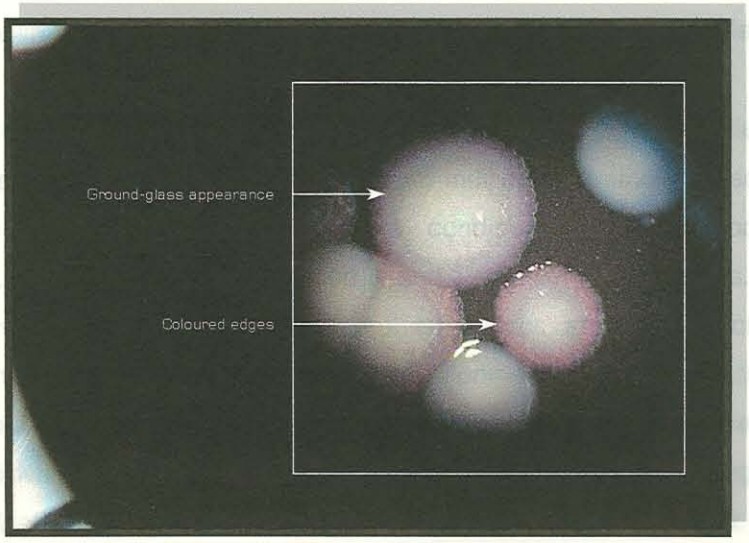

Figure 1.1: Colony morphology on BCYE agar

1.12.9 Cysteine dependence

The first step in Legionella identification is testing morphologically characteristic colonies for cysteine dependence. This is achieved by inoculation onto BCYEα agar (containing cysteine) and blood- or nutrient agar as a negative control. The plates are incubated plates aerobically at 37°C until growth is visible on the BCYEα plate. Colonies growing only on BCYEα are regarded as cysteine dependent and reported as presumptive legionellae.

1.12.10 Direct immunofluorescence

Direct immunofluorescence (DFA) is most commonly used for confirmation of Legionella species from environmental samples. Various polyclonal and monoclonal reagents are available internationally; the reagent available in South Africa is specific for *L pneumophila* SGs 1-6 and *L micdadei.* The test is simple to perform, but interpretation requires a fair amount of experience, especially in highly contaminated samples. Antigen from the sample is fixed to a microscope slide using heat or acetone and covered with fluorescein-isothiocyanate (FITC) labelled globulin. Antigens in the sample bind to the labelled globulin and the resulting antigen-antibody complexes are visible under ultraviolet light.

A number of factors can influence the reactivity of Legionella antigens in surface antigen-dependent tests such as direct and indirect immunofluorescence. These factors include the culture media and culture conditions, the method used for antigen preparation, the fixation method and strain differences. Although direct immunofluorescence (DFA) is very useful to detect antigens in clinical samples when cultures cannot be obtained, its value for environmental samples is controversial although it is used routinely in South Africa (Tobiansky et al 1986). Cross-reactions that may lead to false positive results have been documented to occur between Legionella species and *B fragilis, P alcaligenes, P fluorescens* and *B pertussis* in the DFA test (Grimont 1986, Roig *et al.*1991). Although indirect immunofluorescence (IFA) is routinely used for clinical samples, its value for the identification of Legionella species from environmental samples is not clear.

A typical positive DFA test is illustrated in Figure 1.2.

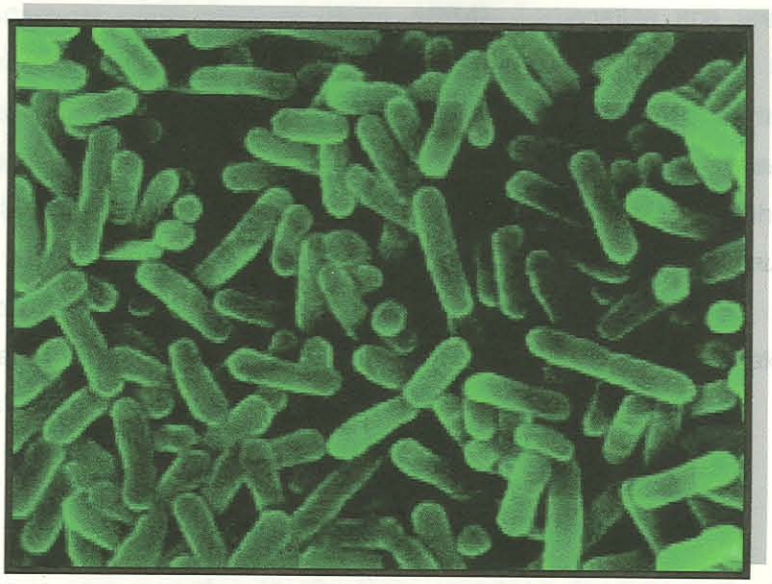

Figure 1.2: Positive DFA

1.12.11 Agglutination tests

A commercially available latex agglutination test is often used in addition to the DFA described above. The three reagents supplied with the test kit commonly used in South Africa are specific for *L pneumophila* SG 1, *L pneumophila* SGs 2-14 and Legionella species respectively. The Legionella species include *L longbeacheae* SGs 1-2, *L bozemanii* SGs 1-2, *L dumoffii, L gormanii, L jordanis, L micdadei* and *L anisa*. The test is recommended for confirmation of morphologically characteristic colonies on agar media.

Several new test kits have recently been developed. Oxoid has brought out a 'dry spot' test range and several agglutination tests are on the market (Hart et al 2000).

1.12.12 Biochemical tests

Legionella species are not easily distinguished by routine biochemical tests. With the exception of starch, the organisms do not ferment or oxidise carbohydrates (Yu 1990). Most strains of *L pneumophila* and *L feeleii* are hippurate hydrolysis positive but the reaction is variable in *L bozemanii, L micdadei, L dumoffii* and some of the other legionella-like organisms (Hébert 1981). Catalase, peroxidase and superopxide dismutase (SOD) is not present in all legionellae (Pine et al 1986). However, according to Yu (1990),

all legionellae contain catalase but the reaction is weak in comparison with other catalase-positive organisms. *L pneumophila* contains only peroxidase, *L bozemanii* only catalase and *L dumoffii* both catalase and peroxidase. The other legionellae are all catalase positive or weak positive (Winn 1984). Legionellae are urease negative and gelatinase positive, nitrate reduction negative and starch positive (Winn 1984). The oxidase reaction is negative in *L pneumophila* but variable in other legionellae whereas beta-lactamase is produced in *L micdadei, L feeleii* and *L maceachernii* and in *L pneumophila* (Thorpe et al 1981, Yu 1990). Extracellular protease, phosphatase, lipase, ribonuclease and deoxyribonuclease have been detected in *L pneumophila*, whereas elastase, collagenase, lecithinase, hyaloronidase, chondroitase, neuramidase and coagulase reactions were absent and alpha-amylase activity was weak in *L pneumophila* during early studies (Thorpe et al 1981).

1.12.13 Molecular methods

Culture is still regarded as the 'gold standard' for Legionella detection, but has several disadvantages. To culture legionellae require several days of incubation, heterotrophic flora from a sample may overgrow the culture media and make plate counts difficult or even impossible, or the organisms may be in a viable but nonculturable (VBNC) state. However, recent developments in the molecular field opened doors for new detection assays of waterborne pathogens such as Legionella; these methods include DNA probe hybridisation (Grimont *et al.* 1985), restriction enzyme digestion (Saunders *et al.* 1990) and the polymerase chain reaction (Bej *et al.* 1991, Mahbubani *et al.* 1990). Although the sensitivity of most of these techniques is insufficient for direct detection of legionellae in environmental samples, PCR has proven to be a sensitive and rapid alternative to culture (Bej *et al.* 1991, Mahbubani *et al.* 1990, Ng *et al.* 1997, Palmer *et al.* 1993).

Many PCR assays have been described, but relatively few of them have been extensively studied on clinical as well as environmental samples and none are routinely used. Polymerase chain reaction (PCR)-based detection methods are normally highly sensitive and specific. In theory, PCR should be able to amplify the number of targets 10^6-fold, in other words it should be able to detect 1-10 bacteria/litre (Tompkins and Loutit 1993). These workers reported a sensitivity of 1-500 bacteria per sample and specificity of 100% following PCR following amplification of Legionella species and other aquatic bacteria. Waters tested from two outbreaks using their PCR method showed a sensitivity

of 57-60% and specificity of 93-100%. Despite this, the sensitivity of PCR is not yet as high as that of culture.

The sensitivity of PCR depends on a number of factors including effective cell lysis, target DNA availability, competition from non-target DNA and the absence of inhibitory substances that might interfere with polymerase enzyme activity (Maiwald *et al.* 1995, Palmer *et al.* 1993). PCR reactions are especially prone to inhibition from organic chemicals such as humic acids that may be present in environmental samples. In order to overcome this inhibition, workers suggest DNA purification and sample dilution.

According to Tompkins and Loutit (1993) amplification of legionellae before probing with specific chromosomal DNA fragments should overcome the problems of 'non-viable' and 'viable but non-culturable' cells. Primers derived from the 5S rRNA gene detect all Legionella species whereas those developed using the *mip*-gene detect only *L pneumophila* strains (Tompkins and Loutit 1993). Most of their studies were done using seeded samples. Whether these methods will work with environmental samples is not clear. According to Bej *et al.* (1993) all the *L pneumophila* serogroups have been identified using the *mip*-gene for PCR. She recommended combining PCR with endonuclease analysis of a portion of the amplified *mip* gene as a potential tool for identification of the various *L pneumophila* serogroups.

Ligase chain reaction (LCR) is another promising molecular method reported by Tompkins and Loutit in 1993. The difference between PCR and LCR is that LCR oligonucleotides are designed to completely cover the target so that one set of oligonucleotides hybridises with the left and one set with the right half. The ligase enzyme then seals the nick between the two halves, thereby creating a full-length complementary sequence. This is followed by the usual amplification. The key to LCR use is that the ligase enzyme will not seal gaps that are not perfect matches, so even if mismatched nucleotides may hibridise to the target they will not be held together to permit subsequent priming for amplification. The advantage of LCR is that very minor mutations (including single base substitutions or deletions) can be detected by using nucleotides that are complementary to the mutation.

Other molecular tools for the epidemiologic characterisation of Legionella strains involve the use of soluble protein patterns, DNA restriction endonuclease profiles, multilocus enzyme analysis or orthogonal-field-alteration gel electrophoresis (Ehret *et al.* 1993). These authors also compared sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE), restriction enzyme cleaviage of genomic bacterial DNA followed by conventional agarose gel electrophoresis (REA) and analysis of the patterns of DNA fragments separated by pulsed-field gel electrophoresis (PFGE). Their results

indicated that these molecular methods were more efficient than the use of monoclonal antibodies for analysis.

Fluorescent in situ hybridization tests are useful for the detection of legionellae in respiratory tract samples. The method makes use of oligonucleotide probes targeting rRNA and offers a rapid and specific alternative to DFA, culture and urine antigen testing in clinical laboratories but has not been extensively tested in environmental samples. However, Grimm *et al.* (2000 conference posters) reported that fluorescent in situ hybridisation is valuable to identify legionellae in their natural environment. They demonstrated that this method is also valuable for identification of the potential host organisms, Hartmanella and Naeglieria species, from the environment.

1.12.14 Typing methods

Legionella identification is becoming more complex as more species and serogroups are being recognised. The diversity of types and subtypes and the ubiquitous nature of the organisms in water and other moist environments make it difficult to identify epidemic strains in particular. In addition, it is not uncommon to detect more than one species or strain from a single plate of primary culture media. Identification of legionellae below the subspecies level is especially necessary in outbreak situations in order to verify associations between patients and sources of infection.

To simplify this process a number of molecular tools have become available recently in addition to microbiological and immunological methods. These methods include arbitrarily primed PCR (AP-PCR), ribotyping, pulsed-field gel electrophoresis (PFGE), multilocus enzyme electrophoresis (MLEE), serologic techniques, plasmid analysis, electrophoretic alloenzyme typing (ET), restriction fragment length polymorphism analysis, RNA or DNA probing of DNA digests and other methods such as pulsed-field electrophoresis of DNA digests. Serologic methods to subtype legionellae include the use of polyvalent [polyclonal] and monoclonal antibodies. A number of monoclonal antibody panels have been developed and are commonly used in the USA, UK and Canada (Barbaree 1993). The majority of these methods have been developed specifically to subtype *L pneumophila* SG 1 (Benson *et al.* 2000 [p29], Lück [030], Lundholm [032], Yu 1990)

- ⇨ Plasmid analysis is of limited use as not all legionellae contain plasmids (Barbaree 1993 book).
- ⇨ Electrophoretic alloenzyme typing (ET), first published by Selander *et al.* in 1985, has been used with some success to subdivide epidemic strains of legionellae but the method is very labour intensive and is not widely used.

- ⇨ Ribotyping involves extracting DNA, nicking it with an endonuclease and probing it with labeled 16S and 23S rRNAs from *E coli* (Grimont *et al.* 1989). This method has been used at the CDC to subtype *L pneumophila* strains implicated in outbreaks (Barbaree 1993).
- ⇨ Restriction endunuclease analysis of total genomic DNA has the advantage of discriminating among strains of *L pneumophila* lacking plasmids, but electrophoretic patterns of the total genomic DNA digests are often difficult to interpret (Bej *et al.* 1993). For this reason an alternative method that uses radiolabeled probe fingerprint analysis following restriction endonuclease digestion of total genomic DNA has been described.
- ⇨ Monoclonal antibodies have been used to screen phenotypic differences between strains of *L pneumophila* (Struelens *et al.* 1993). Several panels of monoclonal antibodies have been developed (Ehret *et al.* 1986, Joly *et al.* 1986).