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Minireview

Legionella: from environmental habitats to disease pathology, detection and control

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Summary

Studies on *Legionella* show a continuum from environment to human disease. Legionellosis is caused by *Legionella* species acquired from environmental sources, principally water sources such as cooling towers, where *Legionella* grows intracellularly in protozoa within biofilms. Aquatic biofilms, which are widespread not only in nature, but also in medical and dental devices, are ecological niches in which *Legionella* survives and proliferates and the ultimate sources to which outbreaks of legionellosis can be traced. Invasion and intracellular replication of *L. pneumophila* within protozoa in the environment play a major role in the transmission of Legionnaires' disease. Protozoa provide the habitats for the environmental survival and reproduction of *Legionella* species. *L. pneumophila* proliferates intracellularly in various species of protozoa within vacuoles studded with ribosomes, as it also does within macrophages. Growth within protozoa enhances the environmental survival capability and the pathogenicity (virulence) of *Legionella*. The growth requirements of *Legionella*, the ability of *Legionella* to enter a viable non-culturable state, the association of *Legionella* with protozoa and the occurrence of *Legionella* within biofilms complicates the detection of *Legionella* and epidemiological investigations of legionellosis. Polymerase chain reaction (PCR) methods have been developed for the molecular detection of *Legionella* and used in environmental and epidemiological studies. Various physical and chemical disinfection methods have been developed to eliminate *Legionella* from environmental sources, but gaining control of *Legionella* in environmental waters, where they are protected from disinfection by growing within protozoa and biofilms, remains a challenge, and one that must be overcome in order to eliminate sporadic outbreaks of legionellosis.

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Environmental sources of legionellosis

Legionnaires' disease or legionellosis, first discovered in 1976 after an outbreak in Philadelphia during a convention of the American Legion, is a serious form of pneumonia caused by *Legionella* species acquired from environmental sources. Several reviews have considered the environmental sources and pathologies of infections with *Legionella* (Meyer and Finegold, 1980; Kramer and Ford, 1994; Stout and Yu, 1997; Abu Kwaik *et al*., 1998). *Legionella pneumophila* causes 4–20% of cases of community-acquired pneumonia and has been ranked as the second or third most frequent cause of pneumonia requiring hospitalization (Rusin *et al*., 1997).

Ever since the initial discovery that the outbreak of Legionnaires' disease in Philadelphia was caused by *Legionella* from a hotel's air conditioning system, there has been an obvious link to environmental waters. *L. pneumophila* is part of the natural aquatic environment, and the bacterium is capable of surviving extreme ranges of environmental conditions (Fliermans *et al*., 1981). Transmission of legionellosis occurs via aerosols generated from environmental sources, and person-to-person transmission does not occur (Fields, 1996). Outbreaks of Legionnaires' disease have been traced to a wide variety of environmental water sources, including cooling towers, whirlpools and spas, fountains, ice machines, vegetable misters and shower heads. These outbreaks have occurred in homes, offices, hotels, hospitals and cruise ships, among other locations. Small cooling towers, particularly when they are started after a period of non-use or during construction, have predominantly been implicated in major outbreaks of legionellosis (Bentham and Broadbent, 1993; Mermel *et al*., 1995).

Relatively little is known about sporadically occurring legionellosis, which accounts for most infections, but correlation analyses indicate that a significant proportion of sporadic cases of Legionnaires' disease may be residentially acquired and associated with domestic potable

water and disruptions in residential plumbing systems (Straus *et al*., 1996). Potable water supplies that harbour *L. pneumophila* are an important source of community-acquired Legionnaires' disease (Stout *et al*., 1992a), and most cases of community-acquired legionellosis probably result from the presence of *Legionella* bacteria in the water distribution systems (Bernander and Kallings, 1998). In a Canadian study, 6.2% of water samples from single-family residences were found to be positive for legionellae compared with 25% for multiple-dwelling apartments (Marrie *et al*., 1994). Even though most sporadic cases of community-acquired Legionnaires' disease have been epidemiologically linked to residential water supplies, the risk of acquiring Legionnaires' disease from exposure to *L. pneumophila* in residential water systems is generally low (Stout *et al*., 1992b).

Legionella pneumophila has been found in 3–33% of drinking water samples, suggesting that drinking water could be a significant source of infection with *Legionella* species (Rusin *et al*., 1997). States *et al*. (1989) showed the potential for *Legionella* growth within municipal systems, and this supports the hypothesis that public water supplies may contaminate the plumbing systems of hospitals and other large buildings. Individual water outlets in hospitals can each serve as its own ecological niche of *L. pneumophila* (Marrie *et al*., 1992). In a study of *Legionella* in the hot water distribution systems of Finnish apartment buildings, Zacheus and Martikainen (1994) found the highest concentrations of legionellae in the shower water. Environmental surveillance in Singapore showed *Legionella* growing in 36% of cooling towers, 15–19% of decorative fountains and waterfalls and 2% of spa pools (Heng *et al*., 1997). In another study of cooling tower water samples, over 90% were positive for *Legionella* spp., and 50% of these contained *L. pneumophila* (Koide *et al*., 1993). In a study of hot water systems in south-eastern Germany, *Legionella* were isolated from 68% of hospitals, 50% of outpatient departments, 58% of dental offices, 85% of public buildings and 65% of private households in large buildings (mostly at concentrations below 100 ml^{-1}; Luck *et al*., 1993).

Biofilms, which are widespread not only in nature but also in medical and dental devices, have been identified as ecological niches in which *L. pneumophila* not only survives but proliferates and lies in wait for susceptible hosts (Barbeau *et al*., 1998). In water piping systems, *L. pneumophila* has been found to be most abundant in biofilms on plastics at 40°C, where it accounted for up to 50% of the total biofilm flora; in contrast, pipes with copper surfaces were inhibitory to total biofouling and included only low numbers of *L. pneumophila* (Rogers *et al*., 1994). Iron limitation leads to greatly reduced virulence of *Legionella* (James *et al*., 1995). Metal plumbing components and associated corrosion products are important factors in providing iron and other metals that support the survival and growth of *L. pneumophila* in plumbing systems and may also be important in related habitats, such as cooling towers and air-conditioning systems (States *et al*., 1985).

Biofilms in dental unit lines are a particular problem, because *Legionella* can proliferate there and become aerosolized during dental procedures (Smith *et al*., 1999). The unique feature of dental chair water lines is the capacity for the rapid development of a biofilm on the dental water supply lines combined with the generation of potentially contaminated aerosols (Pankhurst *et al*., 1998) (Fig. 1). Microbial adherence to the internal surface of dental tubing and the formation of a highly protective biofilm layer is predictable, given the ideal growth conditions in the tubing (Williams *et al*., 1996a). *Legionella* have been found within protozoa growing within dental unit biofilms (Michel and Borneff, 1989; Williams *et al*., 1996a,b) (Fig. 1). *L. pneumophila* serogroup 6 strains were isolated from warm water outlets and dental units at the University of Dresden in Germany (Luck *et al*., 1991). Dentists in Dresden were found to have a higher prevalence of antibodies against legionellae than the general public, suggesting greater exposure to *Legionella* in the dental office (Luck *et al*., 1992). Oppenheim *et al*. (1987) reported widespread *L. pneumophila* contamination of dental stations in a dental school.

Legionella have been found in high concentrations in biofilms in dental unit water lines, where stagnation of the water and a low chlorine residual potentially create a unique niche for this microorganism (Williams *et al*., 1996b). In a study of dental unit contamination by *Legionella*, *Atlas et al*. (1995) detected *Legionella* spp. in 68% of the dental unit water samples, and *L. pneumophila* was detected in 8%. Concentrations of *Legionella* spp. in dental unit water reached 1000 organisms ml^{-1} or more in 36% of the samples, and 19% of the samples were in the category of 10 000 ml^{-1} or above. *L. pneumophila*, when present in dental unit water, never reached concentrations of 1000 ml^{-1} or more. *Legionella* spp. were present in 61% of potable water samples collected for comparative analysis from domestic and institutional taps and drinking fountains; only 4% of the potable water samples had *Legionella* spp. concentrations of 1000 organisms ml^{-1}, and none was in the 10 000 organisms ml^{-1} category. Thus, the health-threatening levels of *Legionella* spp. in potable water were significantly lower than in dental unit water. Control of biofilms in dental unit lines must receive considerable attention (Shearer, 1996).

Although mainly associated with freshwater bodies, *Legionella* has also been shown to be able to survive in marine waters (Heller *et al*., 1998). Ocean waters receiving treated sewage have been found to contain *Legionella* species (Palmer *et al*., 1993). *Legionella* species are present in all phases of sewage treatment, and population

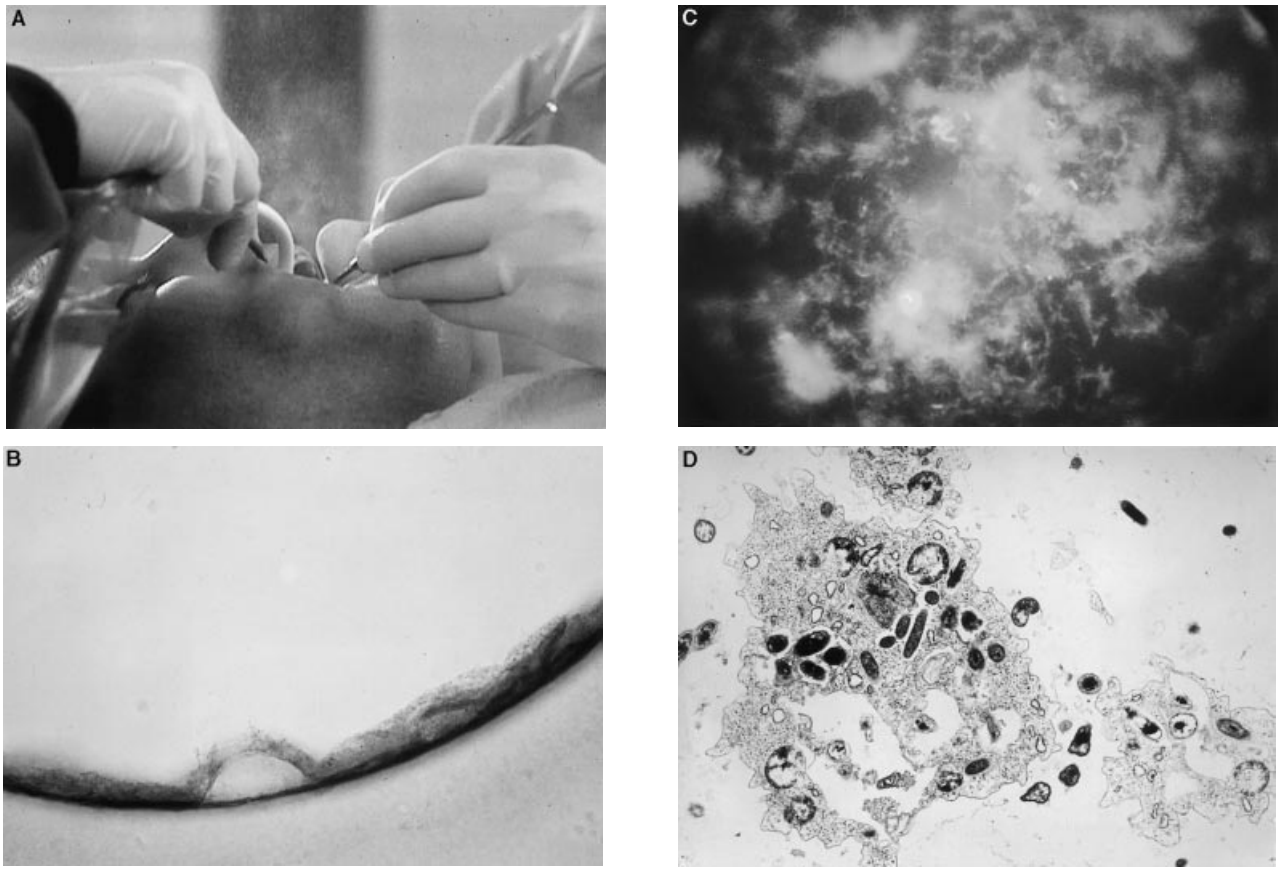

Fig. 1. *Legionella* growing within protozoa in dental unit water line biofilms represent environmental sources for human infection.
A. Extensive aerosols are generated in the dental operation, which can spread *Legionella* from the water lines.
B. Biofilms on the walls of a dental unit water line can slough off carrying *Legionella* into the water. Here, a cross-section of such a dental line is shown with an extensive biofilm on the surface.
C. *Legionella* grows within biofilms, as shown in this micrograph of a sample from a dental unit water supply line after direct fluorescence antibody staining for *Legionella*.
D. Within biofilms, *Legionella* actually grows intracellularly in the vacuoles of amoeboid protozoa, as shown in this electron micrograph.

numbers do not decline significantly through the treatment process (Palmer *et al*., 1993). Reclaimed water is an important resource for areas with inadequate water supplies and may represent a new source of *Legionella* infections (Palmer *et al*., 1995).

Protozoa and pathology of legionellosis

Ecological studies have shown that protozoa provide habitats for the environmental survival of *Legionella* species (Nagington and Smith, 1980; Rowbotham, 1981; 1986; Skinner *et al*., 1983; Barker and Brown, 1994). Various free-living amoebae, such as *Naegleria* and *Acanthamoeba*, can use *L. pneumophila* as a sole food source (Tyndall and Domingue, 1982). *Legionella* can also act as a protozoan parasite, killing the protozoan (Abu Kwaik, 1998). But, while protozoa can ingest *Legionella* and, in some cases, gain nutrition in that way and *Legionella* can kill the host protozoa, in most cases, the *Legionella* survive and replicate within protozoan vacuoles for extended periods.

Reproduction of *Legionella* in the environment is restricted to protozoan cells, and *Legionella* are unable to multiply extracellularly in environmental water bodies (Abu Kwaik *et al*., 1998). Protozoa serve as host cells for the intracellular replication of *Legionella* species, some of which are distinctive, in a variety of environmental settings (Newsome *et al*., 1998). *L. pneumophila* has been observed to multiply in hospital plumbing systems only when amoebae were also present, suggesting that protozoa are essential for providing the habitat in which *Legionella* can multiply (Nahapetian *et al*., 1991). Amoebae have been found to serve as hosts for *Legionella* in a variety of environments, including 38% of warm drinking water samples and 42% of whirlpool waters examined (Henke and Seidel, 1986). *L. pneumophila* has been observed

undergoing binary fission within the intracellular vacuoles of amoebae (Newsome *et al*., 1985). *L. pneumophila* can persist for long periods in the vacuoles of amoebae even under conditions that do not permit its multiplication (Smith-Somerville *et al*., 1991). The ability of *L. pneumophila* to establish the intracellular infection of amoebae is dependent on its capacity to reside and multiply within a phagosome surrounded by the rough endoplasmic reticulum (Abu Kwaik, 1996). This compartment may constitute a rich source of nutrients for the bacteria and is probably recognized as a cellular compartment.

King *et al*. (1988) proposed that resistance to digestion by predatory protozoa was an evolutionary precursor of pathogenicity in bacteria and that today it is a mechanism for the survival of fastidious bacteria in dilute and inhospitable aquatic environments. *L. pneumophila* proliferates intracellularly in various species of protozoa, surviving at some temperatures and proliferating at others within vacuoles studded with ribosomes (Kikuhara *et al*., 1994). *Legionella*-harbouring *Hartmannella* and *Saccamoeba* protozoa from hot water systems have been found to be tolerant of temperatures above 50°C (Rohr *et al*., 1998). *Legionella* species and their protozoan hosts have been found in the waters of many spas. In one study, Kuroki *et al*. (1998a) consistently found free-living amoebae, such as *Hartmannella* and *Vexillifera*, and *L. pneumophila* SG1, SG3, SG4, SG5 and SG6 in water samples from spa bath basins. Kuroki *et al*. (1998b) also reported *L. pneumophila* SG3, SG5 and SG6 and its protozoan hosts *Naegleria*, *Platyamoeba* and *Acanthamoeba* commonly occurring in hot spring bath waters in Japan. High numbers of *Legionella* species occurred when amoebae concentrations were also high.

Naegleria and *Acanthamoeba* have been found to be important hosts for *L. pneumophila* in water (Szenasi *et al*., 1998). Numerous *Legionella*-like slender rods have been observed multiplying within *Acanthamoeba* species isolated from drinking water (Michel *et al*., 1998), which could indicate an iron limitation, as James *et al*. (1995) observed *Legionella* conversion from pleomorphic to thin rod-shaped cells under conditions of iron limitation. The number of *L. pneumophila* ingested per *Acanthamoeba palestinensis* is dependent on the size of the amoeba (Harf *et al*., 1997). *Tetrahymena pyriformis* appears to be an important habitat for the *L. longbeachae* found in potting soil mixes (Steele and McLennan, 1996). *L. pneumophila* will also proliferate as an intracellular parasite in the ciliated protozoan *Tetrahymena pyriformis* in tap water at 35°C (Fields *et al*., 1984). Amoebae and the ciliate protozoan *Tetrahymena* were found to support intracellular multiplication of *L. pneumophila* in cooling tower water and to be involved in the transmission of legionellosis (Barbaree *et al*., 1986).

Entry rather than attachment has been shown to be the limiting step in the infection of *Hartmannella vermiformis* by *L. pneumophila* (Fields *et al*., 1993). A type IV pilus, designated the competence and adherence-associated pilus (CAP), is involved in the initial adherence of *Legionella* to protozoan cells and may provide *Legionella* with a selective advantage in adhering to surfaces and biofilms in the environment (Stone and Abu Kwaik, 1998). *Hartmannella* has been shown to have a lectin receptor that is involved in the attachment and invasion of *Legionella* (Venkataraman *et al*., 1997). Synthesis of *Hartmannella vermiformis* proteins but not of *Acanthamoeba polyphaga* proteins is required for the uptake of *L. pneumophila* (Harb *et al*., 1998). Inhibitors of eukaryotic protein synthesis have been found to have no effect on the uptake of *L. pneumophila* by macrophages but have been found completely to abolish ingestion by the amoeba *Hartmannella vermiformis* (Abu Kwaik *et al*., 1994). The intracellular localization of *L. pneumophila* serogroup 1 within *Acanthamoeba castellanii* can render the bacteria non-culturable even though up to 31% may contain viable *Legionella* (Hay *et al*., 1995). *Acanthamoeba* species have been shown to expel vesicles containing live cells of *L. pneumophila,* even after biocide treatment (Berk *et al*., 1998).

Invasion and intracellular replication of *Legionella pneumophila* within protozoa in the environment plays a major role in the transmission of Legionnaires' disease (Harb *et al*., 1998). After intracellular replication within protozoa, *L. pneumophila* exhibits resistance to conditions of stress, including high temperature, acidity and biocides, which may contribute to its environmental persistence (Abu Kwaik *et al*., 1997). *L. pneumophila*-infected *H. vermiformis* organisms are more pathogenic than an equivalent number of free living *L. pneumophila* cells (Brieland *et al*., 1997). These results demonstrate that *L. pneumophila*-infected amoebae are infectious particles and support the hypothesis that inhaled protozoa may serve as cofactors in the pathogenesis of pulmonary disease induced by inhaled respiratory pathogens. Replication of *L. pneumophila* in protozoans present in domestic water supplies may be necessary to produce bacteria that are competent to enter mammalian cells and produce human disease (Cirillo *et al*., 1994). The outer membrane of *L. pneumophila* growing within *Acanthamoeba* acquires a 15 kDa outer membrane protein and a monounsaturated straight-chain fatty acid, which are found in the host amoeba but not in free-living *Legionella* (Barker *et al*., 1993). Disruption of amoebic membranes, as a result of intra-amoebic infection, may liberate macromolecules, including a 15 kDa polypeptide, a major constituent of the amoebic membrane, which adhere to the surface of the legionellae.

There are important relationships between the replication of *Legionella* within protozoa and the pathology of legionellosis in humans. Comparison of the invasive

strategies of *L. pneumophila* in mammalian and protozoan cells and study of the interactions between *Legionella* and protozoa provide useful information for the development of strategies for the prevention of legionellosis (Fields, 1996). Infection with *L. pneumophila* alone develops multifocal pneumonitis, which resolves with minimal mortality, whereas co-infection of *L. pneumophila* with *Hartmannella vermiformis* develops diffuse pneumonitis, which is associated with diminished intrapulmonary recruitment of lymphocytes and mononuclear phagocytic cells and significant mortality (Brieland *et al*., 1996). Intrapulmonary amoebae potentiate replicative *L. pneumophila* lung infection in both susceptible and resistant hosts; this has significant implications with regard to the potential role of protozoa in the pathogenesis of pulmonary diseases resulting from inhaled pathogens and in the design of strategies to prevent and/or control legionellosis.

The invasion of protozoa by *L. pneumophila* and its role in bacterial ecology and pathogenesis at the molecular level has been reviewed in detail by Abu Kwaik *et al*. (1998). Processing of *L. pneumophila* by the free-living amoeba *Acanthamoeba castellanii* shows many similarities to the processing of *L. pneumophila* by monocytes, including uptake of *L. pneumophila* by coiling phagocytosis, the subsequent confinement of *L. pneumophila* in a ribosome-studded phagosome and inhibition of lysosomal fusion with phagosomes containing *L. pneumophila* (Bozue and Johnson, 1996). Inhibition of phagosome—lysosome fusion is critical for the intracellular survival of *Legionella* (Russell, 1998). The remarkable similarity of the intracellular infections of macrophages and protozoa by *L. pneumophila* strongly supports the hypothesis that adaptation of the bacterium to the intracellular environment of protozoa may be the mechanism for its ability to adapt to the intracellular environment of human alveolar macrophages and cause pneumonia (Abu Kwaik, 1996). Differences in susceptibility to *L. pneumophila* growth between permissive elicited macrophages and non-permissive resident macrophages may be caused by concentrations of intracellular available iron (Gebran *et al*., 1994). The virulence of *L. pneumophila* is significantly attenuated when cultured in an iron-limited environment, because expression of the pathogenicity factor, zinc metalloprotease, is reduced in response to iron limitation (James *et al*., 1997).

Regardless of the capacity of *L. pneumophila* to subvert the microbicidal mechanisms of the macrophage, intracellular *L. pneumophila* is exposed to a high level of stress stimuli throughout the intracellular infection (Abu Kwaik *et al*., 1997). Intracellular *L. pneumophila* manifest a phenotypic modulation and a global stress response to the intracellular environment of the macrophage, involving modulation of multiple regulons that contributes to the survival of *L. pneumophila* within alveolar macrophages (Abu Kwaik *et al*., 1993). Within this ecological niche, *L. pneumophila* alters its gene expression and expresses various virulence-related genes in response to starvation and signalling by (p)ppGpp (Abu Kwaik *et al*., 1998). The Mip (macrophage infectivity potentiator) protein, which belongs to the substance class of FK 506-binding proteins and exhibits peptidyl-prolyl *cis*/*trans* isomerase, represents a factor of *L. pneumophila* necessary for optimal intracellular survival (Wintermeyer *et al*., 1995). *L. pneumophila* is able to replicate within the phagosomes of mammalian cells, because it is not trafficked through the endosomal–lysosomal pathway and is surrounded by the rough endoplasmic reticulum (Abu Kwaik *et al*., 1998).

Pulmonary alveolar epithelial cells may represent an alternative site for replication of *Legionella* species in the terminal airspace and thus clarify some previously unexplained aspects of the pathogenesis of Legionnaires' disease (Mody *et al*., 1993). *Legionella dumoffii* has an ability to invade and proliferate in human alveolar epithelial cells, which may explain the rapid and fulminant progress of the pneumonia it causes. (Maruta *et al*., 1998a). Uptake of *L. dumoffii* within epithelial cell lines requires receptor-mediated endocytosis, in contrast to the uptake of *L. pneumophila*, which mainly uses microfilament-dependent phagocytosis (Maruta *et al*., 1998b). *L. dumoffii* is able to escape from endosomal vacuoles into the cytoplasm during the early stage of infection and proliferate in the cytoplasm surrounded by rough endoplasmic reticula, whereas *L. pneumophila* appear to proliferate only within the ribosome-lined endosome (Maruta *et al*., 1998b).

Legionella pneumophila survives and replicates inside macrophages by preventing phagosome–lysosome fusion based upon the expression of *dot*/*icm* genes that code for a putative large membrane complex, which forms a type IV secretion system used to alter the endocytic pathway (Vogel and Isberg, 1999). *L. pneumophila* requires DotA expression before macrophage uptake in order to establish an intracellular site for replication (Roy *et al*., 1998). The *icm* locus (intracellular multiplication gene) has been shown to be involved in preventing phagosome–lysosome fusion so that *Legionella* can multiply intracellularly within human macrophages (Brand *et al*., 1994).

Detection and disinfection

The association of *Legionella* with protozoa and its occurrence within biofilms complicates its detection and disinfection. Various methods have been developed for the detection of *Legionella* species in environmental samples. Cultivation of viable *Legionella* species remains the standard method, although various factors can interfere with its growth on selective media, including the presence of various other bacterial species. Various media, generally containing amino acid supplements and antimicrobial

inhibitors, can be used to cultivate *Legionella* species (Atlas, 1995). Often, pre-enrichment with various inhibitors is needed to eliminate populations of other bacteria (Roberts *et al*., 1987; Kusnetsov *et al*., 1994a). Kasuga *et al*. (1999), for example, recently reported that combined pretreatment with acid after heating and the addition of polymyxin B and oxytetracycline into the selective cultivation medium is a useful method for the detection of *Legionella* sp. from environmental water samples. Water samples negative for legionellae but positive for amoebae, using standard culture techniques, should be incubated and replicated to maximize the sensitivity of the culture for legionellae (Sanden *et al*., 1992). Enrichment in amoebae can be used for the recovery of viable *L. pneumophila* from clinical and environmental samples (Rowbotham, 1983). Resuscitation of viable but non-culturable *L. pneumophila*, for example, can be achieved by culture in *Acanthamoeba castellani* (Steinert *et al*., 1997).

Because of the difficulty in culturing *Legionella* from various environments, various other methods have been developed for its detection. The direct fluorescent antibody technique has proved to be very useful for detecting *L. pneumophila* in natural aquatic systems (Fliermans *et al*., 1981; Alary and Joly, 1992). A gas chromatographic–mass spectrometric method, based on unique 3-hydroxy and 2,3-dihydroxy fatty acids of the *L. pneumophila* lipopolysaccharides, has been used to detect *L. pneumophila* in biofilms in potable water containing a complex microbial consortium (Walker *et al*., 1993). Various molecular methods have also been developed for detecting *Legionella*. Under certain circumstances, culture of environmental samples should be supplemented with additional tests such as polymerase chain reaction (PCR) to detect non-viable and/or viable but non-culturable legionellae (Miller *et al*., 1993). PCR detection may be superior to the culture and direct fluorescent antibody methods for detecting *Legionella* spp. in environmental water samples, because it can detect viable but non-culturable cells (Palmer *et al*., 1995).

Several PCR methods have been developed for the detection of *Legionella*. Mahbubani *et al*. (1990) found that all species of *Legionella*, including all serogroups of *L. pneumophila*, could be detected by PCR amplification of a 104 bp DNA sequence that codes for a region of 5S rRNA, followed by oligoprobe hybridization to an internal region of the amplified DNA. They also found that strains of *L. pneumophila* (all serogroups) could be specifically detected based upon amplification of a portion of the coding region of the macrophage infectivity potentiator (*mip*) gene. Amplification of the *mip* sequence with PCR has been shown to be specific for *L. pneumophila* (Schlenk *et al*., 1993). This method forms the basis for the commercial EnviroAmp *Legionella* detection kit. Modifications to the EnviroAmp *Legionella* detection system can be made to improve the time and cost efficiency of detection (Saint, 1998).

Villari *et al*. (1998) recommend the use of PCR for initial analysis of water, followed by viable plating of filter-concentrated samples for epidemiological studies on samples that test positive for the presence of significant concentrations of *Legionella*. Bej *et al*. (1991) found a PCR method that detected viable culturable and viable non-culturable *L. pneumophila*. Pabst *et al*. (1997) have developed a PCR method for determining the association between *Legionella* and *Amoeba* species. Nested PCR permits the detection of *L. pneumophila* in samples in which culturing fails, with the advantage of a rapid turnaround time, simplicity and the ability to detect non-culturable cells, fulfilling the requirements of sensitivity and specificity for routine use in an environmental laboratory (Catalan *et al*., 1994; 1997). Nested PCR can overcome PCR inhibitors without loss of sensitivity for the detection of *Legionella* spp. in water (Miyamoto *et al*., 1997).

Analysis of PCR amplicons can be used in epidemiological studies aimed at determining the sources of outbreaks of legionellosis. PCR amplification of the *mip* gene may be useful in such epidemiological investigations of Legionnaires' disease (Lu *et al*., 1997). Fingerprinting of *L. pneumophila* by PCR amplification of variable genomic regions with arbitrary and repeat sequence primers may be useful in epidemiological investigations (van Belkum *et al*., 1993), but it is often difficult to identify the specific environmental source of a community outbreak of Legionnaires' disease, even using molecular profiling methods (Heath *et al*., 1998). Arbitrarily primed (AP)-PCR can be used to differentiate strains of *L. pneumophila* but, as different subpopulations of *L. pneumophila* co-exist, this method has not proved useful for linking an individual *L. pneumophila* strain to the occurrence of a disease outbreak (Ledesma *et al*., 1995).

When high levels of *Legionella* are detectable, disinfection of water is critical for controlling outbreaks of legionellosis. Potential strategies to reduce Legionnaires' disease risk include consistent disinfection of potable water, increasing water heater temperatures and limiting exposure to aerosols after domestic plumbing repairs (Straus *et al*., 1996). Decontamination of water supplies by heat flushing is temporary, and *Legionella* returns to the levels before decontamination within a few months after treatment (Zacheus and Martikainen, 1996; Steinert *et al*., 1998). After an outbreak of Legionnaires' disease *L. pneumophila* serogroup 1 in a renal transplant unit in Sao Paulo, Brazil, periodic hyperchlorination and flushing of pipes were instituted as control measures, but these were only partially effective, and disease outbreaks continued; electric showers were installed, which overcame the problem (Levin *et al*., 1995). Copper and silver ions

can limit the levels of *Legionella* in water systems (Landeen *et al*., 1989; Liu *et al*., 1994a; Lin *et al*., 1995; Rohr *et al*., 1996; Liu *et al*., 1998; States *et al*., 1998; Stout *et al*., 1998). Silver-containing paint can control *Legionella* over short time periods, but replenishment of silver, which leaches from the paint, is necessary for long-term control (Rogers *et al*., 1995). Ultraviolet radiation has been proposed for disinfection of water in distribution systems (Liu *et al*., 1994b; 1995). However, ultraviolet treatment is generally ineffective in controlling *Legionella* in cooling towers because of its growth within biofilms (Kusnetsov *et al*., 1994b).

Legionella are relatively resistant to conventional chlorination (Kuchta *et al*., 1993). *Legionella* spp. have been detected in sewage effluents and reclaimed water even after chlorination (Palmer *et al*., 1995). Neither chlorination nor charcoal filtration deal adequately with the potential hazard of *Legionella* spp. in dental water (Pankhurst *et al*., 1990). Legionellae can survive low levels of chlorine for relatively long periods of time (Kuchta *et al*., 1983). Continuous exposure to high levels of chlorine, however, can eliminate *Legionella* from some waters. No viable *Legionella* were detected in whirlpools with free available chlorine over 0.3 mg l^{-1} (Groothuis *et al*., 1985). Systematic purging of the pipe networks with cold water containing 1–1.5 mg residual chlorine l^{-1} can effectively eliminate *L. pneumophila*, but it can take many months (Moreno *et al*., 1997). Hyperchlorination of shower heads and angle valve strainers has been shown to have only a short-lived effect on legionellae, but regular flushing of showers reduced legionellae to below detectable levels (Makin and Hart, 1990). Kool *et al*. (1999) found that hospitals supplied with drinking water containing free chlorine as a residual disinfectant were more likely to have a reported outbreak of Legionnaires' disease than those using water with monochloramine as a residual disinfectant. Based on this study, 90% of outbreaks associated with drinking water might not have occurred if monochloramine had been used instead of free chlorine for residual disinfection and chloramination of drinking water. Chloramination may thus be a cost-effective method of controlling Legionnaires' disease at the municipal level or in individual hospitals.

The association of *Legionella* with protozoa and its growth within biofilms makes *Legionella* very difficult to eliminate. Cysts of *Acanthamoeba polyphaga* produced from infected trophozoites have been found to protect *Legionella* from at least 50 mg l^{-1} free chlorine (Kilvington and Price, 1990). Cooling tower amoebae containing *Legionella* may adapt to biocides and may even be stimulated by biocides (Srikanth and Berk, 1993; 1994). Polyhexamethylene biguanide has significant killing activity against both host amoeba and *L. pneumophila* (Barker *et al*., 1992). Treatment of cooling tower water with chlorinated phenolic thioether, bromo-nitro-propane-diol and bromo-chloro-dimethylhydantoin failed significantly to lower concentrations of planktonic protozoa harbouring *Legionella* (Bentham and Broadbent, 1995). Ozone and peroxides effectively kill protozoa and *Legionella* in water supplies (Domingue *et al*., 1988). Biocides containing isothiazolinones and dibromonitrilo-proprionamide have been found to be relatively effective against *L. bozemanii* in biofilms (Green, 1993). *Legionella* can survive thermal disinfection of water lines and regrow within a few months within amoebae (Steinert *et al*., 1998). Thus, gaining control of *Legionella* in environmental waters where they are protected from disinfection by growing within protozoa and biofilms remains a challenge, and one that must be overcome in order to eliminate sporadic outbreaks of legionellosis.

References

Abu Kwaik, Y. (1996) The phagosome containing *Legionella pneumophila* within the protozoan *Hartmannella vermiformis* is surrounded by the rough endoplasmic reticulum. *Appl Environ Microbiol* **62:** 2022–2028.

Abu Kwaik, Y.A. (1998) Fatal attraction of mammalian cells to *Legionella pneumophila*. *Mol Microbiol* **30:** 689–695.

Abu Kwaik, Y., Eisenstein, B.I., and Engleberg, N.C. (1993) Phenotypic modulation by *Legionella pneumophila* upon infection of macrophages. *Infect Immun* **61:** 1320–1329.

Abu Kwaik, Y., Fields, B.S., and Engleberg, N.C. (1994) Protein expression by the protozoan *Hartmannella vermiformis* upon contact with its bacterial parasite *Legionella pneumophila*. *Infect Immun* **62:** 1860–1866.

Abu Kwaik, Y., Gao, L.Y., Harb, O.S., and Stone, B.J. (1997) Transcriptional regulation of the macrophage-induced gene (gspA) of *Legionella pneumophila* and phenotypic characterization of a null mutant. *Mol Microbiol* **24:** 629–642.

Abu Kwaik, Y., Gao, L.Y., Stone, B.J., Venkataraman, C., and Harb, O.S. (1998) Invasion of protozoa by *Legionella pneumophila* and its role in bacterial ecology and pathogenesis. *Appl Environ Microbiol* **64:** 3127–3133.

Alary, M., and Joly, J.R. (1992) Comparison of culture methods and an immunofluorescence assay for the detection of *Legionella pneumophila* in domestic hot water devices. *Curr Microbiol* **25:** 19–23.

Atlas, R.M. (1995) *Handbook of Microbiological Media*, 2nd edn. Boca Raton, FL: CRC Press.

Atlas, R.M., Williams, J.F., and Huntington, M.K. (1995) *Legionella* contamination of dental-unit waters. *Appl Environ Microbiol* **61:** 1208–1213.

Barbaree, J.M., Fields, B.S., Feeley, J.C., Gorman, G.W., and Martin, W.T. (1986) Isolation of protozoa from water associated with a legionellosis outbreak and demonstration of intracellular multiplication of *Legionella pneumophila*. *Appl Environ Microbiol* **51:** 422–424.

Barbeau, J., Gauthier, C., and Payment, P. (1998) Biofilms, infectious agents, and dental unit waterlines: a review. *Can J Microbiol* **44:** 1019–1028.

Barker, J., and Brown, M.R. (1994) Trojan horses of the microbial world: Protozoa and the survival of bacterial pathogens in the environment. *Microbiology* **140:** 1253–1259.

Barker, J., Brown, M.R., Collier, P.J., Farrell, I., and Gilbert, P. (1992) Relationship between *Legionella pneumophila* and *Acanthamoeba polyphaga*: physiological status and susceptibility to chemical inactivation. *Appl Environ Microbiol* **58:** 2420–2425.

Barker, J., Lambert, P.A., and Brown, M.R. (1993) Influence of intra-amoebic and other growth conditions on the surface properties of *Legionella pneumophila*. *Infect Immun* **61:** 3503–3510.

Bej, A.K., Mahbubani, M.H., and Atlas, R.M. (1991) Detection of viable *Legionella pneumophila* in water by polymerase chain reaction and gene probe methods. *Appl Environ Microbiol* **57:** 597–600.

van Belkum, A., Struelens, M., and Quint, W. (1993) Typing of *Legionella pneumophila* strains by polymerase chain reaction-mediated DNA fingerprinting. *J Clin Microbiol* **31:** 2198–2200.

Bentham, R.H., and Broadbent, C.R. (1993) A model for autumn outbreaks of Legionnaires' disease associated with cooling towers, linked to system operation and size. *Epidemiol Infect* **111:** 287–295.

Bentham, R.H., and Broadbent, C.R. (1995) Field trial of biocides for control of *Legionella* in cooling towers. *Curr Microbiol* **30:** 167–172.

Berk, S.G., Ting, R.S., Turner, G.W., and Ashburn, R.J. (1998) Production of respirable vesicles containing live *Legionella pneumophila* cells by two *Acanthamoeba* spp. *Appl Environ Microbiol* **64:** 279–286.

Bernander, S., and Kallings, I. (1998) Human parasites and amoeba: Lower warm-water temperature is favourable to *Legionella* bacteria. *Lakartidningen* **95:** 4340–4344.

Bozue, J.A., and Johnson, W. (1996) Interaction of *Legionella pneumophila* with *Acanthamoeba castellanii*: uptake by coiling phagocytosis and inhibition of phagosome-lysosome fusion. *Infect Immun* **64:** 668–673.

Brand, B.C., Sadosky, A.B., and Shuman, H.A. (1994) The *Legionella pneumophila* icm locus: a set of genes required for intracellular multiplication in human macrophages. *Mol Microbiol* **14:** 797–808.

Brieland, J.K., Fantone, J.C., Remick, D.G., LeGendre, M., McClain, M., and Engleberg, N.C. (1997) The role of *Legionella pneumophila*-infected *Hartmannella vermiformis* as an infectious particle in a murine model of Legionnaires' disease. *Infect Immun* **65:** 5330–5333.

Brieland, J., McClain, M., Heath, L., Chrisp, C., Huffnagle, G., LeGendre, M., *et al.* (1996) Coinoculation with *Hartmannella vermiformis* enhances replicative *Legionella pneumophila* lung infection in a murine model of Legionnaires' disease. *Infect Immun* **64:** 2449–2456.

Catalan, V., Garcia, F., Moreno, C., Vila, M.J., and Apraiz, D. (1997) Detection of *Legionella pneumophila* in wastewater by nested polymerase chain reaction. *Res Microbiol* **148:** 71–78.

Catalan, V., Moreno, C., Dasi, M.A., Munoz, C., and Apraiz, D. (1994) Nested polymerase chain reaction for detection of *Legionella pneumophila* in water. *Res Microbiol* **145:** 603–610.

Cirillo, J.D., Falkow, S., and Tompkins, L.S. (1994) Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infect Immun* **62:** 3254–3261.

Domingue, E.L., Tyndall, R.L., Mayberry, W.R., and Pancorbo, O.C. (1988) Effects of three oxidizing biocides on *Legionella pneumophila* serogroup 1. *Appl Environ Microbiol* **54:** 741–747.

Fields, B.S. (1996) The molecular ecology of legionellae. *Trends Microbiol* **4:** 286–290.

Fields, B.S., Shotts, Jr, E.B., Feeley, J.C., Gorman, G.W., and Martin, W.T. (1984) Proliferation of *Legionella pneumophila* as an intracellular parasite of the ciliated protozoan *Tetrahymena pyriformis*. *Appl Environ Microbiol* **47:** 467–471.

Fields, B.S., Fields, S.R., Loy, J.N., White, E.H., Steffens, W.L., and Shotts, E.B. (1993) Attachment and entry of *Legionella pneumophila* in *Hartmannella vermiformis*. *J Infect Dis* **167:** 1146–1150.

Fliermans, C.B., Cherry, W.B., Orrison, L.H., Smith, S.J., Tison, D.L., and Pope, D.H. (1981) Ecological distribution of *Legionella pneumophila*. *Appl Environ Microbiol* **41:** 9–16.

Gebran, S.J., Newton, C., Yamamoto, Y., Widen, R., Klein, T.W., and Friedman, H. (1994) Macrophage permissiveness for *Legionella pneumophila* growth modulated by iron. *Infect Immun* **62:** 564–568.

Green, P.N. (1993) Efficacy of biocides on laboratory-generated *Legionella* biofilms. *Lett Appl Microbiol* **17:** 158–161.

Groothuis, D.G., Havelaar, A.H., and Veenendaal, H.R. (1985) A note on legionellas in whirlpools. *J Appl Bacteriol* **58:** 479–481.

Harb, O.S., Venkataraman, C., Haack, B.J., Gao, L.Y., and Kwaik, Y.A. (1998) Heterogeneity in the attachment and uptake mechanisms of the Legionnaires' disease bacterium, *Legionella pneumophila*, by protozoan hosts. *App Environ Microbiol* **64:** 126–132.

Harf, C., Goffinet, S., Meunier, O., Monteil, H., and Colin, D.A. (1997) Flow cytometric determination of endocytosis of viable labelled *Legionella pneumophila* by *Acanthamoeba palestinensis*. *Cytometry* **27:** 269–274.

Hay, J., Seal, D.V., Billcliffe, B., and Freer, J.H. (1995) Non-culturable *Legionella pneumophila* associated with *Acanthamoeba castellanii*: detection of the bacterium using DNA amplification and hybridization. *J Appl Bacteriol* **78:** 61–65.

Heath, T.C., Roberts, C., Jalaludin, B., Goldthrope, I., and Capon, A.G. (1998) Environmental investigation of a legionellosis outbreak in western Sydney: The role of molecular profiling. *Aust NZ J Public Health* **22:** 428–431.

Heller, R., Holler, C., Sussmuth, R., and Gundermann, K.O. (1998) Effect of salt concentration and temperature on survival of *Legionella pneumophila*. *Lett Appl Microbiol* **26:** 64–68.

Heng, B.H., Goh, K.T., Ng, D.L., and Ling, A.E. (1997) Surveillance of legionellosis and *Legionella* bacteria in the built environment in Singapore. *Ann Acad Med, Singapore* **26:** 557–565.

Henke, M., and Seidel, K.M. (1986) Association between *Legionella pneumophila* and amoebae in water. *Isr J Med Sci* **22:** 690–695.

James, B.W., Mauchline, W.S., Fitzgeorge, R.B., Dennis, P.J., and Keevil, C.W. (1995) Influence of iron-limited continuous culture on physiology and virulence of *Legionella pneumophila*. *Infect Immun* **63:** 4224–4230.

James, B.W., Mauchline, W.S., Dennis, P.J., and Keevil, C.W. (1997) A study of iron acquisition mechanisms of *Legionella pneumophila* grown in chemostat culture. *Curr Microbiol* **34:** 238–243.

Kasuga, O., Takagi, K., Tani, K., and Kinumaki, A. (1999) Studies on detection methods for *Legionella* species from environmental water. *Kansenshogaku Zasshi* **73:** 25–34.

Kikuhara, H., Ogawa, M., Miyamoto, H., Nikaido, Y., and Yoshida, S. (1994) Intracellular multiplication of *Legionella pneumophila* in *Tetrahymena thermophila*. *Sangyo Ika Daigaku Zasshi* **16:** 263–275.

Kilvington, S., and Price, J. (1990) Survival of *Legionella pneumophila* within cysts of *Acanthamoeba polyphaga* following chlorine exposure. *J Appl Bacteriol* **68:** 519–525.

King, C.H., Shotts, Jr, E.B., Wooley, R.E., and Porter, K.G. (1988) Survival of coliforms and bacterial pathogens within protozoa during chlorination. *Appl Environ Microbiol* **54:** 3023–3033.

Koide, M., Saito, A., Kusano, N., and Higa, F. (1993) Detection of *Legionella* spp. in cooling tower water by the polymerase chain reaction method. *Appl Environ Microbiol* **59:** 1943–1946.

Kool, J.L., Carpenter, J.C., and Fields, B.S. (1999) Effect of monochloramine disinfection of municipal drinking water on risk of nosocomial Legionnaires' disease. *Lancet* **353:** 272–277.

Kramer, M., and Ford, T.E. (1994) Legionellosis: Ecological factors of an environmentally 'new' disease. *Zentralblatt Hyg Umweltmedizin* **195:** 470–482.

Kuchta, J.M., States, S.J., McNamara, A.M., Wadowsky, R.M., and Yee, R.B. (1983) Susceptibility of *Legionella pneumophila* to chlorine in tap water. *Appl Environ Microbiol* **46:** 1134–1139.

Kuchta, J.M., Navratil, J.S., Shepherd, M.E., Wadowsky, R.M., Dowling, J.N., States, S.J., *et al.* (1993) Impact of chlorine and heat on the survival of *Hartmannella vermiformis* and subsequent growth of *Legionella pneumophila*. *Appl Environ Microbiol* **59:** 4096–4100.

Kuroki, T., Sata, S., Yamai, S., Yagita, K., Katsube, Y., and Endo, T. (1998a) Occurrence of free-living amoebae and *Legionella* in whirlpool baths. *Kansenshogaku Zasshi* **72:** 1056–1063.

Kuroki, T., Yagita, K., Yabuuchi, E., Agata, K., Ishima, T., Katsube, Y., *et al.* (1998b) Isolation of *Legionella* and free-living amoebae at hot spring spas in Kanagawa, Japan. *Kansenshogaku Zasshi* **72:** 1050–1055.

Kusnetsov, J.M., Jousimies-Somer, H.R., Nevalainen, A.I., and Martikainen, P.J. (1994a) Isolation of *Legionella* from water samples using various culture methods. *J Appl Bacteriol* **76:** 155–162.

Kusnetsov, J.M., Keskitalo, P.J., Ahonen, H.E., Tulkki, A.I., Miettinen, I.T., and Martikainen, P.J. (1994b) Growth of *Legionella* and other heterotrophic bacteria in a circulating cooling water system exposed to ultraviolet irradiation. *J Appl Bacteriol* **77:** 461–466.

Landeen, L.K., Yahya, M.T., and Gerba, C.P. (1989) Efficacy of copper and silver ions and reduced levels of free chlorine in inactivation of *Legionella pneumophila*. *Appl Environ Microbiol* **55:** 3045–3050.

Ledesma, E., Camaro, M.L., Carbonell, E., Sacristan, T., Marti, A., Pellicer, S., *et al.* (1995) Subtyping of *Legionella pneumophila* isolates by arbitrarily primed polymerase chain reaction. *Can J Microbiol* **41:** 846–848.

Levin, A.S., Gobara, S., Scarpitta, C.M., Warschauer, C.L., Sinto, S.I., Rodrigues, E., *et al.* (1995) Electric showers as a control measure for *Legionella* spp. in a renal transplant unit in Sao Paulo, Brazil. *J Hospital Infect* **30:** 133–137.

Lin, Y.E., Vidic, R.D., Stout, J.E., and Yu, V.L. (1995) Effect of copper and silver ionization on *Legionella pneumophila*. *Asce* **1:** 38–46.

Liu, Z., Stout, J.E., Tedesco, L., Boldin, M.M., Hwang, C., Diven, W.F., *et al.* (1994a) Controlled evaluation of copper-silver ionization in eradicating *Legionella pneumophila* from a hospital water distribution system. *J Infect Dis* **169:** 919–922.

Liu, Z., Stout, J.E., Tedesco, L., Boldin, M.M., Hwang, C.C., and Yu, V.L. (1994b) Ultraviolet light irradiation of potable water for *Legionella* colonization in a hospital water distribution system. *Ashrae Trans* **100:** 153–159.

Liu, Z., Stout, J.E., Tedesco, L., Boldin, M., Hwang, C., and Yu, V.L. (1995) Efficacy of ultraviolet light in preventing *Legionella* colonization of a hospital water distribution system. *Water Res* **29:** 2275–2280.

Liu, Z., Stout, J.E., Boldin, M.M., Rugh, J., Diven, W.F., and Yu, V.L. (1998) Intermittent use of copper-silver ionization for *Legionella* control in water distribution systems: a potential option in buildings housing individuals at low risk of infection. *Clin Infect Dis* **26:** 138–140.

Lu, X.H., Wang, L., and Wan, C.Q. (1997) The detection of *Legionella pneumophila* DNA with polymerase chain reaction in water samples. *Chung Hua Liu Hsing Ping Hsueh Tsa Chih* **18:** 345–347.

Luck, P.C., Bender, L., Ott, M., Helbig, J.H., and Hacker, J. (1991) Analysis of *Legionella pneumophila* serogroup 6 strains isolated from a hospital warm water supply over a three-year period by using genomic long-range mapping techniques and monoclonal antibodies. *Appl Environ Microbiol* **57:** 3226–3231.

Luck, P.C., Lau, B., Seidel, S., and Postl, U. (1992) Legionellae in dental units – a hygienic risk? *Deutsches Zahn-, Mund- und Kieferheilkundliches Zentralblatt* **80:** 341–346.

Luck, P.C., Leupold, I., Hlawitschka, M., Helbig, J.H., Carmienke, I., Jatzwauk, L., *et al.* (1993) Prevalence of *Legionella* species, serogroups, and monoclonal subgroups in hot water systems in south-eastern Germany. *Zentralblatt Hyg Umweltmedizin* **193:** 450–460.

Mahbubani, M.H., Bej, A.K., Miller, R., Haff, L., DiCesare, J., and Atlas, R.M. (1990) Detection of *Legionella* with polymerase chain reaction and gene probe methods. *Mol Cell Probes* **4:** 175–187.

Makin, T., and Hart, C.A. (1990) The efficacy of control measures for eradicating legionellae in showers. *J Hospital Infect* **16:** 1–7.

Marrie, T., Haldane, D., Bezanson, G., and Peppard, R. (1992) Each water outlet is a unique ecological niche for *Legionella pneumophila*. *Epidemiol Infect* **108:** 261–270.

Marrie, T., Green, P., Burbridge, S., Bezanson, G., Neale, S.,

Hoffman, P.S., *et al*. (1994) Legionellaceae in the potable water of Nova Scotia hospitals and Halifax residences. *Epidemiol Infect* **112:** 143–150.

Maruta, K., Miyamoto, H., Hamada, T., Ogawa, M., Taniguchi, H., and Yoshida, S. (1998a) Entry and intracellular growth of *Legionella dumoffii* in alveolar epithelial cells. *Am J Respir Crit Care Med* **157:** 1967–1974.

Maruta, K., Ogawa, M., Miyamoto, H., Izu, K., and Yoshida, S.I. (1998b) Entry and intracellular localization of *Legionella dumoffii* in Vero cells. *Microb Pathog* **24:** 65–73.

Mermel, L.A., Josephson, S.L., Giorgio, C.H., Dempsey, J., and Parenteau, S. (1995) Association of Legionnaires' disease with construction: contamination of potable water? *Infect Control Hospital Epidemiol* **16:** 76–81.

Meyer, R.D., and Finegold, S.M. (1980) Legionnaires' disease. *Annu Rev Med* **31:** 219–232.

Michel, R., and Borneff, M. (1989) The significance of amoebae and other protozoa in water conduit systems in dental units. *Zentralblatt Fur Bakteriologie, Mikrobiologie und Hygiene – Serie B, Umwelthygiene, Krankenhaushygiene, Arbeitshygiene, Praventive Medizin* **187:** 312–323.

Michel, R., Muller, K.D., Amann, R., and Schmid, E.N. (1998) *Legionella*-like slender rods multiplying within a strain of *Acanthamoeba* sp. isolated from drinking water. *Parasitol Res* **84:** 84–88.

Miller, L.A., Beebe, J.L., Butler, J.C., Martin, W., Benson, R., Hoffman, R.E., *et al*. (1993) Use of polymerase chain reaction in an epidemiologic investigation of Pontiac fever. *J Infect Dis* **168:** 769–772.

Miyamoto, H., Yamamoto, H., Arima, K., Fujii, J., Maruta, K., Izu, K., *et al*. (1997) Development of a new seminested PCR method for detection of *Legionella* species and its application to surveillance of legionellae in hospital cooling tower water. *Appl Environ Microbiol* **63:** 2489–2494.

Mody, C.H., Paine, III, R., Shahrabadi, M.S., Simon, R.H., Pearlman, E., Eisenstein, B.I., *et al*. (1993) *Legionella pneumophila* replicates within rat alveolar epithelial cells. *J Infect Dis* **167:** 1138–1145.

Moreno, C., de Blas, I., Miralles, F., Apraiz, D., and Catalan, V. (1997) A simple method for the eradication of *Legionella pneumophila* from potable water systems. *Can J Microbiol* **43:** 1189–1196.

Nagington, J., and Smith, D.J. (1980) Pontiac fever and amoebae. *Lancet* **2:** 1241.

Nahapetian, K., Challemel, O., Beurtin, D., Dubrou, S., Gounon, P., and Squinazi, F. (1991) The intracellular multiplication of *Legionella pneumophila* in protozoa from hospital plumbing systems. *Res Microbiol* **142:** 677–685.

Newsome, A.L., Baker, R.L., Miller, R.D., and Arnold, R.R. (1985) Interactions between *Naegleria fowleri* and *Legionella pneumophila*. *Infect Immun* **50:** 449–452.

Newsome, A.L., Scott, T.M., Benson, R.F., and Fields, B.S. (1998) Isolation of an amoeba naturally harboring a distinctive *Legionella* species. *Appl Environ Microbiol* **64:** 1688–1693.

Oppenheim, B.A., Sefton, A.M., Gill, O.N., Tyler, J.E., O'Mahony, M.C., Richards, J.M., *et al*. (1987) Widespread *Legionella pneumophila* contamination of dental stations in a dental school without apparent human infection. *Epidemiol Infect* **99:** 159–166.

Pabst, U., Demuth, J., Gebel, T., and Dunkelberg, H. (1997) Establishment of a method for determining the association between *Legionella* sp. and *Amoeba* sp. using polymerase chain reaction. *Zentralblatt Hyg Umweltmedizin* **199:** 568–577.

Palmer, C.J., Tsai, Y.L., Paszko-Kolva, C., Mayer, C., and Sangermano, L.R. (1993) Detection of *Legionella* species in sewage and ocean water by polymerase chain reaction, direct fluorescent- antibody, and plate culture methods. *Appl Environ Microbiol* **59:** 3618–3624.

Palmer, C.J., Bonilla, G.F., Roll, B., Paszko-Kolva, C., Sangermano, L.R., and Fujioka, R.S. (1995) Detection of *Legionella* species in reclaimed water and air with the EnviroAmp *Legionella* PCR kit and direct fluorescent antibody staining. *Appl Environ Microbiol* **61:** 407–412.

Pankhurst, C.L., Philpott-Howard, J.N., Hewitt, J.H., and Casewell, M.W. (1990) The efficacy of chlorination and filtration in the control and eradication of *Legionella* from dental chair water systems. *J Hospital Infect* **16:** 9–18.

Pankhurst, C.L., Johnson, N.W., and Woods, R.G. (1998) Microbial contamination of dental unit waterlines: the scientific argument. *Int Dental J* **48:** 359–368.

Roberts, K.P., August, C.M., and Nelson, Jr, J.D. (1987) Relative sensitivities of environmental legionellae to selective isolation procedures. *Appl Environ Microbiol* **53:** 2704–2707.

Rogers, J., Dowsett, A.B., Dennis, P.J., Lee, J.V., and Keevil, C.W. (1994) Influence of temperature and plumbing material selection on biofilm formation and growth of *Legionella pneumophila* in a model potable water system containing complex microbial flora. *Appl Environ Microbiol* **60:** 1585–1592.

Rogers, J., Dowsett, A.B., and Keevil, C.W. (1995) A paint incorporating silver to control mixed biofilms containing *Legionella pneumophila*. *J Ind Microbiol* **15:** 377–383.

Rohr, U., Senger, M., and Selenka, F. (1996) Effect of silver and copper ions on survival of *Legionella pneumophila* in tap water. *Zentralblatt Hyg Umweltmedizin* **198:** 514–521.

Rohr, U., Weber, S., Michel, R., Selenka, F., and Wilhelm, M. (1998) Comparison of free-living amoebae in hot water systems of hospitals with isolates from moist sanitary areas by identifying genera and determining temperature tolerance. *Appl Environ Microbiol* **64:** 1822–1824.

Rowbotham, T.J. (1981) Pontiac fever, amoebae, and legionellae. *Lancet* **1:** 40–41.

Rowbotham, T.J. (1983) Isolation of *Legionella pneumophila* from clinical specimens via amoebae, and the interaction of those and other isolates with amoebae. *J Clin Pathol* **36:** 978–986.

Rowbotham, T.J. (1986) Current views on the relationships between amoebae, legionellae and man. *Isr J Med Sci* **22:** 678–689.

Roy, C.R., Berger, K.H., and Isberg, R.R. (1998) *Legionella pneumophila* DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. *Mol Microbiol* **28:** 663–674.

Rusin, P.A., Rose, J.B., Haas, C.N., and Gerba, C.P. (1997) Risk assessment of opportunistic bacterial pathogens in drinking water. *Rev Environ Contamination Toxicol* **152:** 57–83.

Russell, D.G. (1998) What does 'inhibition of phagosome–lysosome fusion' really mean? *Trends Microbiol* **6:** 212–214.

Saint, C.P. (1998) A colony based confirmation assay for *Legionella* and *Legionella pneumophila* employing the EnviroAmp *Legionella* system and seroagglutination. *Lett Appl Microbiol* **26:** 377–381.

Sanden, G.N., Morrill, W.E., Fields, B.S., Breiman, R.F., and Barbaree, J.M. (1992) Incubation of water samples containing amoebae improves detection of legionellae by the culture method. *Appl Environ Microbiol* **58:** 2001–2004.

Schlenk, R., Wildfeuer, A., and Haferkamp, O. (1993) Identification of *Legionella pneumophila* in various specimens by the polymerase chain reaction. *Arzneimittelforschung* **43:** 1249–1252.

Shearer, B.G. (1996) Biofilm and the dental office. *J Am Dental Assoc* **127:** 436.

Skinner, A.R., Anand, C.M., Malic, A., and Kurtz, J.B. (1983) *Acanthamoebae* and environmental spread of *Legionella pneumophila*. *Lancet* **2:** 289–290.

Smith, A.J., Hood, J., Bagg, J., and Burke, F.T. (1999) Water, water everywhere but not a drop to drink? *Br Dental J* **186:** 12–14.

Smith-Somerville, H.E., Huryn, V.B., Walker, C., and Winters, A.L. (1991) Survival of *Legionella pneumophila* in the cold-water ciliate *Tetrahymena vorax*. *Appl Environ Microbiol* **57:** 2742–2749.

Srikanth, S., and Berk, S.G. (1993) Stimulatory effect of cooling tower biocides on amoebae. *Appl Environ Microbiol* **59:** 3245–3249.

Srikanth, S., and Berk, S.G. (1994) Adaptation of amoebae to cooling tower biocides. *Microb Ecol* **27:** 293–301.

States, S.J., Conley, L.F., Ceraso, M., Stephenson, T.E., Wolford, R.S., Wadowsky, R.M., *et al*. (1985) Effects of metals on *Legionella pneumophila* growth in drinking water plumbing systems. *Appl Environ Microbiol* **50:** 1149–1154.

States, S.J., Conley, C.F., Kuchta, J.M., Wolford, R.S., Wadowsky, R.M., and Yee, R.B. (1989) Chlorine, pH, and control of *Legionella* in hospital plumbing systems. *J Am Med Assoc* **261:** 1882–1883.

States, S., Kuchta, J., Young, W., Conley, L., Ge, J., Costello, M., *et al*. (1998) Controlling *Legionella* using copper-silver ionization. *J Am Water Works Assoc* **90:** 122–129.

Steele, T.W., and McLennan, A.M. (1996) Infection of *Tetrahymena pyriformis* by *Legionella longbeachae* and other *Legionella* species found in potting mixes. *Appl Environ Microbiol* **62:** 1081–1083.

Steinert, M., Emody, L., Amann, R., and Hacker, J. (1997). Resuscitation of viable but nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*. *Appl Environ Microbiol* **63:** 2047–2053.

Steinert, M., Ockert, G., Luck, C., and Hacker, J. (1998) Regrowth of *Legionella pneumophila* in a heat-disinfected plumbing system. *Zentralblatt Bakteriologie* **288:** 331–342.

Stone, B.J., and Abu Kwaik, Y. (1998). Expression of multiple pili by *Legionella pneumophila*: identification and characterization of a type IV pilin gene and its role in adherence to mammalian and protozoan cells. *Infect Immun* **66:** 1768–1775.

Stout, J.E., and Yu, V.L. (1997) Legionellosis. *N Engl J Med* **337:** 682–687.

Stout, J.E., Yu, V.L., Muraca, P., Joly, J., Troup, N., and Tompkins, L.S. (1992a) Potable water as a cause of sporadic cases of community-acquired legionnaires' disease. *N Engl J Med* **326:** 151–155.

Stout, J.E., Yu, V.L., Yee, Y.C., Vaccarello, S., Diven, W., and Lee, T.C. (1992b) *Legionella pneumophila* in residential water supplies: Environmental surveillance with clinical assessment for Legionnaires' disease. *Epidemiol Infect* **109:** 49–57.

Stout, J.E., Lin, Y.S., Goetz, A.M., and Muder, R.R. (1998) Controlling *Legionella* in hospital water systems: experience with the superheat-and-flush method and copper-silver ionization. *Infect Control Hospital Epidemiol* **19:** 911–914.

Straus, W.L., Plouffe, J.F., File, Jr, T.M., Lipman, H.B., Hackman, B.H., Salstrom, S.J., *et al*. (1996) Risk factors for domestic acquisition of legionnaires disease. Ohio legionnaires Disease Group. *Arch Intern Med* **156:** 1685–1692.

Szenasi, Z., Endo, T., Yagita, K., and Nagy, E. (1998) Isolation, identification and increasing importance of 'free-living' amoebae causing human disease. *J Med Microbiol* **47:** 5–16.

Tyndall, R.L., and Domingue, E.L. (1982) Cocultivation of *Legionella pneumophila* and free- living amoebae. *Appl Environ Microbiol* **44:** 954–959.

Venkataraman, C., Haack, B.J., Bondada, S., and Abu Kwaik, Y. (1997). Identification of a Gal/GalNAc lectin in the protozoan *Hartmannella vermiformis* as a potential receptor for attachment and invasion by the Legionnaires' disease bacterium *Legionella pneumophila*. *J Exp Med* **186:** 537–547.

Villari, P., Motti, E., Farullo, C., and Torre, I. (1998) Comparison of conventional culture and PCR methods for the detection of *Legionella pneumophila* in water. *Lett Appl Microbiol* **27:** 106–110.

Vogel, J.P., and Isberg, R.R. (1999) Cell biology of *Legionella pneumophila*. *Curr Opin Microbiol* **2:** 30–34.

Walker, J.T., Sonesson, A., Keevil, C.W., and White, D.C. (1993) Detection of *Legionella pneumophila* in biofilms containing a complex microbial consortium by gas chromatography-mass spectrometry analysis of genus-specific hydroxy fatty acids. *FEMS Microbiol Lett* **113:** 139–144.

Williams, H.N., Paszko-Kolva, C., Shahamat, M., Palmer, C., Pettis, C., and Kelley, J. (1996a) Molecular techniques reveal high prevalence of *Legionella* in dental units. *J Am Dental Assoc* **127:** 1188–1193.

Williams, J.F., Molinari, J.A., and Andrews, N. (1996b) Microbial contamination of dental unit waterlines: origins and characteristics. *Comp Continuing Educ Dentistry* **17:** 538–540.

Wintermeyer, E., Ludwig, B., Steinert, M., Schmidt, B., Fischer, G., and Hacker, J. (1995) Influence of site specifically altered Mip proteins on intracellular survival of *Legionella pneumophila* in eukaryotic cells. *Infect Immun* **63:** 4576–4583.

Zacheus, O.M., and Martikainen, P.J. (1994) Occurrence of legionellae in hot water distribution systems of Finnish apartment buildings. *Can J Microbiol* **40:** 993–999.

Zacheus, O.M., and Martikainen, P.J. (1996) Effect of heat flushing on the concentrations of *Legionella pneumophila* and other heterotrophic microbes in hot water systems of apartment buildings. *Can J Microbiol* **42:** 811–818.