Environmental Microbiology (2007) **9**(5), 1267–1277 doi:10.1111/j.1462-2920.2007.01245.x

Acanthamoeba polyphaga resuscitates viable non-culturable *Legionella pneumophila* after disinfection

María Teresa García,[1,2] Snake Jones,[1] Carmen Pelaz,[2] Richard D. Millar[1] and Yousef Abu Kwaik[1]*

[1]*Department of Microbiology and Immunology, University of Louisville, Louisville, KY, USA.*
[2]*Laboratorio de Legionella, Servicio de Bacteriología, Centro Nacional de Microbiología, ISCIII, Majadahonda, Madrid, Spain.*

Summary

Amoebae are the natural hosts for *Legionella pneumophila* and play essential roles in bacterial ecology and infectivity to humans. When *L. pneumophila* colonizes an aquatic installation, it can persist for years despite repeated treatments with disinfectants. We hypothesized that freshwater amoebae play an important role in bacterial resistance to disinfectants, and in subsequent resuscitation of viable non-culturable (VNC) *L. pneumophila* that results in re-emergence of the disease-causing strain in the disinfected water source. Our work showed that in the absence of *Acanthamoeba polyphaga*, seven *L. pneumophila* strains became non-culturable after treatment by 256 p.p.m. of sodium hypochlorite (NaOCl). In contrast, intracellular *L. pneumophila* within *A. polyphaga* was resistant to 1024 p.p.m. of NaOCl. In addition, *L. pneumophila*-infected *A. polyphaga* exhibited increased resistance to NaOCl. When chlorine-sterilized water samples were co-cultured with *A. polyphaga*, the non-culturable *L. pneumophila* were resuscitated and proliferated robustly within *A. polyphaga*. Upon treatment by NaOCl, uninfected amoebae differentiated into cysts within 48 h. In contrast, *L. pneumophila*-infected *A. polyphaga* failed to differentiate into cysts, and *L. pneumophila* was never detected in cysts of *A. polyphaga*. We conclude that amoebic trophozoites protect intracellular *L. pneumophila* from eradication by NaOCl, and play an essential role in resuscitation of VNC *L. pneumophila* in NaOCl-disinfected water sources. Intracellular *L. pneumophila* within trophozoites of *A. polyphaga* block encystation of the amoebae, and the resistance of both organisms to NaOCl is enhanced. To ensure long-term eradication and complete loss of the VNC state of *L. pneumophila*, we recommend that *Legionella*-protozoa co-culture should be an important tool to ensure complete loss of the VNC state of *L. pneumophila*.

Introduction

Legionnaires' disease is a common form of severe pneumonia typical of industrialized countries, caused by legionellae, which are intracellular Gram-negative bacteria that are ubiquitous inhabitants of aquatic environments, where protozoa are considered to be the natural primary hosts (Harb *et al*., 2000; Thomas *et al*., 2006). Fourteen species of amoebae and two species of ciliated protozoa have been identified as potential natural environmental hosts of *Legionella pneumophila* (Fields, 1996; Fields *et al*., 2002). There are at least 50 species of *Legionella* and among the *L. pneumophila* species, serogroup 1 strains are the most frequently associated with outbreaks of Legionnaires' disease and are commonly isolated from aquatic environments (Marston *et al*., 1994). The bacteria are not transmitted between individuals but are transmitted to humans by aerosols from natural and human-made aquatic environments. Upon transmission to humans, *L. pneumophila* invades alveolar macrophages, where the organisms replicate after evasion of lysosomal fusion (reviewed in Molmeret *et al*., 2005). Interestingly, *L. pneumophila* also evades lysosomal fusion within amoebae, and bacterial manipulation of the molecular and cellular bactericidal mechanisms of amoebae and mammalian macrophages is similar. Therefore, it is believed that pathogenesis of *L. pneumophila* to humans has evolved in the environment through interaction with amoebae (Steinert *et al*., 2002).

Different water sources have been associated with Legionnaires' disease (spas, evaporative condensers, respiratory therapy equipments and others), but cooling towers and water systems of large buildings (especially

Received 30 November, 2006; accepted 20 December, 2006. *For correspondence. E-mail abukwaik@louisville.edu; Tel. (+1) 502 852 4117; Fax (+1) 502 852 7531.

hotels and hospitals) are more frequently associated with outbreaks of the disease (Fiore *et al*., 1998; Leoni *et al*., 2005). In the past few years there have been numerous Legionnaires' disease outbreaks around the world, the largest of which affected 650 people in Spain in 2001 (García-Fulgueiras *et al*., 2003). The increasing number of reported Legionnaires' disease outbreaks has raised public concerns, and public health authorities have implemented guidelines of frequent cleaning and disinfection of major water sources to reduce the risk of Legionnaires' disease.

Legionnaires' disease is generally considered a preventable illness, and controlling or eliminating the bacterium from water sources is thought to be the most effective strategy to prevent the disease. However, the optimal strategy for long-term control of *L. pneumophila* in water sources is not yet defined (Hoebe and Kool, 2000). Surprisingly, complete eradication of *L. pneumophila* from water sources over a long period of time has been impossible to achieve (Kool *et al*., 1998). Although *L. pneumophila* becomes undetectable in the disinfected water source, the same strain re-emerges in the same water source within few months of disinfection, but the mechanism of re-colonization is not known. Protection of *L. pneumophila* within biofilms and within amoebae contributes to bacterial survival in disinfected water sources (Kilvington and Price, 1990; Steinert *et al*., 1997; 1998; 2002; Kim *et al*., 2002).

There are a large variety of physical, thermal and chemical methods to disinfect or control *L. pneumophila* in aquatic sources. Metal ions (copper and silver), UV light and oxidizing and non-oxidizing agents are frequently used against *L. pneumophila* (reviewed in Kim *et al*., 2002). Because chlorine is easy to use and cost-effective, hyperchlorination is the most frequently used shock-treatment to eradicate *L. pneumophila* from water sources and cooling towers (Haas and Engelbrecht, 1980). This disinfectant can be applied as chlorine gas, sodium hypochlorite (liquid), or calcium hypochlorite (solid) and has deleterious effects on bacterial membranes, respiration, transport and DNA synthesis. The disadvantages of hyperchlorination include corrosion of pipes, release of carcinogenic by-products into the drinking water and difficulty in maintaining an effective residual disinfectant level.

Similar to other treatments, hyperchlorination has been reported to be ineffective in a long-term elimination of *L. pneumophila* from aquatic environments. For instance, despite 8 months of several treatments with sodium hypochlorite, *L. pneumophila* re-emerged and persisted in a cargo ship in which a Legionnaires' disease outbreak occurred (Caylà *et al*., 2001). Similarly, 2 months after treatment with sodium hypochlorite, *L. pneumophila* re-emerged in the hot water distribution system of a hospital with a Legionnaires' disease outbreak (Borella *et al*., 2004). Rapid re-colonization of water sources by the same strain of *L. pneumophila* has been reported in other outbreaks despite shock-hyperchlorination, with new cases of Legionnaires' disease occurring after disinfection (Heimberger *et al*., 1991). It is not known why there is a re-emergence of the same disease-causing strain after its complete eradication from the same water source. Free-living protozoa, especially cysts, can protect intracellular bacteria against disinfectant (Kilvington and Price, 1990; Barker *et al*., 1992; Barker *et al*., 1995; Nwachuku and Gerpa, 2004; Storey *et al*., 2004). It is not known whether re-emergence of *L. pneumophila* in the water sources after disinfection is due to bacterial protection by the trophozoites and/or the cysts forms of amoebae. It is not known whether amoebae play a role in resuscitation of non-culturable *L. pneumophila* after disinfection of the water sources. Therefore, further studies are needed to determine the effectiveness of disinfectants, such as chlorination, on eradication of intracellular *L. pneumophila* within the trophozoite and cyst forms of amoebae. In addition, the effect of *L. pneumophila* on amoebic encystation and resistance to disinfection should be explored to design better strategies for long-term eradication of *L. pneumophila* from water systems, to prevent re-emergence of the disease-causing strains in the disinfected water source.

The goals of this study were to examine the role of *Acanthamoeba polyphaga* in possible resuscitation and re-emergence of viable non-culturable (VNC) *L. pneumophila* after disinfection. In addition, the effect of chlorination on the ability of infected trophozoites to undergo encystation was examined. Our data indicate reciprocal benefits in the interaction of *A. polyphaga* and *L. pneumophila* in the resistance of both organisms to disinfection, and a critical role for amoebae in resuscitation of VNC *L. pneumophila* after disinfection.

Results

Intracellular replication within A. polyphaga

We examined the intracellular growth kinetics of seven *L. pneumophila* strains (see *Experimental procedures*) within *A. polyphaga*. The infection was performed for 1 h using a multiplicity of infection (moi) of 10, followed by gentamicine treatment for 1 h to kill extracellular bacteria, as described in the *Experimental procedures*. At different time points post infection, the number of bacteria was determined after growth on agar plates.

All the strains exhibited intracellular proliferation within *A. polyphaga* (Fig. 1). Intracellular multiplication of the clinical strain (Cl) and the related environmental strain (EN01) was similar. The environmental strain related to

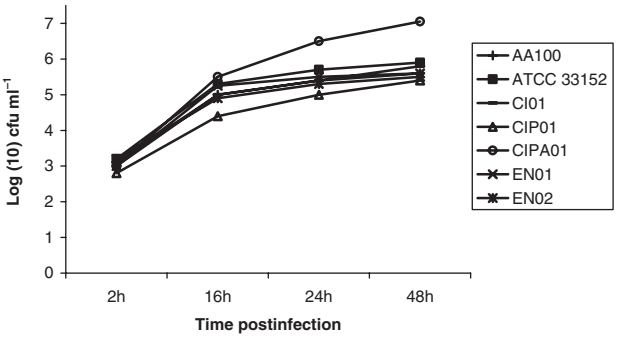

Fig. 1. Growth kinetics of *L. pneumophila* clinical and environmental isolates within *A. polyphaga*. The cells were infected with different strains of *L. pneumophila* at an moi of 10 for 1 h, followed by gentamicine treatment for 1 h followed by removal of the antibiotic. The results are representatives of two independent experiments.

clinical cases (EN01) showed similar intracellular proliferation to the environmental strain not associated with cases (EN02). Therefore, the origin of the strain or its association with clinical cases had no detectable effect on intracellular proliferation within *A. polyphaga*. Moreover, the CIP01 strain that was obtained after 15 passages of the CI strain on agar plates exhibited the lowest intracellular growth, indicating reduced infectivity with passage. On the other hand, the CIPA01 strain, which was derived from the CIP01 strain after five passages within *A. polyphaga*, showed the highest growth within amoebae among the seven strains. These results indicate that proliferation within amoebae enhances bacterial infectivity or selects for clones that are better adapted to proliferate within *A. polyphaga*.

Effect of sodium hypochlorite on L. pneumophila

Chlorine is a frequently used disinfectant for eradication of *L. pneumophila* from water installations. To test susceptibility of the seven *L. pneumophila* strains to sodium hypochlorite, we determined the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) using series of twofold dilutions of disinfectant against 5×10^6 cfu ml^{-1} of each strain. The MIC value, the lowest concentration of sodium hypochlorite (NaOCl) without visible growth, was the same for all the strains and corresponded to 512 p.p.m. The MBC value, the lowest concentration of disinfectant where viable counts were lower than 0.1% compared with untreated samples, was 1024 p.p.m. Therefore, inhibitory and bactericidal activity of chlorine was equivalent for all the strains.

The effect of different concentrations of NaOCl (0–1024 p.p.m.) on the growth kinetics of 5×10^6 cfu per well was determined for each strain (Fig. 2). After treatment with NaOCl, the cultures were incubated at 37°C for 0, 14, 22, or 46 h, and the number of bacteria was determined by colony enumeration after growth on agar plates. Reduced or no growth was exhibited by the CIP01, CIPA01 and EN01 strains treated with 64 p.p.m. of NaOCl and by the rest of the strains treated with 128 p.p.m. At 22 h after treatment, no colonies were detected for the EN02 strain treated with 128 p.p.m. or higher. For the rest of the strains, 256 p.p.m. or higher of NaOCl was essential for sterilization. Therefore, a concentration of 256 p.p.m. was sufficient to reduce viability of *L. pneumophila* to levels undetectable by culture.

Legionella pneumophila that became non-culturable after incubation for 125 days in water was resuscitated upon co-culture with amoebae (Steinert *et al.*, 1997). Therefore, we determined whether *L. pneumophila* that became non-culturable after treatment with more than 128 p.p.m. of NaOCl could be resuscitated upon co-culture with *A. polyphaga*. Aliquots of non-culturable bacteria in NaOCl-treated samples were obtained at 22 or 46 h after treatment and used for co-culture with *A. polyphaga*. Although there was no detectable growth of the EN02 strain after treatment with 128 p.p.m. of NaOCl (Fig. 2), the bacteria were resuscitated when co-cultured with *A. polyphaga* for 24 h. Similarly, in the absence of *A. polyphaga* the rest of the strains did not grow after 22 or 46 h of treatment with 256 p.p.m. of NaOCl (Fig. 2), but when co-cultured with *A. polyphaga* for 48–72 h, there was resuscitation of the strains (Table 1). Even some of the strains treated with high concentrations of NaOCl (1024 p.p.m.) that rendered them non-culturable, such as the CIP01 strain, were resuscitated when they were co-cultured with *A. polyphaga*. Only the CIPA01 strain was not resuscitated when co-cultured with *A. polyphaga* after treatment with up to 256 p.p.m. of NaOCl. We conclude that *A. polyphaga* plays a crucial role in resuscitation of VNC *L. pneumophila* after disinfection by high concentrations of chlorine.

Effect of sodium hypochlorite on survival of A. polyphaga

Because intracellular *L. pneumophila* within *A. polyphaga* were more resistant to, and were protected from chlorine, we examined the effect of NaOCl on the viability of *A. polyphaga*. Monolayers of uninfected *A. polyphaga* or infected by *L. pneumophila* (moi 10), were treated with different concentrations of NaOCl (0–1024 p.p.m.). Survival of *A. polyphaga* was determined by microscopy using trypan blue exclusion at 22 and 46 h after treatment. Results at 22 h (data not shown) and 46 h (Fig. 3) were very similar. Concentrations below or equal to 128 p.p.m. of NaOCl had no effect on uninfected or infected *A. polyphaga*. Treatments with 256 p.p.m. of NaOCl reduced viability of uninfected *A. polyphaga* by $> 80\%$. Interestingly, 256 p.p.m. of NaOCl had significantly less

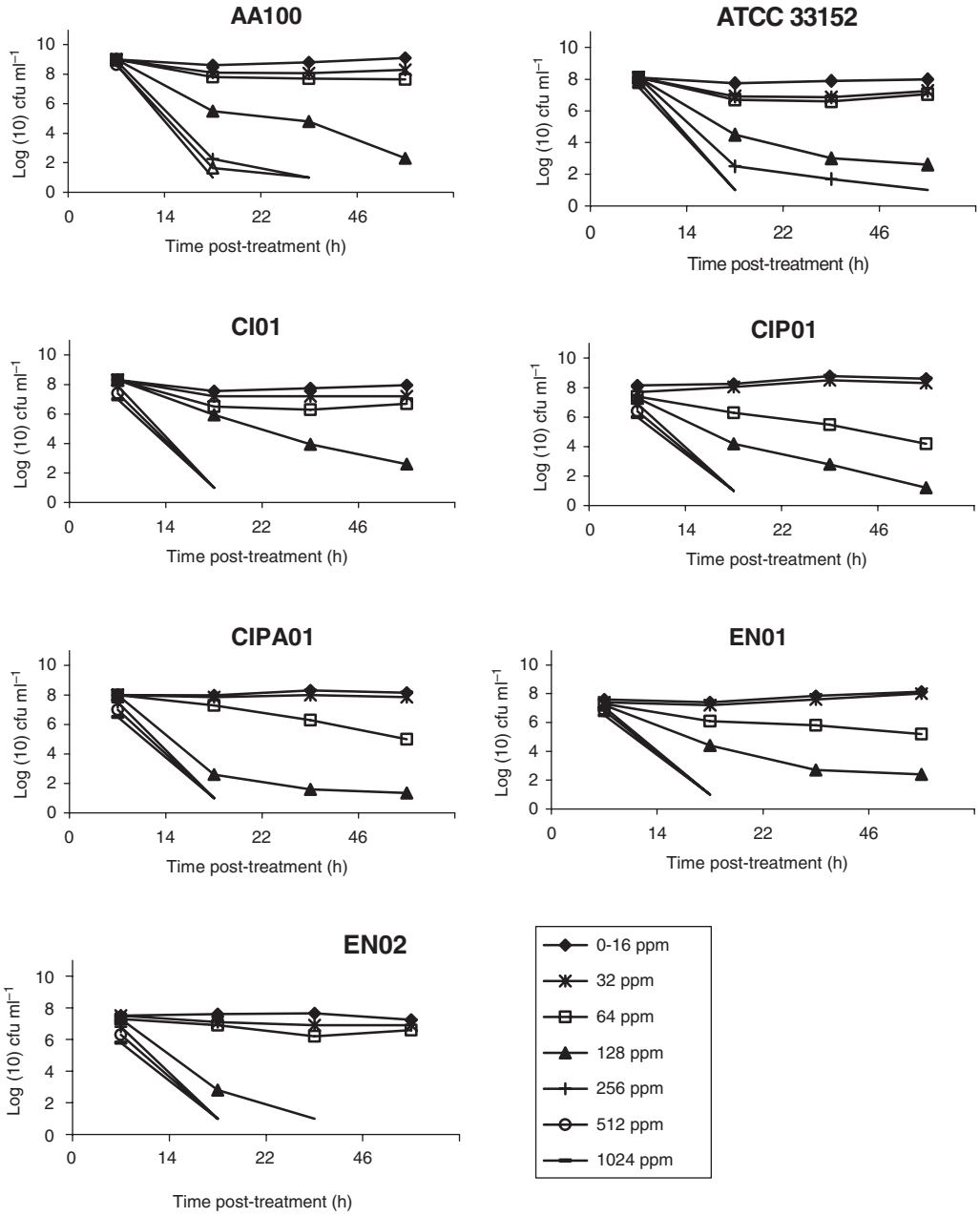

Fig. 2. Growth kinetics of seven *L. pneumophila* strains treated with NaOCl in the absence of *A. polyphaga*. In this study 5×10^6 cfu ml^{-1} of bacteria were incubated for 2 h at 37°C, and cultures were treated with different concentrations of NaOCl. Number of bacteria was determined 0, 14, 22 and 46 h after treatment with NaOCl.

effect on viability of *A. polyphaga* infected with the various strains of *L. pneumophila*, where viability of amoebae was reduced only by 7–40%, and at least 512 p.p.m. of disinfectant was necessary to kill 50% of infected *A. polyphaga*. Therefore, *A. polyphaga* infected by *L. pneumophila* are more resistant to NaOCl. Taken together, our results demonstrate a reciprocal protection of *A. polyphaga* and intracellular *L. pneumophila* from NaOCl.

Effect of NaOCl on L. pneumophila *within* A. polyphaga

We examined whether NaOCl had an effect on intracellular growth kinetics of the seven *L. pneumophila* strains within *A. polyphaga*. Monolayers of *A. polyphaga* were infected for 1 h at an moi of 10 followed by treatment with gentamicine for 1 h to kill extracellular bacteria. Gentamicine has no effect on intracellular organisms, because the antibiotic does not penetrate *A. polyphaga*

Table 1. Resuscitation of NaOCl-sterilized *L. pneumophila* after co-culture with *A. polyphaga*.

Treatment dose	Treatment time	AA100			ATCC 33152			CI01			CIP01			CIPA01			EN01			EN02		
		24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
128 p.p.m.	22 h	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	2	3	4.9
	46 h	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	3	2	4.2
256 p.p.m.	22 h	–	1.3	3.7	*	*	*	2.6	–	–	–	1.5	–	–	–	–	–	2.5	3.9	–	–	–
	46 h	–	1.6	–	–	8	9	2.9	–	–	2.6	2	–	–	–	–	–	–	–	1.3	–	–
512 p.p.m.	22 h	–	1.2	3.2	–	–	4	2.3	–	–	2	–	–	–	–	–	–	–	–	–	–	–
	46 h	–	1.3	–	–	7	8.1	–	–	–	2.3	–	–	–	–	–	–	–	–	–	–	–
1024 p.p.m.	22 h	–	–	–	–	–	1.8	–	–	–	1.6	–	–	–	–	–	–	–	–	–	–	–
	46 h	–	–	–	–	6	6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

–, no growth.
The asterisk (*) indicates bacterial growth was detected after chlorine treatment without co-culture with amoebae.
Bacterial strains were pretreated with serial dilutions of NaOCl for 14, 22 or 46 h (Fig. 2). The treated cultures for each strain that did not show any growth on agar plates at 22 or 46 h (Fig. 2) were co-cultured with *A. polyphaga* to examine whether they can be resuscitated. In this study 30 µl aliquots were co-cultured with 200 µl of fresh axenic *A. polyphaga* for 24, 48 and 72 h, and number of total bacteria (Log_{10}) in co-cultures was determined after growth on agar plates.

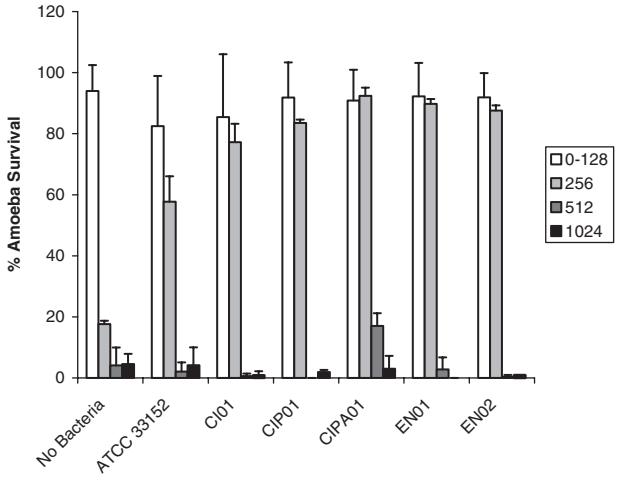

Fig. 3. Survival of *A. polyphaga* after 46 h of treatment by NaOCl. *Acanthamoeba polyphaga* was infected with different strains of *L. pneumophila* at an moi of 10 for 1 h, followed by gentamicine treatment. *Acanthamoeba polyphaga* were treated with different concentrations of NaOCl. Per cent viability of treated and untreated *A. polyphaga* with or without infection was determined at 46 h. The results are representatives of two independent experiments, and error bars represent standard deviation.

(Gao *et al*., 1997). Infected monolayers were treated for 0, 14, 22 or 46 h with different concentrations of NaOCl (0–1024 p.p.m.)and the number of bacteria was determined by colony enumeration after growth on agar plates (Fig. 4). In presence of *A. polyphaga*, the number of viable bacteria decreased only at higher concentrations (256–1024 p.p.m.) of NaOCl, compared with cultures without amoebae (Fig. 2). We conclude that NaOCl is less effective against intracellular *L. pneumophila* within *A. polyphaga*.

Effect of infection and sodium hypochlorite on encystation of A. polyphaga

Free-living amoebae have at least two developmental stages, trophozoite and cyst. Therefore, we examined the differentiation of uninfected and *L. pneumophila*-infected monolayers of *A. polyphaga* in presence or absence of 128 p.p.m. of NaOCl. The data showed that only 1% of control uninfected and untreated *A. polyphaga* developed cysts after 48 h of incubation. In samples treated with 128 p.p.m. of NaOCl, 1% of uninfected control cells differentiated into cysts after 18 or 24 h of incubation but 35% encysted after 48 h. Interestingly, all the infected *A. polyphaga* were in the trophozoite form and no infected cysts were ever detected up to 96 h post infection (Fig. 5A and B). We conclude that NaOCl induces encystment of uninfected trophozoites of *A. polyphaga*. Importantly, *L. pneumophila*-infected *A. polyphaga* fail to differentiate into the cyst form upon treatment with NaOCl.

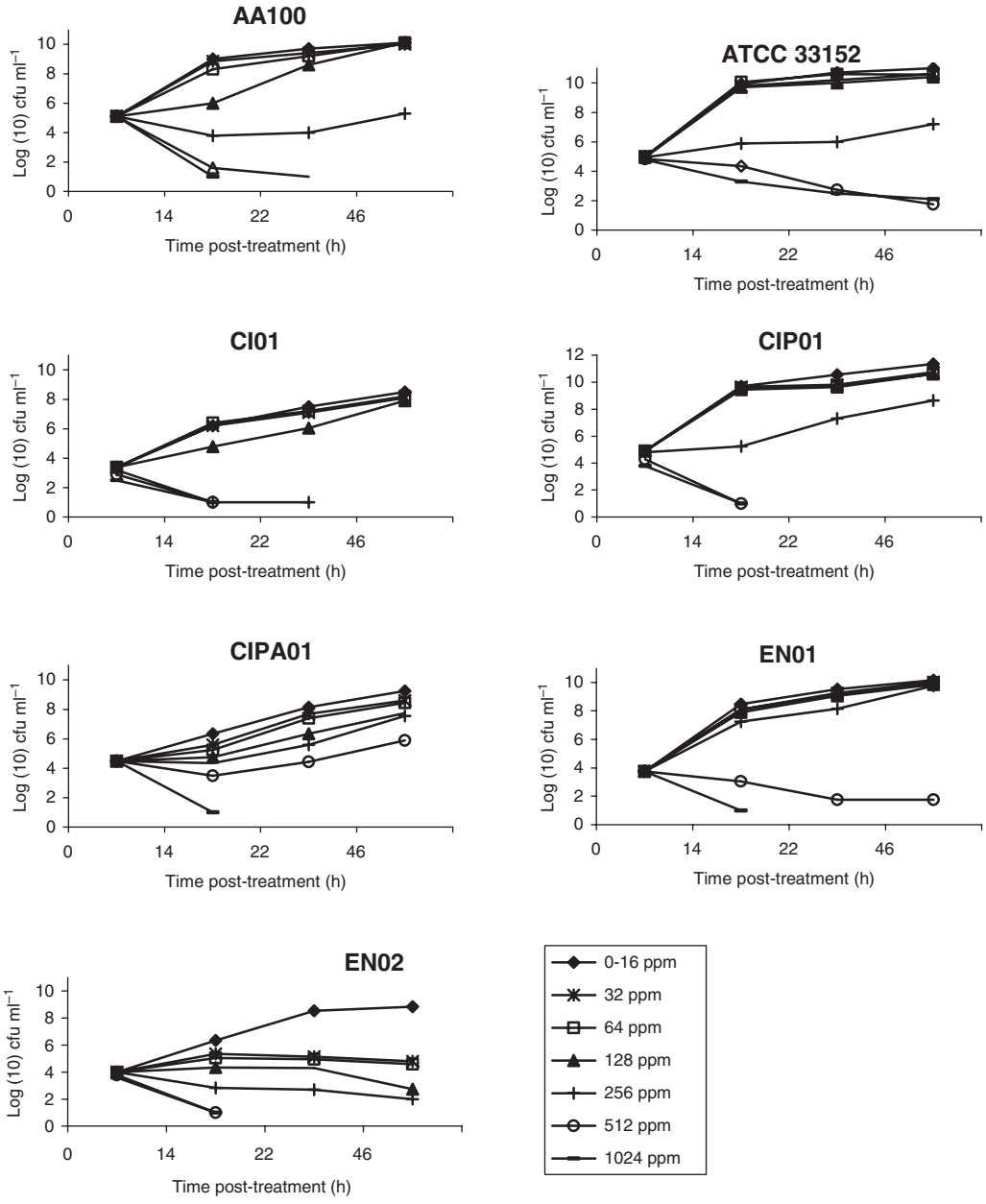

Fig. 4. Growth kinetics of seven *L. pneumophila* strains infecting *A. polyphaga* and treated with NaOCl. In this study 5×10^6 cfu ml^{-1} of bacteria were used to infect *A. polyphaga* at an moi of 10 for 1 h, followed by gentamicin treatment for 1 h and then, cultures were treated with different concentrations of NaOCl. Number of bacteria was determined 0, 14, 22 and 46 h after treatment.

Effect of sodium hypochlorite on secondary infection of A. polyphaga *by* L. pneumophila

During the infection of *A. polyphaga* by *L. pneumophila*, lysis of infected cells is initiated by ~18 h and the released bacteria initiate secondary infections of the cells that have not been infected during the original inoculation (Molmeret *et al.*, 2004). We examined by electron microscopy the effect of 128 p.p.m. of NaOCl on the secondary infection of *A. polyphaga* by released intracellular bacteria (Figs 5 and 6). The data showed that after 2 h of infection, 27% of trophozoites were infected. In untreated cultures, 47%, 68% and 41% of trophozoites were infected at 18, 24 and 48 h respectively (Fig. 6). Because the gentamicine treatment removed extracellular bacteria from the cultures at the beginning of the infection, the increase in the number of infected trophozoites by 24 h post infection demonstrated a secondary infection of cells that were not infected by the original inoculation. In monolayers treated with 128 p.p.m. of NaOCl, 50%, 63% and 41% of

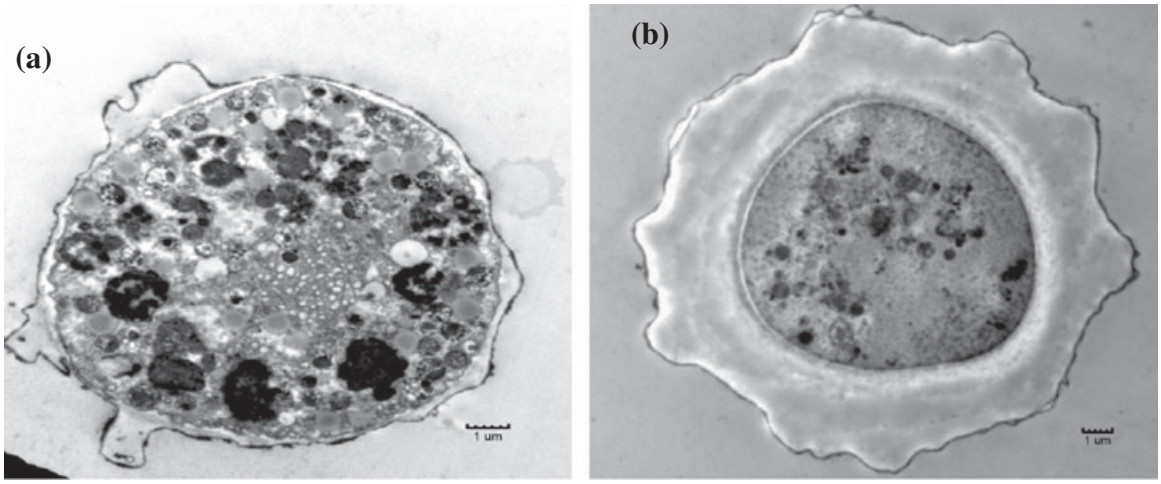

Fig. 5. Representative electron micrographs of *A. polyphaga* cells infected with *L. pneumophila* for 1 h, followed by another hour of gentamicine treatment. Uninfected immature (A) and mature (B) cysts after 48 h of infection are shown.

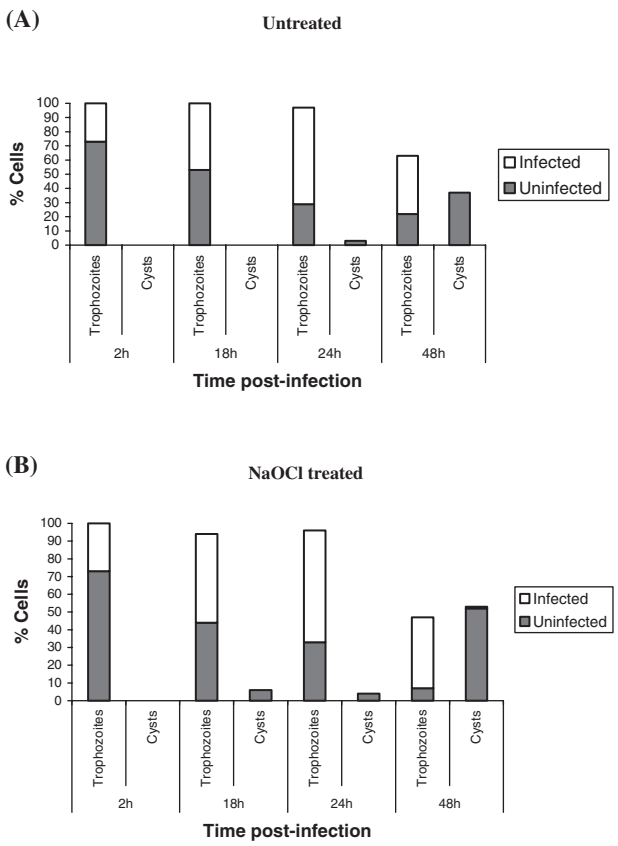

Fig. 6. Re-infection of *A. polyphaga* with *L. pneumophila* in the absence (A) or presence (B) of 128 p.p.m. of NaOCl. *Acanthamoeba polyphaga* was infected with *L. pneumophila* AA100 at an moi of 10 for 1 h, followed by another hour of gentamicine treatment. Per cent of infected and uninfected cysts and trophozoites was determined by electron microscopy by analysis of 100 cells.

trophozoites were infected at 18, 24 and 48 h respectively (Fig. 6). Neither in the absence nor presence of chlorine did encysted *A. polyphaga* harbour intracellular bacteria. We conclude that NaOCl does not affect secondary infection of *A. polyphaga* by *L. pneumophila* released from the primary infected cells.

Effect of sodium hypochlorite on intracellular multiplication

We examined by electron microscopy the intracellular multiplication of *L. pneumophila* strain AA100 within *A. polyphaga* at 2, 18, 24 and 48 h post infection. To exclude secondary infection from our analyses, we excluded infected cells with fewer than 10 or 30 intracellular bacteria at 18 and 24 h respectively (Molmeret *et al.*, 2004). In untreated monolayers, the average number of bacteria per trophozoite at 2, 18 and 24 h post infection was 1, 21 and 83 organisms respectively. In monolayers treated with 128 p.p.m. of NaOCl, the average number of intracellular bacteria at 2, 18 and 24 h was 1, 39 and 56 organisms per trophozoite respectively. Examples of infected trophozoites at 18 and 48 h post infection are shown in Fig. 7A and B. We conclude that *L. pneumophila* multiplies robustly within *A. polyphaga* trophozoites despite treatment with 128 p.p.m. of NaOCl.

Discussion

Frequent hyperchlorination of water sources and installations have failed to eliminate *L. pneumophila* permanently from reservoirs associated with Legionnaires' disease outbreaks (Kilvington and Price, 1990; Borella *et al.*, 2000; Caylà *et al.*, 2001; Perola *et al.*, 2002; Storey *et al.*,

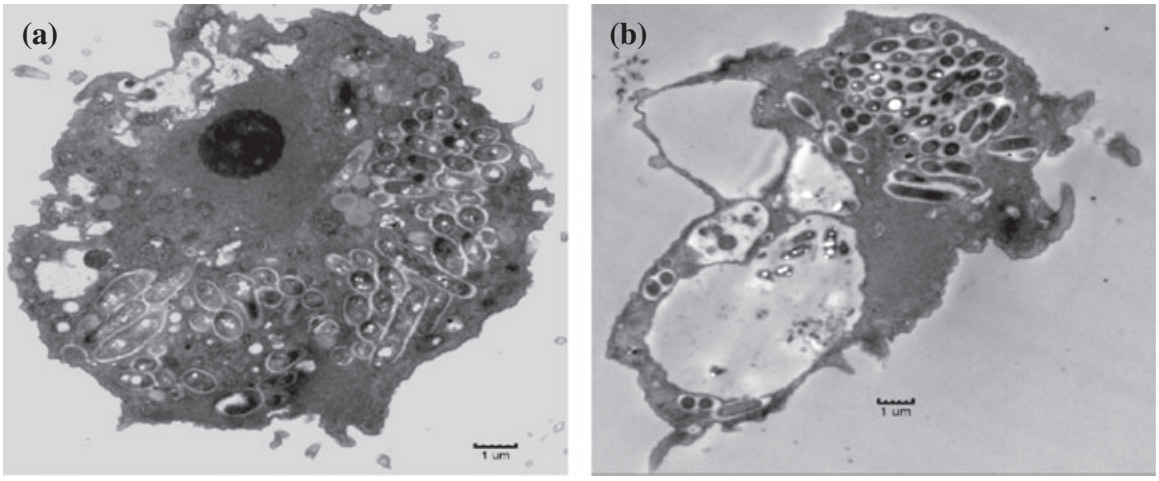

Fig. 7. Representative electron micrographs of *A. polyphaga* cells infected with *L. pneumophila* for 1 h, followed by another hour of gentamicine treatment. Infected trophozoites after 18 h (A) or 48 h (B) of infection are shown.

2004). Intracellular presence of *L. pneumophila* within protozoa protects the bacteria not only from microbial competition and predation, but also from stress stimuli and disinfection (Abu Kwaik *et al*., 1997; Fields *et al*., 2002; Molmeret *et al*., 2005). For instance, it has been demonstrated that *L. pneumophila* can survive within amoebae such as *Acanthamoeba* spp. or *Vahlkampfia* spp. at temperatures up to 70°C in a plumbing system (Steinert *et al*., 1998). Our data show the necessity of higher concentrations of NaOCl for eradication of intracellular *L. pneumophila* within *A. polyphaga.* The effective concentration of disinfectant to kill intracellular bacteria in our study was higher than other studies, which may be due to strain differences or to variations in experimental conditions used in the other studies.

Differentiation into cysts ensures the persistence of protozoa in the environment during adverse conditions. In the natural environment, the viability of protozoa such as *Acanthamoeba* cysts may be as long as 25 years (Mazur *et al*., 1995). It is possible that the cysts are responsible for the protection of intracellular *L. pneumophila* against disinfectants. However, our results show that intracellular *L. pneumophila* are not detectable within cysts of infected monolayers of *A. polyphaga*, suggesting that the trophozoite is the form that protects intracellular *L. pneumophila* against NaOCl. Other studies have been also unable to identify *L. pneumophila* within cysts of *Hartmanella vermiformis* (Greub and Raoult, 2003a). In addition, *L. pneumophila* has been reported within vacuoles of amoebae undergoing encystation (Anand *et al*., 1983) but not within mature cysts (Rowbotham, 1986; Kilvington and Price, 1990). However, *L. pneumophila* within the vacuole or within the two layers of the cyst wall of *A. polyphaga* have been observed (Greub and Raoult, 2003a). Mature cysts are refractory to infection, and encystment is probably a mechanism by which amoebae evade infection by *L. pneumophila* (Rowbotham, 1983). Thus, encystment protects *A. polyphaga* against disinfectants and against infection by *L. pneumophila* (Rowbotham, 1983; 1986; Kilvington and Price, 1990; Mazur *et al*., 1995). Our data indicate reciprocal benefits in the interaction of *A. polyphaga* and *L. pneumophila* in the resistance of both organisms to disinfection.

Four protozoan species isolated from a cooling tower have been found to be resistant to disinfectants (Sutherland and Berk, 1996). Low activity of two biocides against cysts and trophozoites of *A. polyphaga* has been also reported (Greub and Raoult, 2003b). Our results show that resistance of *A. polyphaga* to chlorine is enhanced significantly when the amoebae harbour intracellular *L. pneumophila*. In addition, NaOCl induces the encystment of uninfected but not infected trophozoites. Taken together, it is likely that in order to prevent Legionnaires' disease, treatments of aquatic installations must apply disinfectants at concentrations effective not only against *L. pneumophila* but also against protozoa. Because the interaction of *A. polyphaga* with *L. pneumophila* enhances the resistance of both organisms to disinfection, it is necessary to develop standard tests to assess the effectiveness of disinfectants against *L. pneumophila*-infected protozoa in aquatic environments.

Legionella pneumophila that become non-culturable after 125 days of incubation in water is resuscitated upon co-culture with amoebae (Steinert *et al*., 1997). It is not known whether *L. pneumophila* that are rendered non-culturable after disinfection can be resuscitated upon co-culture with amoebae. Our data show that although *L. pneumophila* become non-culturable after chlorine treatment, co-culture with *A. polyphaga* resuscitates the VNC bacteria, with subsequent robust intracellular prolif-

eration within *A. polyphaga*. Interestingly, co-cultures of *L. pneumophila* with protozoa have been used to isolate *Legionella*-like amoebal pathogens (LLAPs), which are *Legionella*-related bacterial agents of pneumonia (Adeleke *et al*., 1996; Marrie *et al*., 2001) that cannot be grown on routine bacteriological media used to grow *L. pneumophila* (Rowbotham *et al*., 1993). Amoebae co-culture is an effective tool for the recovery of fastidious intracellular bacteria such as the LLAPs (Rowbotham, 1998) and other potential emerging bacterial pathogens (Greub and Raoult, 2004). Because *L. pneumophila* that become non-culturable after disinfection, even at 1024 p.p.m. of NaOCl, can be resuscitated upon co-culture with amoebae, repeated water treatments with disinfectant are not sufficient for long-term eradication of *L. pneumophila* from the aquatic systems. To ensure long-term eradication and complete loss of the VNC state of *L. pneumophila*, we recommend that *Legionella*-protozoa co-culture should be an important tool to ensure complete loss of the VNC state of *L. pneumophila*.

We conclude that because of the reciprocal protection of *L. pneumophila* and amoebae against disinfectants, protozoa should be monitored during environmental investigations of Legionnaires' disease outbreaks, and disinfectants should be effective against both *L. pneumophila* and infected protozoa. In addition, loss of bacterial viability after disinfection should be confirmed by co-culture of disinfected water samples with protozoa to ensure complete loss of the VNC state of *L. pneumophila*.

Experimental procedures

Legionella *strains and disinfectant*

Seven strains of *L. pneumophila* serogroup 1 were used: (i) AA100, a clinical isolate (Abu Kwaik and Engleberg, 1994); (ii) ATCC 33152, a clinical strain; (iii) Cl01, a clinical isolate from a patient of the outbreak that occurred in Spain in 2001 (García-Fulgueiras *et al*., 2003); (iv) CIP01, derived from the Cl01 strain after 15 passages on buffered charcoal yeast extract (BCYE) agar plates; (v) CIPA01, derived from the CIP01 strain after five cycles of infection of *A. polyphaga*; (vi) EN01, the environmental strain epidemiologically related to Cl01 (García-Fulgueiras *et al*., 2003); and (vii) EN02, an environmental strain frequently recovered from Spanish water installations but not associated with clinical cases (García-Fulgueiras *et al*., 2003). Bacteria were frozen at −80°C until use. Bacteria were grown on BCYE agar plates for 72 h at 37°C before use. Chlorine, as NaOCl, was used as a disinfectant. Stock solutions were prepared in distilled water and sterilized by filtration before its use.

Protozoan cells

Axenic cultures of *A. polyphaga* (ATCC 50998) was prepared as adherent cells in PYG medium, as previously described (Gao *et al*., 1997). Monolayers were developed in 96-well tissue culture plates using PY medium (PYG without glucose). *Legionella pneumophila* were grown for 72 h at 37°C on BCYE plates and were subsequently re-suspended in PY for infections, as described below for each experiment.

Intracellular growth kinetics of L. pneumophila *within* A. polyphaga

In this study, 1×10^5 cfu per well of *A. polyphaga* in 96-well plates were infected with 200 μl of *L. pneumophila* at 5×10^6 cfu ml^{-1} in triplicate (moi 10). The plates were centrifuged at 500 *g* for 5 min followed by incubation for 1 h at 37°C. At the end of this infection period, monolayers were washed three times with PY medium to remove non-adherent cells. Subsequently, monolayers were incubated for 1 h at 37°C in the presence of 50 μg ml^{-1} gentamicine to kill extracellular bacteria, and then antibiotic was removed by washing three times with tissue culture medium. Monolayers were incubated for several time intervals (2, 16, 24 and 48 h after infection) and were lysed with 0.05% Triton X-100 (Gao *et al*., 1997; 1998). The supernatants prior to lysis of the monolayers were combined with the ones after lysis, and aliquots were plated on BCYE agar plates for colony enumeration.

Susceptibility tests

Minimal inhibitory concentration was determined by the conventional microdilution method in broth, using 96-well plates (García *et al*., 2000). Briefly, cells grown on BCYE agar for 3 days at 37°C were suspended at 5×10^6 cfu ml^{-1} in buffered yeast extract (BYE) broth containing a series of twofold dilutions of the disinfectant. Minimal inhibitory concentration value (expressed in p.p.m.) was the lowest concentration of sodium hypochlorite without visible growth after 48 h of incubation at 37°C.

Minimal bactericidal concentration was evaluated following the method previously described (García *et al*., 2000). The MBC values, expressed in p.p.m. and determined for treated cultures without visible growth, corresponded to the lowest concentration of disinfectant where reduced viable counts were more than 99.9% of untreated samples.

Resuscitation of NaOCl-sterilized L. pneumophila *by co-culture with* A. polyphaga

In this study, 5×10^6 cfu per well of bacteria in 96-well plates were incubated in PY medium for 2 h at 37°C. Cultures were treated with different concentrations of NaOCl (0–1024 p.p.m.) and incubated at 37°C for 0, 14, 22 or 46 h. The number of bacteria was determined by colony enumeration on BCYE agar plates. For cultures that were sterilized by treatment for 22 and 46 h, 30 μl aliquots were used to infect 200 μl of amoeba suspension for 24, 48 and 72 h.

Intracellular growth kinetics of L. pneumophila *within amoebae in the presence of disinfectant*

In this study, 1×10^5 cfu per well of amoebae were infected with *L. pneumophila* in PY medium as described above. After

gentamicine treatment, different concentrations of NaOCl (0–1024 p.p.m.) were added. Number of bacteria was determined at 0, 14, 22 and 46 h after treatment. Per cent survival of amoebae was determined at 22 and 46 h after treatment by counts and trypan blue exclusion.

Electron microscopy

Monolayers in 12-well plates were infected with *L. pneumophila* strain AA100 in PY medium at an moi of 10 for 1 h, followed by 1 h of treatment with gentamicin and three washes. Some cultures were treated with 128 p.p.m. of sodium hypochlorite and others were untreated. After 2, 18, 24 and 48 h of incubation at 37°C, monolayers were washed with 0.1 M Sorenson's phosphate buffer and incubated for 30 min, at 4°C, in 0.1 M Sorenson's phosphate buffer containing 3.5% glutaraldehyde (pH 7.4). After three washes in Sorenson's phosphate buffer for 5 min each time, cells were post-fixed with 1% osmium tetroxide in the same buffer for 30 min. Samples were dehydrated and processed as described previously (Gao *et al*., 1998). Sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-7000/STEM electron microscope (Hitachi, Tokyo, Japan) at 80 kV as described previously (Gao *et al*., 1998).

Acknowledgements

M.T. García is supported by a grant from the Ramon Areces Foundation from Spain. Y.A.K. is supported by the Commonwealth of Kentucky Research Challenge Trust Fund. We thank Maëlle Molmeret and Rexford Asare and all the members of the Abu Kwaik laboratory for technical advice and helpful discussions.

References

Abu Kwaik, Y., and Engleberg, N.C. (1994) Cloning and molecular characterization of a *Legionella pneumophila* gene induced by intracellular infection and by various *in vitro* stress stimuli. *Mol Microbiol* **13:** 243–251.

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.

Adeleke, A., Pruckler, J., Benson, R., Rowbotham, T., Halablab, M., and Fields, B. (1996) *Legionella*-like amoebal pathogens-phylogenetic status and possible role in respiratory disease. *Emerg Infect Dis* **2:** 225–230.

Anand, C.M., Skinner, A.R., Malic, A., and Kurtz, J.B. (1983) Interaction of *L. pneumophilia* and a free living amoeba (*Acanthamoeba palestinensis*). *J Hyg (Lond)* **91:** 167–178.

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. Erratum in: *Appl Environ Microbiol* 1992; **58:** 4089.

Barker, J., Scaife, H., and Brown, M.R. (1995) Intraphagocytic growth induces an antibiotic-resistant phenotype of *Legionella pneumophila*. *Antimicrob Agents Chemother* **39:** 2684–2688.

Borella, P., Montagna, M.T., Romano-Spica, V., Stampi, S., Stancanelli, G., Triassi, M., *et al.* (2004) Legionella infection risk from domestic hot water. *Emerg Infect Dis* **10:** 457–464.

Caylà, J.A., Maldonado, R., González, J., Pellicer, T., Ferrer, D., Pelaz, C., *et al.* (2001) A small outbreak of Legionnaires' disease in a cargo ship under repair. *Eur Respir J* **17:** 1322–1327.

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

Fields, B.S., Benson, R.F., and Besser, R.E. (2002) *Legionella* and Legionnaires' disease: 25 years of investigation. *Clin Microbiol Rev* **15:** 506–526.

Fiore, A.E., Pekka, N.J., and Levine, O.S. (1998) Epidemic Legionnaires' disease two decades later: old sources, new diagnostic methods. *CID* **26:** 426–433.

Gao, L.-Y., Harb, O.S., and Abu Kwaik, Y. (1997) Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant hosts, mammalian and protozoan cells. *Infect Immun* **65:** 4738–4746.

Gao, L.-Y., Harb, O.S., and Abu Kwaik, Y. (1998) Identification of macrophage-specific infectivity loci (*mil*) of *Legionella pneumophila* that are not required for infectivity of protozoa. *Infect Immun* **1998:** 883–892.

García, M.T., Pelaz, C., Giménez, M.J., and Aguilar, L. (2000) *In vitro* activities of gemifloxacin versus five quinolones and two macrolides against 271 Spanish isolates of *Legionella pneumophila*: influence of charcoal on susceptibility test results. *Antimicrob Agents Chemother* **44:** 2176–2178.

García-Fulgueiras, A., Navarro, C., Fenoll, D., García, J., González-Diego, P., Jiménez-Buñuales, T., *et al.* (2003) Legionnaires' disease outbreak in Murcia, Spain. *Emerg Infect Dis* **9:** 915–921.

Greub, G., and Raoult, D. (2003a) Morphology of *L. pneumophila* according to their location within *H. vermiformis*. *Res Microbiol* **154:** 619–621.

Greub, G., and Raoult, D. (2003b) Biocides currently used for bronchoscope decontamination are poorly effective against free-living amoebae. *Infect Control Hosp Epidemiol* **24:** 784–786.

Greub, G., and Raoult, D. (2004) Microorganisms resistant to free-living amoebae. *Clin Microbiol Rev* **2:** 413–433.

Haas, C.N., and Engelbrecht, R.S. (1980) Physiological alterations of vegetative microorganisms resulting from aqueous chlorination. *J Water Pollut Control Fed* **52:** 1976–1989.

Harb, O.S., Gao, L.-Y., and Abu Kwaik, Y. (2000) From protozoa to mammalian cells: a new paradigm in the life cycle of intracellular bacterial pathogens. *Environ Microbiol* **2:** 251–265.

Heimberger, T., Birkhead, G., and Bornstein, D. (1991) Control of nosocomial Legionnaires' disease through hot water flushing and supplemental chlorination of potable water. *J Infect Dis* **163:** 413.

Hoebe, C.J., and Kool, J.L. (2000) Control of *Legionella* in drinking water systems. *Lancet* **355:** 2093–2094.

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

Kim, B.R., Anderson, J.E., Mueller, S.A., Gaines, W.A., and Kendall, A.M. (2002) Literature review-efficacy of various disinfectants against *Legionella* in water systems. *Water Res* **36:** 4433–4444.

Kool, J.L., Fiore, A.E., Kioski, C.M., Brown, E.W., Benson, R.F., Pruckler, J.M., *et al.* (1998) More than 10 years of unrecognized nosocomial transmission of Legionnaires' disease among transplant patients. *Infect Control Hosp Epidemiol* **19:** 898–904.

Leoni, E., De Luca, G., Legnani P.P., Sacchetti, R., Stampi, S., and Zanetti, F. (2005) Legionella waterline colonization: detection of *Legionella* species in domestic, hotel and hospital hot water systems. *J Appl Microbiol* **98:** 373–379.

Marrie, T.J., Raoult, D., La Scola, B., Birtles, R.J., and de Carolis, E. (2001) *Legionella*-like and other amoebal pathogens as agents of community-acquired pneumonia. *Emerg Infect Dis* **7:** 1026–1029.

Marston, B.J., Lipman, H.B., and Breiman, R.F. (1994) Surveillance for Legionnaires' disease. Risk factors for morbidity and mortality. *Arch Intern Med* **154:** 2417–2422.

Mazur, T., Hadas, E., and Iwanicka, I. (1995) The duration of the cyst stage and the viability and virulence of *Acanthamoeba* isolates. *Trop Med Parasitol* **46:** 102–108.

Molmeret, M., Bitar, D.M., Han, L., and Abu Kwaik, Y. (2004) Disruption of the phagosomal membrane and egress of *Legionella pneumophila* into the cytoplasm during the last stages of intracellular infection of macrophages and *A. polyphaga*. *Infect Immun* **72:** 4040–4051.

Molmeret, M., Horn, M., Wagner, M., Santic, M., and Abu Kwaik, Y. (2005) Amoebae as training grounds for intracellular bacterial pathogens. *Appl Environ Microbiol* **71:** 20–28.

Nwachuku, N., and Gerpa, C.P. (2004) Health effects of *Acanthamoeba* spp and its potential for waterborne transmission. *Rev Environ Contam Toxicol* **180:** 93–131.

Perola, O., Kauppinen, J., Kusnetsov, J., Heikkinen, J., Jokinen, C., and Katila, M.L. (2002) Nosocomial *Legionella pneumophila* serogroup 5 outbreak associated with persistent colonization of a hospital water system. *APMIS* **110:** 863–868.

Rowbotham, T.J. (1983) Isolation of *L. 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.

Rowbotham, T.J. (1993) *Legionella*-like amoebal pathogens. In *Legionella: Current Status and Emerging Perspectives.* Barbaree, J.M., Breiman, R.F., and Dufour, A.P. (eds). Washington, DC, USA: American Society for Microbiology, pp. 137–140.

Rowbotham, T.J. (1998) Isolation of *L. pneumophila* serogroup 1 from human feces with use of amebic cocultures. *Clin Infect Dis* **26:** 502–503.

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 *L. pneumophila* in a heat-disinfected plumbing system. *Zentrall Bakteriol* **288:** 331–342.

Steinert, M., Henstchel, U., and Hacker, J. (2002) *Legionella pneumophila*: an aquatic microbe goes astray. *FEMS Microbiol Rev* **26:** 149–162.

Storey, M.V., Winiecka-Krusnell, J., Ashbolt, N.J., and Stenström, T.-A. (2004) The efficacy of heat and chlorine treatment against thermotolerant Acanthamoebae and Legionellae. *Scand J Infect Dis* **36:** 656–662.

Sutherland, E.E., and Berk, S.G. (1996) Survival of protozoa in cooling tower biocides. *J Ind Microbiol* **16:** 73–78.

Thomas, V., Herrera-Rimann, K., Blanc, D.S., and Greub, G. (2006) Biodiversity of amoebae and amoeba-resisting bacteria in a hospital water network. *Appl Environ Microbiol* **72:** 2428–2438.

