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REVIEW ARTICLE

The role of biofilms and protozoa in *Legionella* pathogenesis: implications for drinking water

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Summary

Current models to study *Legionella* pathogenesis include the use of primary macrophages and monocyte cell lines, various free-living protozoan species and murine models of pneumonia. However, there are very few studies of *Legionella* spp. pathogenesis aimed at associating the role of biofilm colonization and parasitization of biofilm microbiota and release of virulent bacterial cell/vacuoles in drinking water distribution systems. Moreover, the implications of these environmental niches for drinking water exposure to pathogenic legionellae are poorly understood. This review summarizes the known mechanisms of *Legionella* spp. proliferation within *Acanthamoeba* and mammalian cells and advocates the use of the amoeba model to study *Legionella* pathogenicity because of their close association with *Legionella* spp. in the aquatic environment. The putative role of biofilms and amoebae in the proliferation, development and dissemination of potentially pathogenic *Legionella* spp. is also discussed. Elucidating the mechanisms of *Legionella* pathogenicity development in our drinking water systems will aid in elimination strategies and procedural designs for drinking water systems and in controlling exposure to *Legionella* spp. and similar pathogens.

Introduction

Legionellosis is a bacterial infection caused by species of the genus *Legionella* and is the most common waterborne disease reported in the United States (Liang *et al.* 2006; Yoder *et al.* 2008). Surveillance data from 1990 to 2005 indicate that legionellosis cases have dramatically increased in recent years underscoring the importance of understanding this disease and its environmental sources (Neil and Berkelman 2008). Legionellosis has two clinically distinct forms: Legionnaires' disease, a severe type of infection, which includes pneumonia (Tsai *et al.* 1979) and Pontiac fever, a milder self-limiting illness (Glick *et al.* 1978). There are an estimated 8000–18 000 reported cases of legionellosis requiring hospitalization in the United States each year with a case mortality rate of about 8·8% (Marston *et al.* 1997). However, the total number of cases may actually be under-reported because of variances in diagnostic and reporting procedures. Currently, there are over 50 species with 70 distinct serogroups in the genus *Legionella*. About half of those species are associated with clinical cases, largely as opportunistic pathogens that include *Legionella pneumophila* serogroup 1, *Legionella micdadei*, *Legionella longbeachae*, *Legionella dumoffii*, and *Legionella bozemanii* (Yu *et al.* 2002; Bartram *et al.* 2007). *Legionella pneumophila* serogroup 1 is the causative agent in at least 70% of all Legionnaire's disease cases in the United States and Europe, making it the most clinically relevant and thus, well-studied species in the entire genus (Benin *et al.* 2002; Yu *et al.* 2002). *Legionella micdadei* causes fewer infections than *L. pneumophila* but it is the second most common causative agent of Legionnaire's disease in the United States (Reingold *et al.* 1984) and in the UK and Europe (Roig *et al.* 2003). In Australia, *L. longbeachae* is responsible for about half of all *Legionella* pneumonia cases and exposure is attributed to inhalation of aerosols from contaminated soil and wood wastes (Doyle and Heuzenroeder 2002).

The pathology of legionellosis is very similar for many *Legionella* spp. including heavy inflammatory infiltrate

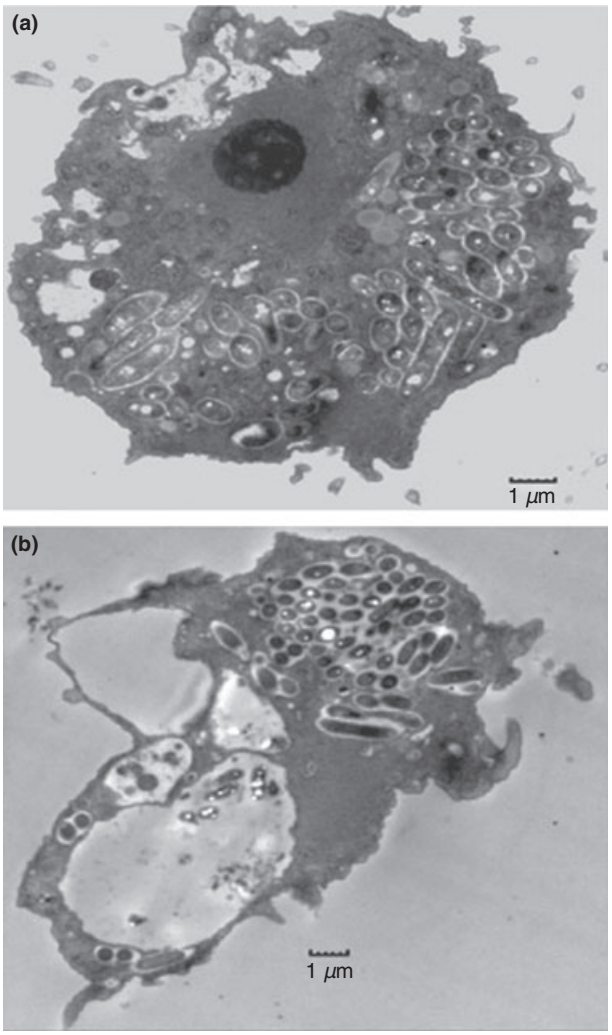

Figure 1 *Acanthamoeba polyphaga* trophozites infected with *Legionella pneumophila*. The intracellular multiplication of *L. pneumophila* strain AA100 within *A. polyphaga* was examined by electron microscopy. Infected trophozites after 18 h (a) and 48 h (b) are shown. This figure was originally published in Garcia *et al.* (2007) and shown here with permission. Bar = 1 μm.

consisting of neutrophils and macrophages, necrosis, abscess formation and inflammation of small blood vessels (Winn and Myerowitz 1981). Exposure to *Legionella* spp. occurs via the inhalation of contaminated aerosols from devices such as cooling towers, showers and faucets and aspiration of contaminated water (Bornstein *et al.* 1986; Breiman *et al.* 1990a, 1990b). Several studies have shown that *L. pneumophila* serogroup 1 can multiply within various species of free-living protozoa isolated from those same sources suspected in disease transmission (Barbaree *et al.* 1986; Fields *et al.* 1989; Breiman *et al.* 1990b). Figure 1 illustrates the robust intracellular multiplication (Icm) of *L. pneumophila* within *Acanthamoeba polyphaga* cells at early and late time points following infection. Therefore, free-living protozoa may serve as a critical source for the dissemination of *Legionella* spp. causing legionellosis by providing an intracellular environment for multiplication of pathogenic strains. Further, there may be an obligate need for intracellular growth within protozoa to maintain/select for virulent strains, rather than free-living growth of nonpathogenic legionellae within biofilm.

Legionellosis is a significant health concern because of its morbidity and mortality rates, as first reported at the conference of Legionnaires in 1978 (Brenner *et al.* 1979). While a disease now well understood from exposure to cooling tower aerosols, legionellosis is increasingly being identified from direct exposures to drinking water, particularly within health-care settings (Liang *et al.* 2006; Yoder *et al.* 2008). Therefore, understanding how *Legionella* propagates and persists in man-made water systems, and the role of amoebae in that process, is seen as critical to the development of more effective control strategies for pathogenic legionellae in drinking water. As several other bacterial pathogens, i.e. *Mycobacterium* and *Helicobacter* spp. as well as various *Chlamydiae* (Thomas *et al.* 2006), are engaged in intracellular lifestyles, improving our knowledge of *Legionella*–amoeba interactions is also relevant to potentially reducing exposure to a wide diversity of human pathogens via exposures to drinking water (Loret *et al.* 2008).

The role of amoebae in *Legionella* pathogenicity

Legionella spp. are Gram-negative, facultative, intracellular bacteria that are ubiquitous in freshwater (Fliermans *et al.* 1981) and man-made water systems (Wadowsky *et al.* 1982; Colbourne and Dennis 1985). Because of the selective pressure on bacteria to thrive in these variable and low-nutrient environments, they have developed mechanisms to acquire nutrients by residing in relatively nutrient-rich biofilms (Singh and Coogan 2005; Declerck *et al.* 2007b, 2007c). However, in these environments, *Legionella* spp. are subjected to protozoan predation and therefore, have countered this act by developing means of parasitizing and residing within at least 20 species of amoebae, two species of ciliated protozoa and one species of slime mould (Table 1).

Rowbotham (1980) was the first to report the growth of *L. pneumophila* within *Acanthamoeba* and *Naegleria* as a method to enrich for environmental strains of *L. pneumophila*. Interestingly, others have shown that growth of *L. pneumophila* in potable water occurs only in the presence of amoebae (Wadowsky *et al.* 1988) and *L. pneumophila* may remain culturable for up to 6 months in a medium containing *Acanthamoeba castellanii* (Bouyer *et al.* 2007), whereas free-living *Legionella* within biofilms

Table 1 Protozoan species found to harbour intracellular *Legionella* spp.

Type	References
Amoeba	
Acanthamoeba castellani	Rowbotham (1980)
Acanthamoeba culbertsoni	Fields *et al.* (1989)
Acanthamoeba hatchetti	Breiman *et al.* (1990b)
Acanthamoeba polyphaga	Rowbotham (1980, 1986)
Acanthamoeba palestinensis	Rowbotham (1986)
Acanthamoeba royreba	Tyndall and Domingue (1982)
Amoeba proteus strain x D	Park *et al*. (2004)
Comandonia operculata	Breiman *et al.* (1990b)
Echinamoeba exudans	Fields *et al.* (1989)
Filamoeba nolandi	Breiman *et al.* (1990b)
Hartmannella spp.	Fields *et al.* (1989)
Hartmannella cantabrigiensis	Rowbotham (1986); Breiman *et al.* (1990b)
Hartmannella vermiformis	Rowbotham (1986); Fields *et al.* (1989); Breiman *et al.* (1990b)
Naegleri fowleri	Newsome *et al.* (1985)
Naegleri gruberi	Rowbotham (1980)
Naegleri jadini	Rowbotham (1980)
Naegleri lovaniensis	Tyndall and Domingue (1982)
Paratetramitus jugosis	Breiman *et al.* (1990b)
Vahlkampfia spp.	Breiman *et al.* (1990b)
Vahlkampfia jugosa	Rowbotham (1986)
Vahlkampfia ustiana	Breiman *et al.* (1990b)
Ciliate	
Tetrahymena pyriformis	Fields *et al.* (1984)
Tetrahymena thermophila	Kikuhara *et al.* (1994)
Slime Mould	
Dictyostelium discoideum	Hagele *et al.* (2000)

may be inactivated within a few weeks (Murga *et al.* 2001; Declerck *et al.* 2007b). Furthermore, active but nonculturable *Legionella* species have only been propagated or 'resuscitated' culture via co-culture with *A. polyphaga* and *A. castellanii* (Hay *et al.* 1995; Steinert *et al.* 1997; Ohno *et al.* 2003; Seno *et al.* 2006; Garcia *et al.* 2007). Two new species of *Legionella* have been characterized as strictly obligate intracellular pathogens of protozoa and thus cannot be cultivated or axenically grown in cell-free media. *Legionella drancourtii* has only been reported to replicate within *A. polyphaga* and human lung tissues (La Scola *et al.* 2004) and *Legionella jeonii* appears to be an obligate intracellular symbiont of *Amoeba proteus* strain xD (Park *et al.* 2004). However, in the aforementioned studies, the absence of growth or persistence in other amoeba species was not investigated, leaving open the question of whether *Acanthamoeba* spp. are the preferred host for human infectious *Legionella* spp. What is clear is that *A. castellanii* and *A. polyphaga* provide an intracellular niche in which environmental strains of *Legionella* can proliferate.

In addition to the intracellular niche provided by *Acanthamoeba* species, intra-amoeba grown *Legionella* spp. have distinct properties different from their broth grown counterparts. Barker *et al.* (1993) reported the presence of *A. polyphaga* antigens, a 15-kDa outer membrane protein and mono-unsaturated straight-chain fatty acids, coating the entire *L. pneumophila* cell only after intra-amoeba passage. Mixing *L. pneumophila* cells with amoeba lysate did not have the same effect. The same group also showed that two biocides, polyhexamethylene biguanide and benzisothiazolone, both of which compromise the integrity of the bacterial cell membrane, were not as effective against *A. polyphaga*-grown *L. pneumophila* cells compared with pure cultures (Barker *et al.* 1992). This suggests that amoebal proteins coating legionellae, after intra-amoeba growth, confer biocide resistance.

Planktonic-grown *L. pneumophila* re-suspended in water are susceptible to 2 mg l^{-1} of free chlorine (sodium hypochlorite) judged by the lack of detectable viable cells after 3 min of exposure (Miyamoto *et al.* 2000). However, *L. pneumophila* contained within *A. polyphaga* cysts were shown to survive exposures of up to 50 mg l^{-1} of free chlorine for 18 h (Kilvington and Price 1990). The presence of disinfectants may therefore aid in the selection of *Legionella* strains that prefer to grow and persist within amoebae and thus have the potential to be pathogenic. However, there is also evidence that nonpathogenic *Legionella erythra* can survive within amoebae cysts and resist disinfectant exposure at a similar extent to *L. pneumophila* (Storey *et al.* 2004).

Intra-amoeba-grown *Legionella* spp. may also have significant impacts on their invasiveness and human pathogenicity. Intracellular replication of *Legionella gormanii*, *L. micdadei*, *Legionella steigerwaltii*, *L. longbeachae* and *L. dumoffii* within human monocytic leukaemia cells (MM6), was greatly enhanced after passage through *A. castellanii* (Neumeister *et al.* 2000). Furthermore, others demonstrated that intra-*A. castellanii*-grown *L. pneumophila* cells were more virulent in murine models of pneumonia and exhibited enhanced entry into several cell lines including human acute monocytic leukaemia cells (THP-1), human peripheral blood monocytes (hPBM), human epidermoid carcinoma cells (HEp-2) and mouse leukaemic monocyte macrophage cells (RAW 264.7) (Cirillo *et al.* 1994, 1999). These results support the notion that environmental factors such as growth within free-living protozoans, notably *Acanthamoeba*, can give rise to invasive microbes such as *Legionella* spp. by triggering expression of its invasive phenotype. Nagl *et al.* (2000) emphasized this by demonstrating that after multiple passages of *L. pneumophila* on agar, the ability to replicate within *A. polyphaga* was lost.

Collectively, these studies strongly suggest that free-living protozoa are natural hosts for *Legionella* spp., which not only function as environmental reservoirs but also could be involved in selecting for, protecting and maintaining potentially pathogenic *Legionella* spp. in the environment. It should be noted that free-living amoebae share many common features with mammalian phagocytes such as alveolar macrophages (see below). It has been hypothesized that the evolutionary interactions between bacteria and amoeba promote the acquisition and expression of genes that confer resistance to the bactericidal mechanisms of mammalian phagocytes. This is emphasized by the fact that other clinically relevant pathogens such as members of the genera *Vibrio*, *Mycobacterium*, *Helicobacter*, *Afipia*, *Bosea* and *Pseudomonas*, as well as mimiviruses, are associated with protozoa in the environment (reviewed in (Greub and Raoult 2004; Thomas *et al.* 2007; Corsaro *et al.* 2009)). Thus, the ability of *Legionella* spp. to survive and grow within protozoa has been implicated in the selection of virulent bacterial strains well suited for causing human disease.

Similarities and differences among the host phagocytes of *Legionella* spp.

Microbicidal mechanisms of macrophages and amoebae

There are a number of mechanisms utilized by host cells in an attempt to evade the effects of the pathogens that traffic through them. For example, professional phagocytes (specialized phagocytes that target pathogens) of the innate immune system undergo respiratory burst to rapidly release reactive oxygen species (ROS) and reactive nitrogen species (RNO) that degrade and eliminate ingested pathogens. Early studies used the reduction of nitroblue tetrazolium (NBT) to blue–black formazan as a measure of respiratory burst, which was then visualized as a dark ring around microbes within phagosomes (Schopf *et al.* 1984). Jacobs *et al.* (1984) demonstrated that phagocytosis of virulent *L. pneumophila* by primary primate alveolar macrophages results in respiratory burst as indicated by the localized reduction of NBT within the phagosome. These and other studies suggest that generation of ROS and RNO are mammalian microbicidal mechanisms that can be effective against *L. pneumophila* (Gebran *et al.* 1994), and which appears to be activated via Toll-like receptor 9 in mice (Bhan *et al.* 2008).

Acanthamoeba castellanii possesses a superoxide (O_2^-) generating respiratory burst oxidase, which is both active during phagocytosis of latex beads and heat-killed yeast and analogous to the superoxide-generating NADPH oxidase of neutrophils and macrophages (Brooks and Schneider 1985; Davies and Edwards 1991; Davies *et al.* 1991). A virulent *L. pneumophila* strain cocultured with *A. polyphaga* or human polymorphonuclear leucocytes (PMN) showed a faster reduction rate of NBT compared with an avirulent strain suggesting that respiratory burst against *L. pneumophila* in both amoebae and mammalian phagocytes is similar (Halablab *et al.* 1990). Interestingly, *L. pneumophila* induces caspase 3-dependent apoptosis in U937 macrophages (human leukaemic monocyte lymphoma cell line) but induces necrotic cell death in *A. polyphaga* cells (Gao and Abu Kwaik 1999, 2000). This difference in *Legionella*-mediated host-cell death illustrates the specialized adaptation of *Legionella* spp. to parasitize the amoebal host. However, the significance of this in regard to *Legionella* pathogenicity remains to be elucidated.

Attachment/uptake of *Legionella* by host cells

Phagocytosis by mammalian macrophages is receptor mediated and required for the induction of intracellular microbicial mechanisms, as reviewed in Linehan *et al.* (2000). The recognition and initial events in *Acanthamoeba* spp. phagocytosis are similar to those in mammalian macrophages, notably the presence of a D(+)-mannose inhibitable receptor and the presence of degradation products indicative of phosphoinositide metabolism (Brown *et al.* 1975; Lock *et al.* 1987; Allen and Dawidowicz 1990a, 1990b). Although *L. pneumophila* has a higher affinity for the α1-3-D-mannobiose binding site of the mannose receptor in *A. castellanii*, there is still a strong affinity for the D-mannose binding receptor (Cao *et al.* 1998). Interestingly, the importance of this receptor in *Legionella* uptake may not be specific for the entire *Acanthamoeba* genus and may explain why *L. micdadei* can reportedly grow within *A. castellanii* (Neumeister *et al.* 1997), but not *A. polyphaga* (Gao *et al.* 1999). The addition of the same concentration of D-mannose to the culture medium will block *A. castellanii* uptake of *L. pneumophila,* but not *A. polyphaga* (Harb *et al.* 1998; Declerck *et al.* 2007a). Furthermore, addition of cyclohexamide (protein synthesis inhibitor) and cytocholasin D (microfilament disrupter) will inhibit *A. castellanii* but not *A. polyphaga* uptake of *L. pneumophila* (Harb *et al.* 1998; Declerck *et al.* 2007a).

Uptake mechanisms via coiling phagocytosis of *L. pneumophila* by human monocytes, macrophages and PMNs and by *A. castellanii* are similar (Horwitz 1984; Bozue and Johnson 1996). Coiling phagocytosis was therefore hypothesized to play a role in virulence by facilitating the growth and evasion of intracellular degradation. However, virulent *L. pneumophila* strains and *L. micdadei* are phagocytosed by human monocytes and macrophages in a noncoiling manner suggesting that

virulence is not dependent on this unique uptake mechanism (Rechnitzer and Blom 1989). In summary, the role of these uptake mechanisms and lectin receptors in *Legionella* spp. virulence is still poorly understood.

Intracellular trafficking of *Legionella*

In both amoebae (*A. castellanii* and *A. polyphaga*) and macrophage [hPBM, U937 and primary murine bone marrow-derived monocytic cells (pMBMM)] hosts, the *L. pneumophila*-containing vacuole (LpCV) inhibits fusion of the phagosome with lysosomes and forms a ribosome-studded phagosome (Horwitz 1983; Horwitz and Maxfield 1984; Bozue and Johnson 1996; Harb *et al.* 1998). Within minutes of uptake, smooth vesicles and mitochondria are recruited to the newly formed vacuole, which is subsequently remodelled to be indistinguishable from endogenous rough-endoplasmic reticulum (RER) (Swanson and Isberg 1995; Bozue and Johnson 1996; Harb *et al.* 1998; Tilney *et al.* 2001). However, there are differences in host-cell trafficking among *L. pneumophila*, *L. micdadei* and *L. longbeachae*. For example, virulent *L. longbeachae* strains traffick into RER-associated phagosomes in MM6 cells and in the alveolar macrophages of guinea pigs, where the phagosomes contain cellular debris and appear to fuse with lysosomes (Gerhardt *et al.* 2000; Doyle *et al.* 2001). *Legionella micdadei* replicates in a ribosome-free vacuole in U937 and *Hartmannella vermiformis* cells and is significantly less cytotoxic compared with *L. pneumophila*, even though intracellular growth rates of *L. pneumophila* and *L. micdadei* in these host cells are similar (Gao *et al.* 1999). Weinbaum *et al.* (1984) also reported that *L. micdadei*-containing phagosomes do not associate with host ribosomes. Although the *L. micdadei*-containing phagosomes appear not to be surrounded by a RER-derived membrane, the phagosome has been reported to co-localize with calnexin, a resident ER marker (Gerhardt *et al.* 2000). As stated earlier, *A. castellanii,* but not *A. polyphaga* cells, can support the growth of *L. micdadei*, and *L. longbeachae* cannot grow within *A. castellanii* cells (Neumeister *et al.* 1997; Gao *et al.* 1999). Thus, there may be a correlation between the growth patterns in *Acanthamoeba* spp. and the mechanisms of mammalian intracellular trafficking for various *Legionella* spp., a possibility that warrants further investigation.

Release of *Legionella* from host cells

After intracellular replication within the LpCV, bacteria may become cytotoxic and lyse the host cell. This pore-forming activity and cytotoxicity of *L. pneumophila* were demonstrated in pMBMM from A/J mice, U937 cells and *A. polyphaga* (Byrne and Swanson 1998; Kirby *et al.* 1998; Gao and Abu Kwaik 1999). Lysis from host cells is mediated by the expression of Icm-T and is not dependent on the type II secretion system (Molmeret *et al.* 2002, 2004; Lammertyn and Anne 2004; Albert-Weissenberger *et al.* 2007). However, nonlytic release from *A. castellanii* and *A. polyphaga* has also been reported. Rowbotham (1983) first noted that after intracellular replication, the LpCV and amoeba cell will rupture to release motile bacteria; however, two to three intact LpCV can also be released from the lysed amoebae. This was later confirmed by Berk *et al.* (1998) who showed that the LpCV were: (i) expelled from *A. castellanii* and *A. polyphaga* just prior to encystment and (ii) were resistant to killing by cooling tower biocides and freeze-thawing procedures. Interestingly, LepA and LepB, which are secreted into the host cell in an Icm/Dot (defective in organelle trafficking)-dependent fashion were shown to be involved in the release of LpCV from host cells. Furthermore, LepA- and LepB-mediated release of LpCV was seen only in *A. castellanii* cells but not hPBM (Chen *et al.* 2004, 2007). These data suggest that the nonlytic release of *Legionella*-containing vesicles may be amoeba specific and could be both a source of legionellosis transmission and a way for *Legionella* to persist and survive in the environment including drinking water systems.

Legionellosis and drinking water systems

Role of biofilms

Legionella spp. are ubiquitous in nature with water being the major reservoir for these organisms. However, to date, outbreaks of legionellosis have not been associated with natural freshwater lakes. Rather, legionellosis has been associated with exposure to warm/hot drinking water systems such as cooling towers, showers and faucets in numerous independent studies (Barbaree *et al.* 1986; Bornstein *et al.* 1986; Breiman *et al.* 1990b; Azara *et al.* 2006). In these artificial water systems, microbial growth is detected almost exclusively in biofilms covering the interior of pipe walls, in-premise plumbing fixtures and heating, ventilation and air-conditioning systems. *Legionella* spp. are fastidious and therefore, have developed mechanisms to acquire nutrients by residing in these relatively nutrient-rich biofilms (Rogers and Keevil 1992; Murga *et al.* 2001; Declerck *et al.* 2007b, 2007c). Lehtola *et al.* (2007) demonstrated that pathogenic microbes such as *Mycobacterium avium*, *L. pneumophila*, *Escherichia coli* and Caliciviruses that are spiked into artificial biofilms can remain viable/infectious for several weeks under high shear and turbulent flow conditions. Furthermore, *L. pneumophila* has been shown to survive and grow on dead biofilm-associated microbial cells such as heat-killed

Pseudomonas putida, *E. coli*, *Bacillus subtilis*, *Lactobacillus plantarum*, *A. castellanii* and *Saccharomyces boulardii* (Temmerman *et al.* 2006).

Pryor *et al.* (2004) reported that *L. pneumophila* and *Legionella* spp., identified via 16S rRNA and direct culture, were present in hot water heater and shower-head biofilms during chlorine or monochloramine treatment with the latter resulting in less *Legionella* species diversity. Interestingly, in the same study, it was reported that *L. pneumophila* numbers remained unaffected by changes in disinfectant regimes. In contrast, culture-based viable *Legionella* species and serogroups differed in their distribution according to type of hot-water heater, water temperature and free chlorine (Borella *et al.* 2004). Thus drinking water systems not only serve as a reservoir for potential pathogens, but also their interactions and development in biofilm communities have the potential to select for strains that are more fit to thrive in that type of environment.

Role of free-living protozoa

Although it was previously thought that microbial formation of biofilm communities provided protection against protozoan predation, Huws *et al.* (2005) and others have demonstrated that certain protozoa, such as *A. castellanii* and the ciliate *Colpoda maupasi*, are able to graze on biofilm material and, thus, play a pivotal role in biofilm development. In the absence of protozoa, *L. pneumophila* are able to persist and remain viable for about 15 days within artificial biofilms constructed from filter-sterilized tap water or distilled water (Rogers and Keevil 1992; Murga *et al.* 2001; Declerck *et al.* 2007b). However, addition of *H. vermiformis* or *A. castellanii* in these systems is necessary for *L. pneumophila* replication (Murga *et al.* 2001; Declerck *et al.* 2007b). In two independent studies, between 30% and 40% of biofilm samples isolated from various hospital water supply sources and dental unit and taps were positive for *Acanthamoeba* spp. (Barbeau and Buhler 2001; Carlesso *et al.* 2007). Thus, because microbial and protozoan diversity within biofilms is poorly understood and characterized, it remains unclear as to which protozoan species are contributing to the maintenance and propagation of these pathogens in drinking water systems. In a 2008 study of domestic water in South Florida, 19·4% of all tap water samples were positive for *Acanthamoeba*, *Hartmannella* and *Vahlkampfia* spp. (Shoff *et al.* 2008), all of which have been shown to harbour intracellular *Legionella* spp. (Table 1). This underscores the need for further research to understand the putative link between protozoa and drinking water exposure to these opportunistic pathogens.

Future of drinking water research and concluding remarks

The recognized emergence of legionellosis in the last half of the century has occurred within an environment of increasing drinking water quality standards (USEPA 2000). As stated previously, outbreaks of legionellosis have not been associated with natural freshwater lakes but rather from exposure to warm/hot drinking and cooling tower water systems. Therefore, biofilm formation and association within engineered water systems can be interpreted as a microbial survival and selection mechanism to avoid elimination by biocides in cooling towers and

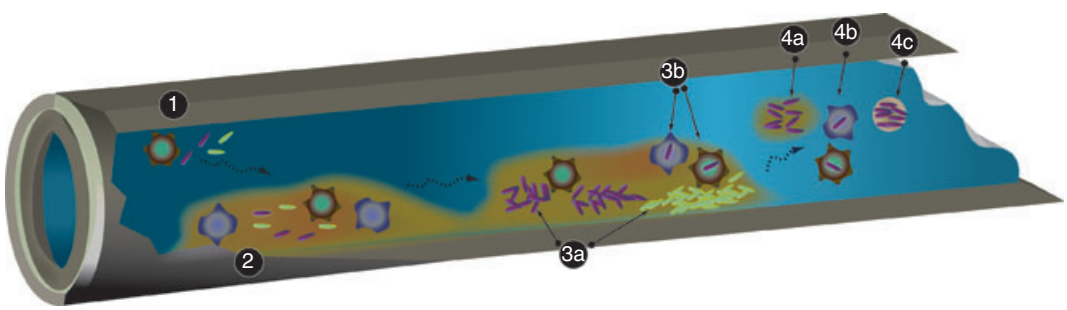

Figure 2 Development and release of *Legionella* spp. in drinking water systems. Both nonpathogenic and pathogenic *Legionella* spp. along with various protozoa species enter drinking water systems (1) and are absorbed into biofilms (2). Pathogenic *Legionella* spp. either colonize (3a) or are ingested by grazing protozoa (3b). The intracellular fate of *Legionella* spp. after ingestion can vary: they are either digested by the protozoa, the legionellae can parasitize and eventually kill the protozoan host, or the protozoa can encyst while containing intracellular legionellae. *Legionella* spp. are then released from the biofilm in a variety of ways: *Legionella* spp. that have colonized and proliferated within the biofilm can be released as this material sloughs off (4a), they can be found either within the trophozite or cyst form of certain protozoa (4b), or *Legionella* spp. can be contained within the replication vacuole (vesicle) derived from their protozoan host (4c). After initial release from the biofilm, the various forms of released *Legionella* spp. can enter into the drinking water or recolonize biofilms downstream. (, Non-Pathogenic *Legionella* spp.; , Pathogenic *Legionella* spp.; , Grazing protozoa (trophozite); , protozoa (cyst); , Biofilm material; , *Legionella* - containing vesicles expelled from protozoa).

various chlorinated disinfectants introduced into drinking water distribution systems. Furthermore, the types of disinfectant residuals in drinking water systems have been shown to directly influence both the composition of biofilm communities and the physical development of biofilms (Williams *et al.* 2005). Therefore, methods implemented to remove disease-carrying faecal microbes are potentially selecting for native biofilm microbiota that have developed mechanisms to survive, proliferate and disseminate opportunistic pathogens from the distribution system.

We propose a model in which the propagation and dissemination of pathogenic *Legionella* spp. in drinking water systems occur via their colonization and interactions with protozoa within biofilms present along surfaces in drinking water systems (Fig. 2). The high surface area and amount of water within the distribution network and in buildings only serve to concentrate potentially pathogenic microbes and increase the chances for selection and development of those specialized survival mechanisms. The ability of strains of *Legionella* spp. to persist in biofilms and replicate via parasitization of amoeba cells can be seen as a survival mechanism, but one with potentially severe consequences for human health. Currently, the concentration of *Legionella* spp. in drinking water systems and the infectious dose to humans is poorly defined (Armstrong and Haas 2008), as is the diversity and density of *Legionella* spp.-propagating protozoa (Thomas *et al.* 2008). Thus, future research should aim to understand the relationships between *Legionella* spp. and their natural host(s), such as *Acanthamoeba* spp., and the putative role the latter may play in selecting and releasing opportunistic pathogens into drinking waters. The results of this research may well change our views on the preferred disinfection strategies and procedural designs for drinking water systems in controlling exposure to *Legionella* spp. and similar pathogens.

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