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ORIGINAL ARTICLE

Effect of flow regimes on the presence of *Legionella* within the biofilm of a model plumbing system

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Abstract

Aims: Stagnation is widely believed to predispose water systems to colonization by *Legionella*. A model plumbing system was constructed to determine the effect of flow regimes on the presence of *Legionella* within microbial biofilms.
Methods and Results: The plumbing model contained three parallel pipes where turbulent, laminar and stagnant flow regimes were established. Four sets of experiments were carried out with Reynolds number from 10 000 to 40 000 and from 355 to 2000 in turbulent and laminar pipes, respectively. *Legionella* counts recovered from biofilm and planktonic water samples of the three sampling pipes were compared with to determine the effect of flow regime on the presence of *Legionella*. Significantly higher colony counts of *Legionella* were recovered from the biofilm of the pipe with turbulent flow compared with the pipe with laminar flow. The lowest counts were in the pipe with stagnant flow.
Conclusions: We were unable to demonstrate that stagnant conditions promoted *Legionella* colonization.
Significance and Impact of the Study: Plumbing modifications to remove areas of stagnation including deadlegs are widely recommended, but these modifications are tedious and expensive to perform. Controlled studies in large buildings are needed to validate this unproved hypothesis.

Introduction

The water distribution system is the source for Legionnaires' disease occurring in hospitals. Factors that have been linked to *Legionella* colonization in water distribution systems include hot water temperature, age and configuration of the system and calcium concentration (Plouffe *et al.* 1983; Vickers *et al.* 1987; Colbourne *et al.* 1988; Alary and Joly 1992). Stagnation is also widely believed to be a predisposing factor for colonization (Ciesielski *et al.* 1984). For example, Ciesielski *et al.* found that the concentration of *Legionella* was reduced from 100 CFU ml^{-1} to an undetectable level (<10 CFU ml^{-1}), when stagnant hot water tanks were brought back into service. The authors suggested that *Legionella* concentrations might be minimized in water systems by eliminating stagnation (Ciesielski *et al.* 1984). Therefore, numerous authorities and publications have recommended the avoidance of conditions promoting stagnation within water distribution systems to minimize the growth of *Legionella* (Harper 1988; Anon 1996, 2000, 2001). However, the assumption that stagnation predisposes to *Legionella* colonization has not been rigorously assessed. Moreover, in a controlled disinfection evaluation in a hospital, plumbing modifications with the removal of deadlegs had no effect on *Legionella* colonization (Sidari *et al.* 2004).

Thus, a model plumbing system that produced three-flow regimens was constructed to evaluate the effect of

varying flow regimes (turbulent, laminar and stagnation) on the presence of *Legionella* in microbial biofilm within this plumbing model system.

Materials and methods

Plumbing model

The plumbing model was designed as a partially open system (5% of water continuously flowed through the system while 95% of the water was recirculated within the system) (Fig. 1). The solution in the feed tank contained bulk *Legionella pneumophila* and was pumped to a mixing tank that dispensed water into the plumbing system. The turbulent and laminar flows were regulated by ball-valves at both the ends of each pipe. Flow velocities in the pipe were determined by individual flow metres incorporated in each section. The plumbing model was operated at room temperature (24°C) with intermittent flow (8:00 a.m.–10:00 p.m. on, 10:00 p.m.–8:00 a.m. off) to simulate conditions in a water distribution system. Three parallel transparent PVC pipes (1″ × 10′) with turbulent, laminar and stagnant flow conditions, respectively, were used as sampling devices

Inoculum

Legionella-contaminated filter cartridges (Model No. WBRS-5, Culligan Water Conditioning Co., Pittsburgh, PA, USA) removed from the hot water system of a VA hospital building in Pittsburgh were used as the inoculum. For each experiment, one cartridge was mixed with fresh nonsterile cold tap water in the mixing tank and one cartridge was mixed in the feed tank. As a result of using this 'natural' source of inoculum, the starting *Legionella* concentration in the inoculum solution was different for each experiment.

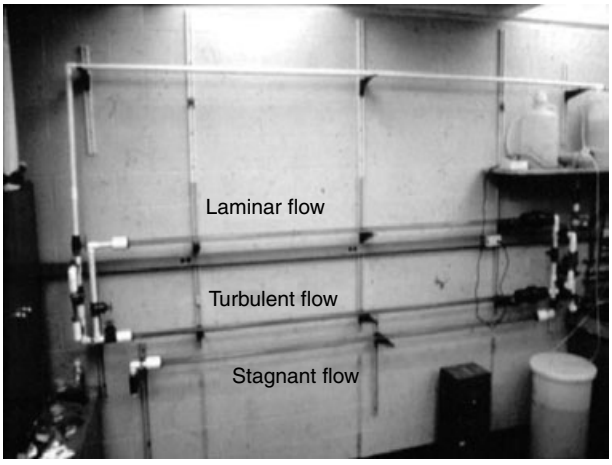

Figure 1 This plumbing model system was designed to study the effect of flow regimes on *Legionella* presence within biofilm. The test section consisted of three parallel clear PVC sampling pipes (from top to bottom: laminar, turbulent and stagnant) of 1 inch in diameter. The four transition ports (4 inch diameter) were connected to inlets and outlets of laminar and turbulent flow sampling pipes to create smooth entry and exit conditions.

Legionella species and serogroup confirmation

A direct fluorescent antibody (DFA) testing kit was used for *Legionella* speciation and serogroups (m-Tech Alpharetta, GA, USA).

Planktonic water sampling

A 250-ml planktonic (bulk water) sample was collected from each sampling pipe by disconnecting the sampling pipe from the exit port. 0·1 ml of the water sample was plated directly and after dilution to 10^{-1}, 10^{-2} and 10^{-3} onto two buffered charcoal yeast extract agar (BCYE) plates and two plates with *Legionella* selective media containing dyes, glycine, vancomycin and polymyxin (DGVP) (Ta *et al.* 1995).

Biofilm sampling

Sessile (surface-adherent) samples were taken by swabbing the inner surface of a section (total surface area = 10·8 cm^2) of pipe starting from both ends (inlet and outlet) of each sampling pipe. The swab was first vortexed for 1 min in 5 ml of sterile water. 0·1 ml of the solution was plated directly and after dilution to 10^{-1}, 10^{-2} and 10^{-3} onto two BCYE and two DGVP plates to culture *Legionella*. 1·0 ml of the solution was mixed with 1·0 ml of HCl-KCl acid for 2 min. 0·1 ml of this acid-treated solution was plated directly and after dilution to 10^{-1}, 10^{-2} and 10^{-3} onto two BCYE and two DGVP plates to culture *Legionella*. Samples were taken pre-start-up and weekly after start-up of the experiments for 5 weeks.

Total suspended solids

Total suspended solids (TSS) in biofilm and planktonic samples were also used as a measure of organic and inorganic content in the system. TSS was measured according to Standard Method 2540B (Anon 1994).

Reynolds number

Reynolds number (Re) was used to classify the fluid property using the following expression:

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$$\mathrm{Re} = \frac{V \times D_{\mathrm{Pipe}}}{\upsilon} \quad (1)$$

where, V is the fluid velocity, D_{pipe} the diameter of pipe, υ the kinematic viscosity of water, which is equal to 0·01 $cm^2\ s^{-1}$ at 25°C.

For pipe flow, Reynolds number below 1000 was classified as laminar flow and Reynolds number above 10 000 was classified as turbulent flow.

Flow regimens

The following Reynolds numbers were tested in the laminar flow sampling pipe and turbulent flow sampling pipe in four different experiments: (1) $Re_1 = 750$ *vs* $Re_2 = 40\ 000$; (2) $Re_1 = 355$ *vs* $Re_2 = 34\ 825$; (3) $Re_1 = 1400$ *vs* $Re_2 = 10\ 000$ and (4) $Re_1 = 2000$ *vs* $Re_2 = 25\ 000$; where Re_1 is the Reynolds number in the laminar flow pipe and Re_2 the Reynolds number in the turbulent flow pipe.

Statistical analysis

Legionella concentrations in planktonic and biofilm samples were compared using the mean colony forming units (CFU) from the four culture plates. The culture results from turbulent, laminar and stagnation flow pipes were compared by mean CFU (*t*-test of unequal variance).

Results

Effect of flow regimes on *Legionella* within biofilm

Legionella pneumophila serogroups 1 and 6 were recovered from the biofilm of all three flow pipes using DFA test. The lowest concentration of *Legionella* was recovered from a pipe with no flow (stagnation, Re = 0) in each experiment (Table 1; Fig. 2). The concentration of *Legionella* (mean CFU cm^{-2}) recovered from the biofilm samples of the turbulent sampling pipe was significantly higher than that recovered from the laminar flow pipes ($P < 0{\cdot}05$) (Table 2). The ratio of *Legionella* CFUs recovered from turbulent and laminar flow pipes in four experiments ranged from 2·0 to 10·4. For example, in experiment 1, *Legionella* concentrations (mean CFU) from the biofilm in a turbulent flow pipe (Re = 40 000) were 6591 CFU cm^{-2} at the inlet and 8759 cfu cm^{-2} at the outlet (Table 1). However, *Legionella* concentrations recovered from the laminar flow pipe (Re = 750) were only 635 CFU cm^{-2} at the inlet and 927 CFU cm^{-2} at the outlet. *Legionella* concentrations recovered from the inlet and outlet of the stagnant flow pipe inlet were 200 and 328 CFU cm^{-2}, respectively. See Table 1 for data for the other three experiments.

Higher suspended solids in the biofilm were also observed in turbulent flow sampling pipes compared with laminar flow and stagnant flow sampling pipes. In experiment 1, the concentration of suspended solids recovered from the biofilm established at the inlet of the turbulent, laminar and stagnant flow pipes were $3{\cdot}8 \times 10^{-4}$, $2{\cdot}9 \times 10^{-4}$ and $2{\cdot}3 \times 10^{-4}$ g ml^{-1}, respectively (data for other experiments are shown in Table 3; Fig. 3).

Effect of flow regimes on *Legionella* in planktonic sample

Table 1 shows that the lowest concentration of *Legionella* was always recovered from the biofilm established in the

Table 1 *Legionella* concentrations in biofilm and planktonic samples from turbulent, laminar and stagnation flow pipes after 5 weeks of recirculation

Experiment ($Re_{laminar}$ *vs* $Re_{turbulent}$)	*Legionella* in inlet biofilm (CFU cm^{-2})	*Legionella* in outlet biofilm (CFU cm^{-2})	*Legionella* in planktonic sample (CFU ml^{-1})
1 (750 *vs* 40 000)			
Turbulent	6591	8759	1545
Laminar	635	927	4257
Stagnant	200	328	631
2 (355 *vs* 34 825)			
Turbulent	564	734	5067
Laminar	276	142	11 865
Stagnant	57	25	1938
3 (1400 *vs* 10 000)			
Turbulent	15000	14167	2125
Laminar	4183	2242	3900
Stagnant	392	1358	1074
4 (2000 *vs* 25 000)			
Turbulent	5408	4300	339
Laminar	2183	1817	633
Stagnant	1308	617	164

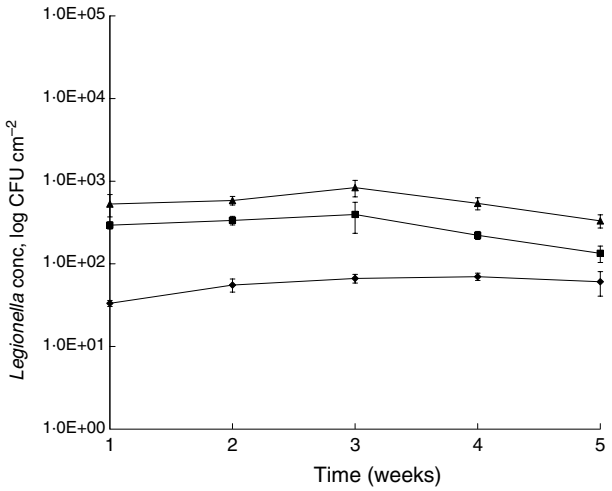

Figure 2 Comparison of *Legionella* concentration in inlet biofilm samples (Re 1 = 355, Re 2 = 34 825). Significantly higher number of *Legionella* counts were recovered from the biofilm of turbulent flow pipe than that from the laminar flow pipe,while the lowest counts were recovered from the stagnant flow pipe ($P = 0{\cdot}01$). Legends: ▲, Turbulent flow; ■, Laminar flow; ●, Stagnant flow.

stagnant pipe (Re = 0) when compared with turbulent and laminar flow pipes. However, the highest concentration of *Legionella* was recovered in the planktonic samples of the laminar flow pipes, which was contrary to the biofilm results. The ratio of planktonic *Legionella* recovered from turbulent to laminar flow pipe for four experiments ranged from 0·36 to 0·54 (Table 2).

Effect of shear stress on *Legionella* within biofilm

Shear stress analysis was performed for experiment 1 only. The concentration of *Legionella* recovered from the turbulent sampling pipe was a maximum of ten times that recovered from the laminar sampling pipe at the 3-week sampling point, while the shear stresses in the turbulent sampling pipe at the inlet site were 10 000 times that of the laminar sampling pipe (0·0063 dyne cm^{-2}). Thus, for Reynolds number of 40 000 or lower, shear stress had no significant effect on *Legionella* presence in microbial biofilms. The shear stress at the inlet of the turbulent sampling pipe (Re = 40 000) was 50·8 dyne cm^{-2},

Experiment ($Re_{laminar}$ *vs* $Re_{turbulent}$)	*Legionella* in inlet biofilm (CFU cm^{-2})	*Legionella* in outlet biofilm (CFU cm^{-2})	*Legionella* in planktonic sample (CFU ml^{-1})
1 (750 *vs* 40 000)	10·4*	5·2	0·36
2 (355 *vs* 34 825)	2·0	9·45	0·43
3 (1400 *vs* 10 000)	3·58	6·32	0·54
4 (2000 *vs* 25 000)	2·47	2·36	0·54

*All values are ratios of *Legionella* concentration in turbulent flow pipes to that in laminar flow pipes. Values greater than 1 indicate that more *Legionella* is recovered from turbulent flow pipe than from laminar and vice versa.

Table 2 Ratio of *Legionella* concentrations in biofilm and planktonic samples from turbulent and laminar flow pipes

Experiment ($Re_{laminar}$ *vs* $Re_{turbulent}$)	TSS in inlet biofilm (g cm^{-2})	TSS in outlet biofilm (g cm^{-2})	TSS in planktonic sample (g ml^{-1})
1 (750 *vs* 40 000)			
Turbulent	$3{\cdot}8 \times 10^{-4}$	$3{\cdot}4 \times 10^{-4}$	$6{\cdot}5 \times 10^{-4}$
Laminar	$2{\cdot}9 \times 10^{-4}$	$2{\cdot}4 \times 10^{-4}$	$1{\cdot}2 \times 10^{-3}$
Stagnant	$2{\cdot}3 \times 10^{-4}$	$1{\cdot}9 \times 10^{-4}$	$3{\cdot}1 \times 10^{-4}$
2 (355 *vs* 34 825)			
Turbulent	$5{\cdot}7 \times 10^{-4}$	$4{\cdot}4 \times 10^{-4}$	$3{\cdot}6 \times 10^{-6}$
Laminar	$5{\cdot}1 \times 10^{-4}$	$3{\cdot}6 \times 10^{-4}$	$8{\cdot}2 \times 10^{-6}$
Stagnant	$4{\cdot}0 \times 10^{-4}$	$1{\cdot}6 \times 10^{-4}$	$1{\cdot}8 \times 10^{-6}$
3 (1400 *vs* 10 000)			
Turbulent	$3{\cdot}9 \times 10^{-4}$	$3{\cdot}3 \times 10^{-4}$	$5{\cdot}8 \times 10^{-4}$
Laminar	$3{\cdot}5 \times 10^{-4}$	$2{\cdot}7 \times 10^{-4}$	$9{\cdot}2 \times 10^{-4}$
Stagnant	$3{\cdot}0 \times 10^{-4}$	$2{\cdot}1 \times 10^{-4}$	$3{\cdot}3 \times 10^{-4}$
4 (2000 *vs* 25 000)			
Turbulent	$4{\cdot}1 \times 10^{-4}$	$3{\cdot}4 \times 10^{-4}$	$6{\cdot}1 \times 10^{-4}$
Laminar	$3{\cdot}6 \times 10^{-4}$	$2{\cdot}6 \times 10^{-4}$	$1{\cdot}0 \times 10^{-3}$
Stagnant	$3{\cdot}0 \times 10^{-4}$	$1{\cdot}9 \times 10^{-4}$	$3{\cdot}3 \times 10^{-4}$

Table 3 Total suspended solids (TSS) in biofilm and planktonic samples from turbulent, laminar and stagnant flow pipes

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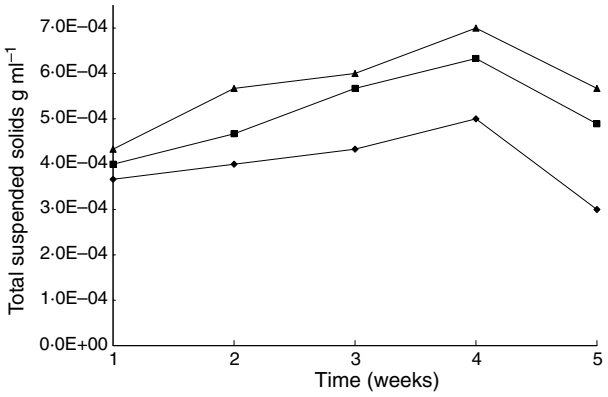

Figure 3 Comparison of total suspended solids (TSS) in inlet biofilm samples (Re 1 = 355, Re 2 = 34 825). More TSS were recovered from the biofilm of the turbulent flow pipe than that from the laminar flow pipe, while the lowest amount was recovered from the stagnant flow pipe. Legends: ▲, Turbulent flow; ■, Laminar flow; ●, Stagnant flow.

which was different from that at its outlet (37·6 dyne cm^{-2}). However, *Legionella* recovered from the inlet and outlet of the turbulent flow pipe were not significantly different (Table 1).

Discussion

Stagnation within water systems has been cited by numerous authors as a condition favouring *Legionella* replication (Ciesielski *et al.* 1984; Harper 1988; Anon 1996). However, the effect of low flow conditions on the presence of *Legionella* in a water system has not been scientifically evaluated. Therefore, we investigated the effect of flow dynamics on the presence of *Legionella* in a model plumbing system under controlled conditions.

Turbulent, laminar and stagnant flow conditions were created by regulating flow velocities through identical PVC pipes. The lowest concentration of *Legionella* was recovered in biofilm samples from the stagnant pipe in each experiment compared with turbulent and laminar flow pipes. It was also visually apparent that turbulent flow resulted in the greatest accumulation of biofilm in the sampling pipe. Measurements of TSS and *Legionella* quantification by culture confirmed this observation (Table 1; Fig. 3).

Reynolds number (Re) was used in this study to describe the flow properties. Using the definition of Reynolds number, the pipe flow can be classified as turbulent (Re > 10 000), laminar (Re < 1000) or stagnant (Re = 0; zero flow). Higher velocity of water flow will result in higher Reynolds number at the same pipe diameter, which then causes higher shear stress. Shear stress is a force due to the friction between the flow and the pipe surface. Theoretically, higher shear stress will result in the detachment of biofilm from the pipe surface (i.e. less biofilm formation). We expected that the *Legionella* recovered from the turbulent flow pipes would be less than that recovered from the laminar flow pipes due to biofilm detachment caused by shear stress. However, our data showed that the formation of biofilm was greater under turbulent flow conditions (Table 1). The biofilm detachment due to shear stress may have been offset by faster biofilm accumulation due to other factors such as mass transfer (Duddrige *et al.* 1982; Rittmann 1982; Kays and Crawford 1966). Alternatively, the shear stress tested in this model was insufficient to cause biofilm detachment.

This apparent contradiction might be explained by the fact that turbulent flow would result in a higher overall mass transfer rate compared with laminar flow. Mass transfer can be described as the efficiency of suspended solids (nutrient) delivery from the bulk phase (flowing water) to the attached phase (biofilm). A higher mass transfer rate would result in greater particle deposition onto the pipe surface, which was indicated by higher TSS found in turbulent flow pipes (Table 3). Leon Ohl *et al.* (2004) showed that increasing the flow velocity in the bulk phase leads to higher biofilm density and higher maximum substrate flux. So it is possible that the turbulent conditions increased oxygen and nutrient availability at the attachment surface, which in turn may have led to the observed increase in *Legionella* under turbulent conditions. The lowest concentration of *Legionella* recovered from stagnant flow pipes may be explained by the limited availability of oxygen and nutrients under these conditions.

The results from our model failed to show that stagnation promoted growth of *Legionella*. Similarly, in a small controlled study of disinfection in a hospital colonized with *Legionella*, removal of deadlegs had no effect on *Legionella* colonization (Sidari *et al.* 2004a,b). Plumbing modifications to remove areas of stagnation including deadlegs are widely recommended, but these modifications are tedious and expensive to perform. Controlled studies in large buildings are indicated to validate this unproved hypothesis.

References

Alary, M. and Joly, J.R. (1992) Factors contributing to the contamination of hospital water distribution systems. *Appl Environ Microbiol* **165**, 565–569.

Anon. (1994) *Standard Methods for the Examination of Water and Wastewater*, 16th edn. Washington DC: American Public Health Association, Inc.

Anon. (1996) *Control of Legionellosis. II:7-1-II:7-41*. Washington DC: Occupational Safety and Health Administration.

Anon. (2000) *ASHRAE Guideline 12: Minimizing the Risk of Legionellosis Associated with Building Water Systems.*

Atlanta, GA: American Society of Heating, Refrigerating and Air-Conditioning Engineers, Inc.

Anon. (2001) *The Control of Legionellosis Including Legionnaires' Disease*. London: Health and Safety Executive, Her Majesty's Stationary Office.

Ciesielski, C.A., Blaser, M.J. and Wang, W.L. (1984) Role of stagnation and obstruction of water flow in isolation of *Legionella pneumophila* from hospital plumbing. *Appl Environ Microbiol* **48**, 984–987.

Colbourne, J.S., Dennis, P.J., Trew, R.M., Berry, C. and Vesey, G. (1988) *Legionella* and public water supplies. *Water Sci Technol* **20**, 5–10.

Duddrige, J.E., Kent, C.A. and Laws, J.F. (1982) Effect of surface shear stress on the attachment of *Pseudomonas fluorescence* to stainless steel under defined flow conditions. *Biotechnol Bioeng* **24**, 153–164.

Harper, D. (1988) The Legionnaires' disease outbreaks – the engineering implications. *J Hosp Infect* **11**(Suppl A), 201–208.

Kays, W.M. and Crawford, M.E. (1966) Momentum transfer: turbulent flow in tubes. In *Convective Heat and Mass Transfer*, 2nd edn. pp. 196–203. New York: McGraw-Hill.

Leon Ohl, A., Horn, H. and Hempel, D.C. (2004) Behaviour of biofilm systems under varying hydrodynamic conditions. *Water Sci Technol* **49**, 345–351.

Plouffe, J.F., Webster, L.R. and Hackman, B. (1983) Relationship between colonization of hospital building with *Legionella pneumophila* and hot water temperatures. *Appl Environ Microbiol* **46**, 769–770.

Rittmann, B.E. (1982) The effect of shear stress on biofilm loss rate. *Biotechnol Bioeng* **24**, 501–506.

Sidari, F.P., Stout, J.E., VanBriesen, J.M., Bowman, A.M., Grubb, D., Neunere, A., Wagener, M.M. and Yu, V.L. (2004) Keeping *Legionella* out of water systems. *Am Water Works Assoc* **96**, 111–119.

Ta, A.C., Stout, J.E., Yu, V.L. and Wagener, M.M. (1995) Comparison of culture methods for monitoring *Legionella* species in hospital potable water systems and recommendations for standardization of such methods. *J Clin Microbiol* **33**, 2118–2123.

Vickers, R.M., Yu, V.L., Hanna, S.S, Muraca, P., Diven, W., Carmen, N. and Taylor, F.B. (1987) Determinants of *L. pneumophila* contamination of water distribution systems: 15 hospital prospective study. *Infect Control* **8**, 357–363.