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ORIGINAL ARTICLE

Heterogeneity in chlorine susceptibility for *Legionella pneumophila* released from *Acanthamoeba* and *Hartmannella*

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Abstract

Aims: To assess chlorine susceptibility of *Legionella pneumophila* grown from two amoebic hosts, *Acanthamoeba castellanii* and *Hartmannella vermiformis*.
Methods and Results: After being released from amoebae, *Leg. pneumophila* were chlorinated at 2 and 5 mg l^{-1} for 5 min–24 h. Bacterial culturability and cytoplasmic membrane deterioration were quantified by culture assay on BCYEα agar and BacLight stains coupled with a fluorescent microscope, respectively. Chlorination reduced the culturability of *Leg. pneumophila* by 2·93–4·59 log CFU ml^{-1} and damaged cellular membrane by 53·8–99·2%. Moreover, cells released from *H. vermiformis* exhibited significantly lower degrees in culturability reduction ($P = 0{\cdot}0008$) and membrane deterioration ($P < 0{\cdot}0001$) when compared with those from *A. castellanii*. The amoebic genus is the most significant parameter affecting cytoplasmic membrane integrity of chlorinated *Legionella* ($P < 0{\cdot}0001$), followed by free chlorine concentration ($P = 0{\cdot}042$).
Conclusions: *Legionella pneumophila* replicated from *H. vermiformis* possess greater chlorine resistance than the cells from *A. castellanii*.
Significance and Impact of the Study: This study shows the heterogeneity of amoebae-grown *Leg. pneumophila* in chlorine susceptibility, which should be considered in the control of legionellae proliferation, particularly in the systems where *H. vermiformis* is dominant, e.g. hot water plumbing.

Introduction

Free-living amoebae (FLA) are ubiquitous in aquatic environments and may serve as microniches and transmission carriers for *Legionella pneumophila* (Greub and Raoult 2004), a human pathogen causing Pontiac fever and severe pneumonia named Legionnaires' disease. *Legionella pneumophila* are reported to be harboured in at least 13 species of amoebae, in which the bacteria multiply intracellularly (Fields 1996). *Acanthamoeba* and *Hartmannella*, the most predominant amoebae in water sources (Molmeret *et al.* 2005), are natural hosts for *Leg. pneumophila*. Both the genera have been isolated from water networks where *Leg. pneumophila* grow (Breiman *et al.* 1990; Thomas *et al.* 2006). In addition, both the genera have been found in chlorine-treated swimming pools (Rivera *et al.* 1993). Trophozoites and cysts of *Hartmannella vermiformis* may survive at 4 mg l^{-1} of chlorine treatment and still support intracellular multiplication and growth of *Leg. pneumophila* (Kuchta *et al.* 1993). Similarly, cysts of *Acanthamoeba castellanii* and *Acanthamoeba polyphaga* can retain their viability at 10 mg l^{-1} of chlorination (Kilvington and Price 1990; Storey *et al.* 2004).

Acanthamoeba and *Hartmannella* are not only resistant to chlorine, but may also interfere with the chlorine efficacy against *Leg. pneumophila*. Previous studies have demonstrated that legionellae located in cysts of

A. polyphaga exhibit an increased resistance to chlorination (Kilvington and Price 1990). The susceptibility of *Leg. pneumophila* to free chlorine (FC) is also reduced when *Legionella* are associated with *H. vermiformis* in a model biofilm when compared with that determined in absence of *H. vermiformis* (Donlan *et al.* 2005). In addition to FC, intracellular replication within *A. polyphaga* also enhances the resistance of *Leg. pneumophila* to other chemical biocides (Barker *et al.* 1992). These investigations provide evidence that *Leg. pneumophila* grown from *Acanthamoeba* or *Hartmannella* are changed phenotypically in their susceptibility to disinfectants. However, to our knowledge, the difference in chlorine resistance of *Leg. pneumophila* grown from different FLA hosts has not been thoroughly researched. It remains unknown whether the chlorine efficacy against *Leg. pneumophila* replicated and released from various amoebic genera is significantly different.

The objectives of the present study were to characterize the susceptibility of *Leg. pneumophila* released from two common hosts, *A. castellanii* (Rivera *et al.* 1989; Tsvetkova *et al.* 2004) and *H. vermiformis* (Rohr *et al.* 1998; Thomas *et al.* 2006) to chlorine at 2 and 5 mg l^{-1}. The chlorine concentrations selected were based on the ranges suggested by US Environmental Protection Agency (EPA) for continuous hyperchlorination against *Legionella* (2–6 mg l^{-1}) (EPA 1999) and by the European Surveillance Scheme for Travel Associated Legionnaires' Disease (EWGLINET) and the European Working Group for *Legionella* Infections (EWGLI) for hot water system (1–2 mg l^{-1}) (EWGLINET and EWGLI 2005).

Materials and methods

Bacterial and amoebic strains

Legionella pneumophila serogroup 1 ATCC 33152 was cultured on BCYEα agar (Sigma Chemical Co., St Louis, MO, USA) at 37°C in an atmosphere of 5% CO_2 for 2 days. The cells were collected, washed with phosphate-buffered saline and suspended in sterile distilled water.

Following the instructions of American Type Culture Collection (ATCC), *A. castellanii* ATCC 30234 and *H. vermiformis* ATCC 50237 were grown in ATCC 712 medium (PYG) and ATCC 1034 medium (modified PYNFH), respectively, at 25°C in a 75-cm^2 tissue culture flask (NUNC, Roskilde, Denmark). After 2-day incubation, amoebic suspensions were centrifuged at 200 ***g*** for 6 min at 25°C. The supernatant was replaced with Page's Amoeba Saline (PAS; 120 mg NaCl, 4 mg $MgSO_4$ $7H_2O$, 4 mg $CaCl_2$, 142 mg Na_2HPO_4 and 136 mg KH_2PO_4 in 1 l ultrapure water), and amoebic cultures were equilibrated at 37°C for 1 h.

Co-culture of *Legionella pneumophila* with amoebae

After 1 h of equilibrium, amoebic cultures were votexed briefly to bring the cells into suspension. Amoebic concentrations were then determined by a haemocytometer (Marienfeld, Lauda-Konigshofen, Germany) and adjusted to a titre of 10^6 cells ml^{-1} with PAS. *Legionella pneumophila* cells in sterile distilled water were also measured by a haemocytometer, and an aliquot of bacterial cells was added to the amoebae-containing flask to achieve a multiplicity of infection (MOI) of 500 *Leg. pneumophila* per amoeba. After incubation at 37°C for 2 h, uptake of *Leg. pneumophila* by amoebae occurred in the co-culture. The extra-amoebic *Leg. pneumophila* was then removed by centrifugation at 200 ***g*** for 6 min and careful replacement of the supernatants with 10 ml of PAS five times, a procedure modified from previous studies (Holden *et al.* 1984; Bozue and Johnson 1996). The concentrations of extra-amoebic *Leg. pneumophila* before and after five centrifugations were determined by a haemocytometer, which resulted in an average removal rate of 98·5% (±1·7%). The co-culture samples were then adjusted to 5×10^4 amoebae ml^{-1} with PAS, and 1 ml of co-culture was pipetted into 24-well infection plates (NUNC). Following 48 h of incubation at 37°C, extensive release of *Leg. pneumophila* cells from amoebae was confirmed by staining the cells with LIVE/DEAD BacLight kit (L-7012; Molecular Probe –Invitrogen – CA, USA) and observing them under a fluorescent microscope (Model DMR; Leica, Wetzlar, Germany) (details in 'membrane integrity' section). The significant release of *Leg. pneumophila* from amoebae was in agreement with the report by Gao and Abu Kwaik (2000).

Chlorination experiments

Free chlorine solutions, freshly prepared by dilution of sodium hypochlorite (Sigma Chemical Co.) with PAS, were added to 48-h co-culture samples (i.e., *Leg. pneumophila* with *A. castellanii* and *H. vermiformis* denoted as Lp-Ac and Lp-Hv, respectively) and to PAS alone without cells at a volume ratio of 9 : 1 to achieve an initial FC concentration of 2 and 5 mg l^{-1}. Both types of samples were then gently shaken at 25°C for 5 min, 1 h and 24 h. At the beginning and the end of chlorination, residual FC concentrations were determined by a pocket colorimeter analysis system (Hach Test Kit; HACH Company, Loveland, CO, USA), the accuracy of which was confirmed by the dialkyl-p-phenylene-diamine method (i.e. relative error <5%) (Chang *et al.* 2007). Cell suspensions were then neutralized with 0·1 ml of 5% (w/v) sterile sodium thiosulfate, and residual FC was confirmed to be nondetectable. In addition to chlorinated samples, nonchlorinated controls were prepared by adding PAS, instead of FC solution, to the co-culture samples, and analysed for cellular culturability and membrane integrity along with the chlorine-treated samples. All experiments were performed six times.

Culturability

Six replicates of chlorinated samples and nonchlorinated controls were serially diluted with PAS. The culturability of *Leg. pneumophila* was determined by inoculatng 0·1-ml aliquot of diluted and undiluted samples on each of triplicate plates of BCYEα agar and incubating them for 10 days at 37°C with 5% CO_2. The number of culture-negative samples in six replicates was recorded. For culture-positive ones, the log difference in culturability (log CFU ml^{-1}) between the chlorinated samples and nonchlorinated controls was determined, and presented as the mean log culturability reduction of chlorinated samples when compared with nonchlorinated ones.

Membrane integrity

Membrane integrity of *Leg. pneumophila* was determined by fluorogenic stains using LIVE/DEAD BacLight kit (Invitrogen) coupled with a fluorescent microscope (Leica). The BacLight kit contains two nucleic acid fluorochromes, namely, SYTO9 and propidium iodide (PI). SYTO9 is a green intercalating stain that is membrane permeable, while PI is a red intercalating stain penetrating compromised membranes only (Stocks 2004). The experiment was conducted as described previously (Chang *et al.* 2007) with modification of the volumes of stains used. Three microlitres of BacLight stains containing SYTO9 and PI in 1 : 1 were applied to 1 ml of nonchlorinated controls, whereas 10 μl of the stains was mixed with chlorinated samples in order to optimize the stain quality (final concentration: 6·68 $\mu g\ ml^{-1}$ for SYTO9 and 66·84 $\mu g\ ml^{-1}$ for PI). With the aid of a fluorescent microscope (Leica) equipped with a CCD (Leica DC 300F; Leica), the numbers of membrane-intact and membrane-compromised cells, observed as green and red cells, respectively, were determined from the images. The number of total cells was calculated as the sum of membrane-intact and membrane-damaged cells. The numbers of microscopic fields enumerated for Lp-Ac and Lp-Hv samples averaged 24 and 51, respectively; this difference was due to less bacterial density in Lp-Hv suspensions. Overall, the average number of counted *Leg. pneumophila* was 1257 cells per sample and in total 108 samples were analysed by BacLight staining and cell enumeration. A few *A. castellanii* and *H. vermiformis* were observed in co-culture samples during bacterial enumeration; however, amoebic presence did not interfere with bacterial counting due to relatively low quantity and distinguishable cell morphology in size and shape. Concentrations of membrane-intact, membrane-damaged and total cells were calculated based on the cell counts in 1 ml BacLight-stained samples, and further log-transformed and expressed as the log mean of six replicates. The percentage of membrane-damaged cells, determined as the number of membrane-compromised cells relative to that of total cells in 1 ml BacLight-stained sample, was also presented as the mean of the percentage values and used as one of the indicators of chlorine efficacy.

Statistical analysis

The nonparametric Wilcox rank-sum (Mann–Whitney) test was performed to examine the differences of the measured parameters between *A. castellanii* and *H. vermiformis* co-cultured samples, between PAS-only and cell-containing samples and between nonchlorinated and chlorinated samples. A linear regression model was conducted to assess the effects of FC concentration, FC contact time and amoebic genus from which *Leg. pneumophila* were released on chlorine efficacy against *Legionella*. All statistical analyses were undertaken using SAS software version 9.1 (SAS Institute Inc., Cary, NC, USA). Statistical significance was considered $P < 0{\cdot}05$.

Results

Residual Free Chlorine

In challenge with 2 and 5 mg l^{-1} chlorine over a 24-h period, the residual FC concentrations in the suspensions of *Leg. pneumophila* released from *A. castellanii* (Lp-Ac) and *H. vermiformis* (Lp-Hv) were lower than those in PAS alone (both $P < 0{\cdot}0001$), indicating chlorine demand by micro-organisms (Table 1). Residual FC concentrations were generally greater in Lp-Hv than in Lp-Ac; however, no statistically significant difference was found. Free chlorine concentrations decreased immediately right after adding chlorine solution to Lp-Ac and Lp-Hv samples, followed by a rapid FC consumption during the first 5-min period with averaged decrease rates of $-2{\cdot}4 \times 10^{-1}$ mg Cl_2 min^{-1} and $-4{\cdot}7 \times 10^{-1}$ mg Cl_2 min^{-1} for chlorination at 2 and 5 mg l^{-1}, respectively. Afterwards, residual FC continuously declined at slower rates: $-5{\cdot}7 \times 10^{-3}$ mg Cl_2 min^{-1} (2 mg l^{-1} chlorination) and -2×10^{-2} mg Cl_2 min^{-1} (5 mg l^{-1} chlorination) between 5 and 60 min, and at a rate less than -3×10^{-4} mg Cl_2 min^{-1} between 1 and 24 h. While decreasing, residual FC remained detectable at the end of 24-h chlorine contact in both Lp-Hv and Lp-Ac suspensions.

Table 1 Residual free chlorine in Page's Amoeba Saline (PAS) and in suspensions of *Legionella pneumophila* co-cultured with *Acanthamoeba castellanii* (Lp-Ac) and *Hartmannella vermiformis* (Lp-Hv)

	Residual free chlorine (mg l^{-1})		
Chlorination	Lp-Ac	Lp-Hv	PAS
2 mg l^{-1}, 0 min	1·14 (0·40)†	1·34 (0·47)	2·11 (0·22)
2 mg l^{-1}, 5 min	0·70 (0·30)	0·88 (0·59)	1·89 (0·31)
2 mg l^{-1}, 1 h	0·29 (0·36)	0·67 (0·63)	1·64 (0·49)
2 mg l^{-1}, 24 h	0·14 (0·13)	0·50 (0·33)	0·91 (0·42)
5 mg l^{-1}, 0 min	2·98 (0·94)	3·73 (0·96)	5·10 (0·23)
5 mg l^{-1}, 5 min	2·19 (0·98)	3·08 (0·91)	4·59 (0·41)
5 mg l^{-1}, 1 h	1·66 (1·13)	1·38 (1·18)	4·59 (0·52)
5 mg l^{-1}, 24 h	1·13 (0·75)	1·56 (0·99)	3·73 (0·18)

Chlorination at 2 and 5 mg l^{-1} was applied to Page's Amoeba Saline (PAS) and cell suspensions (Lp-Ac and Lp-Hv) for up to 24 h at 25°C ($N = 6$).

†Standard deviation of the mean of residual free chlorine values in the parenthesis.

Total cell concentrations and membrane integrity

Following co-culture with *A. castellanii* and 10-folded dilution of cell suspensions, the average of log total cell ml^{-1} of *Leg. pneumophila* without chlorination ranged between 5·41 and 5·66 (Table 2). For those co-cultures chlorinated at 2 and 5 mg l^{-1}, the concentrations of total cells averaged 5·52–5·76 log cell ml^{-1}, which was not statistically different from those without chlorination. As for cytoplasmic membrane integrity, high concentrations of membrane-intact cells (5·37–5·64 log cell ml^{-1}) relative to membrane-damaged ones (3·94–4·04 log cell ml^{-1}) in nonchlorinated controls indicated that most of *Leg. pneumophila* released from *A. castellanii* possessed cellular membrane integrity. However, exposure to 2 mg l^{-1} of FC for 5 min significantly damaged cell membrane, resulting in an increase of membrane-damaged cells from 4·04 log cell ml^{-1} to 5·24 log cell ml^{-1}. An increase in FC from 2 to 5 mg l^{-1} caused further membrane deteriorations, which was revealed at all of the tested contact periods.

Regarding *Leg. pneumophila* released from *H. vermiformis* (Table 3), evident damage of cellular membranes was observed at 2 and 5 mg l^{-1} chlorination in comparison with nonchlorinated cells. Such membrane deterioration was aggravated with increasing FC concentration and contact time in most cases. In contrast, total bacterial concentrations were comparable between nonchlorinated controls and chlorinated samples, similar to the findings of *Leg. pneumophila* released from *A. castellanii*.

Although total cell concentrations between chlorinated and nonchlorinated groups were comparable for both Lp-Ac and Lp-Hv co-cultures, the total concentrations of *Leg. pneumophila* released from *A. castellanii* (5·41–5·76 log cell ml^{-1}, Table 2) were significantly greater than those released from *H. vermiformis* (4·87–5·10 log cell ml^{-1}, Table 3) ($P < 0{\cdot}0001$). The percentage of membrane-damaged *Leg. pneumophila* was determined and used to evaluate the chlorine efficacy in order to adjust for the difference in total cell concentration. As shown in Table 4, in absence of chlorination, only 4·3–7·5% *Leg. pneumophila* cells released from *A. castellanii* were membrane-damaged. In contrast, 79–88·4% and 97·4–99·2% of the cells were membrane-compromised after exposure to 2 and 5 mg l^{-1} chlorine, respectively, significantly different from cells without chlorination ($P < 0{\cdot}0001$). As for *Leg. pneumophila* released from *H. vermiformis*, 53·8–73·6% and 78·6–83·6% of the cells were membrane-compromised by 2 and 5 mg l^{-1} chlorination, respectively, which were also statistically greater than those without chlorination (i.e. 8·5–17·8%) ($P < 0{\cdot}0001$). The percentages of membrane-damaged cells were significantly

Table 2 Effects of chlorination on membrane integrity of *Legionella pneumophila* released from *Acanthamoeba castellanii*

Chlorination	Total cells* (log cell ml^{-1})	Membrane-intact cells (log cell ml^{-1})	Membrane-damaged cells (log cell ml^{-1})
0 mg l^{-1}, 5 min	5·41 (0·38)†	5·37 (0·39)	4·04 (0·55)
0 mg l^{-1}, 1 h	5·57 (0·37)	5·54 (0·39)	3·95 (0·40)
0 mg l^{-1}, 24 h	5·66 (0·12)	5·64 (0·10)	3·94 (0·84)
2 mg l^{-1}, 5 min	5·52 (0·22)	4·41 (0·73)	5·24 (0·48)
2 mg l^{-1}, 1 h	5·64 (0·19)	4·31 (0·71)	5·47 (0·35)
2 mg l^{-1}, 24 h	5·76 (0·14)	4·11 (0·94)	5·70 (0·19)
5 mg l^{-1}, 5 min	5·70 (0·11)	4·04 (0·21)	5·68 (0·12)
5 mg l^{-1}, 1 h	5·53 (0·43)	3·63 (0·22)	5·51 (0·45)
5 mg l^{-1}, 24 h	5·76 (0·16)	3·59 (0·35)	5·76 (0·16)

*The sum of membrane-intact and membrane-damaged cells determined by the BacLight staining with a fluorescent microscope and expressed as the mean of the log-transformed values from six replicates.

†Standard deviation of the mean of log-transformed values in the parenthesis.

Table 3 Effects of chlorination on membrane integrity of *Legionella pneumophila* released from *Hartmannella vermiformis*

Chlorination	Total cells* (log cell ml^{-1})	Membrane-intact cells (log cell ml^{-1})	Membrane-damaged cells (log cell ml^{-1})
0 mg l^{-1}, 5 min	5·10 (0·43)†	5·06 (0·40)	3·89 (0·72)
0 mg l^{-1}, 1 h	4·90 (0·35)	4·79 (0·28)	3·73 (0·67)
0 mg l^{-1}, 24 h	4·87 (0·40)	4·83 (0·36)	3·59 (0·84)
2 mg l^{-1}, 5 min	5·07 (0·43)	4·62 (0·36)	4·72 (0·57)
2 mg l^{-1}, 1 h	4·93 (0·27)	4·30 (0·34)	4·76 (0·37)
2 mg l^{-1}, 24 h	5·02 (0·28)	4·36 (0·38)	4·88 (0·31)
5 mg l^{-1}, 5 min	4·94 (0·25)	4·12 (0·15)	4·87 (0·29)
5 mg l^{-1}, 1 h	5·01 (0·42)	4·08 (0·26)	4·94 (0·45)
5 mg l^{-1}, 24 h	4·87 (0·40)	4·14 (0·33)	4·75 (0·44)

*The sum of membrane-intact and membrane-damaged cells determined by the BacLight staining with a fluorescent microscope and expressed as the mean of the log-transformed values from six replicates.

†Standard deviation of the mean of log-transformed values in the parenthesis.

Table 4 Percentage of membrane-damaged *Legionella pneumophila* released from *Acanthamoeba castellanii* and *Hartmannella vermiformis* in challenge with chlorination

	Membrane-damaged cells* (%)	
Chlorination	*Leg. pneumophila* from *A. castellanii*	*Lleg. pneumophila* from *H. vermiformis*
0 mg l^{-1}, 5 min	7·5 (7·9)†	8·5 (6·6)
0 mg l^{-1}, 1 h	4·3 (4·8)	17·8 (25·0)
0 mg l^{-1}, 24 h	5·1 (7·0)	9·4 (10·6)
2 mg l^{-1}, 5 min	79·0 (37·5)	53·8 (30·7)
2 mg l^{-1}, 1 h	82·8 (35·0)	70·7 (21·2)
2 mg l^{-1}, 24 h	88·4 (17·1)	73·6 (16·8)
5 mg l^{-1}, 5 min	97·4 (1·3)	83·6 (7·9)
5 mg l^{-1}, 1 h	97·5 (3·7)	86·6 (7·1)
5 mg l^{-1}, 24 h	99·2 (0·4)	78·6 (12·4)

*The number of membrane-damaged cells relative to that of total cells in 1 ml BacLight-stained sample, expressed as the mean of the percentage values from six replicates.

†Standard deviation of the mean of percentage values in the parenthesis.

greater in chlorinated *Leg. pneumophila* released from *A. castellanii* than in the cells from *H. vermiformis* ($P < 0·0001$). The results from the stepwise linear regression model indicated that the amoebic genus was the most statistically significant parameter ($P < 0·0001$) affecting the percentage of chlorine-induced membrane damage, followed by FC concentration ($P = 0·042$) with an adjusted R^2 of 0·9 for the model. The contact time with chlorine was not statistically significant, and therefore excluded from the model.

Culturability

In addition to its propensity to damage cellular membranes, chlorine efficacy was evaluated by the number of culture-negative samples over six replicates and by the reduced culturability of chlorinated *Leg. pneumophila* relative to nonchlorinated cells. Table 5 shows that 3–5 samples out of six replicates became culture-negative when *A. castellanii*-releasing *Leg. pneumophila* was challenged with 2 mg l^{-1} chlorine. For the replicate(s) remaining culturable, culturability was decreased by 3·83–4·46 log CFU ml^{-1}. Increasing chlorine concentration to 5 mg l^{-1} resulted in further culturability reductions. For the cells released from *H. vermiformis*, chlorination caused 1–3 nonculturable samples over six replicates, and the culturability was declined by 2·93–3·72 log CFU ml^{-1} and 3·33–3·81 log CFU ml^{-1} at 2 and 5 mg l^{-1} chlorination, respectively. Overall, chlorinated *Leg. pneumophila* released from *A. castellanii* suffered greater culturability loss than the cells from *H. vermiformis* ($P = 0·0008$).

Discussion

Given the same MOI and initial amoebic concentration at the beginning of a 48 h co-culture period, this study shows that the concentrations of *Leg. pneumophila* released from *A. castellanii* were significantly greater than those from *H. vermiformis* ($P < 0·0001$), suggesting *Leg. pneumophila* tends to infect and/or replicate more efficiently within *A. castellanii* than *H. vermiformis.* Released *Leg. pneumophila* were subjected to FC for a 24-h period during which residual FC was continuously detectable. Residual FC levels were generally higher in Lp-Hv than in Lp-Ac suspensions, suggesting that *H. vermiformis*-releasing *Leg. pneumophila* possibly were exposed to more residual FC than the cells from *A. castellanii*. Assuming that amoeba-grown *Leg. pneumophila* possess equal chlorine susceptibility regardless of their amoebic hosts, more *H. vermiformis*-releasing bacteria would be attacked by FC due to more residual FC available for less amount of

Table 5 Effects of chlorination on culturability of *Legionella pneumophila* released from *Acanthamoeba castellanii* and *Hartmannella vermiformis*

Chlorination	*Leg. pneumophila* from *A. castellanii*		*Leg. pneumophila* from *H. vermiformis*	
	Culture-negative* (*n/N*)	Reduction in culturability† (log CFU ml^{-1})	Culture-negative (*n/N*)	Reduction in culturability (log CFU ml^{-1})
0 mg l^{-1}, 5 min	0/6	–	0/6	–
0 mg l^{-1}, 1 h	0/6	–	0/6	–
0 mg l^{-1}, 24 h	0/6	–	0/6	–
2 mg l^{-1}, 5 min	3/6	3·83 (0·50)‡	1/6	2·93 (0·62)
2 mg l^{-1}, 1 h	5/6	4·34	3/6	3·10 (0·75)
2 mg l^{-1}, 24 h	4/6	4·46 (0·16)	2/6	3·72 (0·78)
5 mg l^{-1}, 5 min	4/6	4·18 (1·41)	2/6	3·33 (0·24)
5 mg l^{-1}, 1 h	4/6	4·59 (0·60)	3/6	3·54 (0·71)
5 mg l^{-1}, 24 h	4/6	4·52 (0·65)	3/6	3·81 (0·62)

*Number of culture-negative samples in six replicates.
†By taking the log value of culturability in nonchlorinated controls as the reference, the log difference in culturability between the chlorinated samples and nonchlorinated controls was determined and presented as the mean log culturability reduction. Only the culture-positive samples were included in this calculation (number of chlorinated and culture-positive samples = 1–5).
‡Standard deviation of the mean of log-reduction values in the parenthesis.

Leg. pneumophila in Lp-Hv suspensions. In other words, the culturability reduction and cellular membrane damage to *H. vermiformis*-grown bacteria would be predicted to be greater compared with damages to *A. castellanii*-grown *Legionella*. Nevertheless, the statistically significant findings did not support this prediction (Tables 4 and 5). Furthermore, the statistical results of stepwise linear regression model indicated that amoebic genus was the most significant parameter affecting the chlorine efficacy against *Legionella*. This evidence indicates heterogeneity in chlorine susceptibility of amoeba-grown *Leg. pneumophila*. Indeed, *Leg. pneumophila* released from *H. vermiformis* are more resistant to FC than the cells from *A. castellanii*. While previous investigations demonstrated that *Leg. pneumophila* has increased tolerance to FC and other stresses after the cell infects a suitable amoebic host (Abu Kwaik *et al.* 1997; Donlan *et al.* 2005), there is limited comparative research exploring the disinfectant-responding characteristics of *Legionella* grown within different FLA. The current study provides evidence that *Leg. pneumophila* replicating within either *A. castellanii* or *H. vermiformis* have different characteristics in chlorine susceptibility.

Legionella pneumophila has been shown to attach and invade *A. polyphaga* and *H. vermiformis* by different mechanisms (Harb *et al.* 1998). Co-inoculation of *Leg. pneumophila* with *H. vermiformis* significantly enhances intrapulmonary growth of *Leg. pneumophila* in mice (Brieland *et al.* 1996), whereas co-cultures of *Leg. pneumophila* and *Acanthamoeba royreba* do not (Tyndall and Domingue 1982). These results support the premise that *Leg. pneumophila* interact differently with *Acanthamoeba* and *H. vermiformis* and may produce more virulent phenotypes associated with *H. vermiformis*. In this study, we found *H. vermiformis*-grown *Leg. pneumophila* possessed an increased capacity to resist chlorination compared with that of *A. castellanii*-grown bacteria. We also observed less *Leg. pneumophila* cells replicated from *H. vermiformis* than from *A. castellanii*. Taking the intracellular replication rate as an indication of the preferred growth environment for *Leg. pneumophila* (Cirillo *et al.* 1994), our results suggest *H. vermiformis* is less favourable than *A. castellanii* for bacterial growth. As amoebae are considered as training grounds for *Leg. pneumophila* to survive in harsh environmental conditions (Molmeret *et al.* 2005), *Legionella* growing in a hostile host, e.g. *H. vermiformis*, may be trained to become more resistant to a variety of stresses than the cells replicated within hospitable amoebae such as *A. castellanii*. Interestingly, increased chlorine resistance in tap water-adapted *Leg. pneumophila* when compared with agar medium-passed strains (Kuchta *et al.* 1985) appears to support this consideration. We hypothesize that *Leg. pneumophila* growing in *H. vermiformis* have faced strict challenges and undergone genetic, physiological and structural modulations (Abu Kwaik *et al.* 1994, 1997; Greub and Raoult 2003) different from those occurring in *A. castellanii*. This difference may result in *Leg. pneumophila* with distinct properties in response to FC, e.g. expression of stress-induced genes and proteins (Abu Kwaik *et al.* 1997) to cross-protect bacteria from chlorine attack and/or thickening of cell wall to hinder chlorine uptake by bacterial membrane and cytoplasm (Chang *et al.* 2007). Heterogeneity in chlorine susceptibility of amoeba-grown *Leg. pneumophila* may be due to alternations in bacterial structure and physiology as a result of the communication between *Leg. pneumophila* and its amoebic hosts. However, the exact mechanism remains unclear, and warrants further investigation.

In addition to the heterogeneity in chlorine susceptibility, the present study also indicates chlorination caused more profound impact on cell culturability (reduction by 2·93–4·59 log CFU ml^{-1}, Table 5) than on the degradation of the cytoplasmic membrane (impairment by 53·8–99·2%, Table 4). Considering cellular membrane integrity as an indicator of bacterial viability (Stocks 2004), this finding suggests the presence of viable but nonculturable *Leg. pneumophila* when challenged with FC. Such characteristics have been observed in other chlorinated waterborne micro-organisms, including *Escherichia coli* O157:H7 (Lisle *et al.* 1999) and *Stenotrophomonas maltophilia* collected from a water treatment plant (Hoefel *et al.* 2005).

While chlorination reduces culturability and attacks the cytoplasmic membrane of amoeba-grown *Leg. pneumophila*, the similar concentrations of total cells between chlorinated and nonchlorinated samples indicate no significant bacterial lysis by chlorination. The comparable total cell concentrations also support that there is no interference of FC with the staining performance of PI, one of the dual fluorochromes in BacLight kit. Previous investigations reported that, when 10 $\mu g\ ml^{-1}$ of PI was solely used to stain chlorinated drinking water bacteria, the number of PI-positively stained cells (i.e. cells with damaged membrane) detected by a flow cytometer progressively decreased with increasing chlorine concentration from 0·2 to 3 mg l^{-1} because chlorine appeared to damage nucleic acids leading to the failure of PI staining (Phe. *et al.* 2005). In the present study, if the nucleic acids of *Leg. pneumophila* were severely impaired by chlorine so that both PI and SYTO9 failed to stain, the total cell concentrations in chlorinated suspensions would be erroneously determined as decreasing. Alternatively, if SYTO9 could still intercalate on nucleic acids whereas PI failed, the membrane-compromised cells would be falsely stained as green cells, resulting in no difference in membrane-damaged percentages between chlorinated and nonchlorinated samples. Nevertheless, the present study indicates that neither of these potential errors occurred; total cell concentrations were comparable regardless of chlorination, and 53·8–99·2% of chlorinated *Leg. pneumophila* were successfully stained with PI as red cells in contrast to 4·3–17·8% in nonchlorinated suspensions. Moreover, FC concentration was shown to be a significant determinant positively affecting the percentages of PI-positively stained cells. Therefore, no data in the present study support that FC would affect the staining efficiency of BacLight fluorochromes or bias the assessment of chlorine efficacy against *Leg. pneumophila*. In fact, BacLight stains have been successfully used in chlorine-associated studies to assess disinfection efficacy and/or chlorine resistance (Arana *et al.* 1999; Lisle *et al.* 1999; Ramirez *et al.* 2000; Moreno *et al.* 2004; Hoefel *et al.* 2005; Chang *et al.* 2007). The disagreement between the current study and Phe. *et al.* (2005) investigation could be attributed to the differences in tested fluorochromes (dual stains *vs* PI only), PI concentration (66·84 $\mu g\ ml^{-1}$ *vs* 10 $\mu g\ ml^{-1}$), bacterial strain (*Leg. pneumophila vs* drinking water bacteria) and/or cell enumeration system (microscope *vs* flow cytometer).

This is the first study to illustrate the heterogeneity in chlorine susceptibility for amoeba-grown *Leg. pneumophila* using BacLight dual stains and culture assay. As *Leg. pneumophila* replicated from *H. vermiformis* exhibit an increased resistance to FC, chlorine efficacy against legionellae might be attenuated in the hot water systems where *H. vermiformis* predominate (Rohr *et al.* 1998; Thomas *et al.* 2006). *Hartmannella vermiformis* can tolerate a higher temperature (53°C) compared with *Acanthamoeba* (Rohr *et al.* 1998). Moreover, *H. vermiformis* may survive a 4 mg l^{-1} of chlorine treatment and further support the intracellular multiplication of legionellae (Kuchta *et al.* 1993). Because chlorination is usually adopted for *Legionella* control in the hot water systems (Breiman *et al.* 1990; Wiedenmann *et al.* 2001; Nakamura *et al.* 2003), *H. vermiformis*, the dominant species with great heat tolerance and chlorine resistance, may have a better chance than *Acanthamoeba* to serve as protective and replicative microenvironments for the growth of *Leg. pneumophila* (Rohr *et al.* 1998). Once released from *H. vermiformis*, chlorine-resistant phenotypes of *Leg. pneumophila* could persist in the chlorinated plumbing system. Moreover, the nutrient deprivation, common in tap water, has been shown to enhance chlorine resistance of planktonic *Leg. pneumophila* (Chang *et al.* 2007). The additive effects of intra-amoebic growth and starvation stress on bacterial chlorine resistance might occur and dramatically reduce the disinfection efficacy. Further study on this hypothesis is warranted.

Maintenance of residual FC in water is commonly recommended for *Legionella* control (EPA 1999; EWGLINET and EWGLI 2005). However, it should be noted that higher residual FC levels in water may not always guarantee greater disinfection efficacy. In comparison with the results from Lp-Ac suspensions, the residual FC level was higher in Lp-Hv suspensions although the chlorine efficacy against *Legionella* was significantly lower in terms of culturability reduction and cellular membrane damage.

This inverse relationship between residual FC concentration and disinfection efficacy against *Legionella* accords with the findings of a recent investigation on hot waters of 40 hotels where *Leg. pneumophila* serogroup 1 colonization was positively associated with a higher FC concentration (Borella *et al.* 2005). The personnel responsible for water disinfection in the places such as hospitals and hotels should note this phenomenon and routinely monitor *Legionella* in addition to maintaining residual FC in the waters, as suggested by EWGLINET and EWGLI (EWGLINET and EWGLI 2005).

In conclusion, chlorination at 2 and 5 mg l^{-1} significantly reduces the culturability of *Leg. pneumophila* released from *A. castellanii* and *H. vermiformis*, and damages cellular membrane to a lesser extent. When compared with *Leg. pneumophila* released from *H. vermiformis*, *Leg. pneumophila* replicated from *A. castellanii* experience a much greater culturability reduction and cellular membrane damage under chlorination, indicating heterogeneity in chlorine susceptibility for amoeba-grown *Leg. pneumophila*. The above-mentioned characteristics could help to improve our understanding of the persistence of *Leg. pneumophila* in chlorinated waters, and should be taken into account in the control of Legionellae proliferation particularly in the water systems where *H. vermiformis* is predominant.

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