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Source / Izvornik: **Annals of Agricultural and Environmental Medicine**, 2016, 23, 452 - 455

Journal article, Published version

Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

<https://doi.org/10.5604/12321966.1219186>

Permanent link / Trajna poveznica: <https://urn.nsk.hr/urn:nbn:hr:184:739411>

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Download date / Datum preuzimanja: **2025-01-16**



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Environmental factors affecting the survival of soil dwelling *Legionella longbeachae* in water

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Potočnjak M, Magdalenic Z, Dijan M, Rebić D, Gobin I. Environmental factors affecting the survival of soil dwelling *Legionella longbeachae* in water. Ann Agric Environ Med. 2016; 23(3): 452–455. doi: 10.5604/12321966.1219186

Abstract

Introduction. *Legionella longbeachae*, a causative agent of Legionnaire's disease, has often been associated with potting soil and gardening, a feature quite distinct from other *Legionella* species. The precise transmission mechanism is still unknown, although due to the ecological coherence of the soil and water there is a potential risk of infection by contaminated stagnant water in the garden.

Objective. The aim of the study was to explore the ability of *L. longbeachae* to survive in stagnant tap water usually used for watering in gardens. The influence of different factors (temperature, pH and NaCl concentration) on *L. longbeachae* survival in stagnant tap water was also tested.

Results. The result showed that *L. longbeachae* is viable in stagnant tap water over 100 days at 4 °C and 25 °C. The survival of *L. longbeachae* exposed to different pH and NaCl concentration suggests resistance to low pH values (pH2 and pH5) and all tested NaCl concentrations at temperatures lower than 25 °C. The ability of *L. longbeachae* to persist in stagnant tap water should be taken seriously in the risk assessments as a possible hidden reservoir of infection.

Key words

Legionella, survival, stagnant tap water, temperature

INTRODUCTION

Legionella longbeachae is a significant human pathogen that causes legionellosis in Australia and New Zealand, and is an emerging pathogen worldwide [1, 2, 3, 4]. Two clinical manifestations of legionellosis are Pontiac fever and Legionnaires' disease; the latter presents a far more serious health threat in a form of pneumonia that can be fatal [5]. Recent studies have reported an increased number of infections in Europe [5, 6, 7]. It is important to notice that the diagnosis of infection with *Legionella* spp. other than *Legionella pneumophila* has been a persistent challenge. *L. longbeachae* would not be distinguished from *L. pneumophila* with the routine *Legionella* urinary antigen tests; therefore, infections caused by this species of *Legionella* occur are under- detected [8]. Despite causing a clinically indistinguishable infection, they have distinct environmental niches: *L. pneumophila* is an aquatic organism, found in both natural and human-made aquatic environments, and *L. longbeachae* is predominantly found in soil environments, with most human infections associated with contaminated potting soil, particularly with dripping hanging pots and poor garden hygiene [6, 9]. The main route of transmission of Legionnaires' disease is the inhalation or aspiration of water aerosols contaminated with *L. pneumophila*. For infection linked to gardening and compost use, it is still questionable whether *L. longbeachae* is present in the dust or aerosol.

Although belonging to the same genus and sharing some basic common characteristics, different studies have shown significant differences in the intracellular life cycle in amoeba and human cells, as well as in the genome of *L. longbeachae* and *L. pneumophila* [7, 10]. *L. longbeachae* also

shows distinction in its virulence factors which might help its survival in the potting soil environment – production of a capsule, the chemotaxis system and cellulolytic enzymes [10].

The water environment presents a natural habitat for most *Legionella* species where these bacteria live in a biofilm and survive and multiply within free-living amoebae [11]. The risk of infection caused by the *L. pneumophila*, has been well investigated throughout the years [12]. Factors affecting the survival of *L. pneumophila* in water have been well explored, and the optimal range of pH for survival is between 6.0–8.0 and temperatures between 25 °C and 42 °C [13]. Also, *L. pneumophila* showed resistance to chlorine, which is routinely used to disinfect water [14]. Previously, we have shown that both *Legionella* species, *L. pneumophila* and *L. longbeachae*, survive for more than 30 days in rainwater samples [15]. Taking this under consideration, we now explore the environmental conditions in stagnant tap water that might pose a potential reservoir of *L. longbeachae* infection, shedding new light on this under- investigated *Legionella* strain.

MATERIALS AND METHOD

Bacterial strain. In this the study, *L. longbeachae* serogroup I (A5H5 strain), clinical isolate from Australia, kindly provided by Elizabeth L. Hartland, Department of Microbiology and Immunology, University of Melbourne, Australia was used. Bacteria were stored at -80 °C in glycerol broth (Biolife, Italy) and were cultured by standard procedures on buffered charcoal yeast extract agar (BCYE agar pH 6.9) (Oxoid, England), supplemented with sterile ferric acid and L-cystein, at a temperature of 35±2 °C.

Stagnant tap water. In all experiments, tap water from the public water supply of the city of Rijeka, Croatia, was used. The salinity, pH values and conductivities of tap water used in

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Received: 14 September 2015; accepted: 09 March 2016

this study in the test period was: salinity amounted to 0, pH ranged from 7.5–8.0, and conductivity from 216–300 $\mu\text{S}/\text{cm}$. According to these characteristics, the water was medium hard. The tap water was sterilized by autoclaving and kept in a glass bottle at room temperature for 2 days for the evaporation of free residual chlorine prior to testing.

Inoculum. *L. longbeachae* was cultured on BCYE for 3 days at $35\pm 2^\circ\text{C}$ and then subcultured in AYE broth at $35\pm 2^\circ\text{C}$ for a further 2 days. Bacteria were harvested by centrifugation at 5,000 g for 10 min, the pellet was washed twice in a sterile stagnant tap water and bacterial suspension, adjusted to an OD600 nm of 1 (1×10^9 CFU/ml). Further dilution was prepared and suspensions of approximately 10^6 CFU/ml and 10^8 CFU/ml in 10 mL stagnant tap water were used in survival studies. The viable bacterial counts were confirmed retrospectively by cultivating serial ten-fold dilution on BCYE agar plates. All the experiments were performed in triplicate.

Temperatures. The effects of different temperatures (4°C , 25°C , 37°C and 42°C) on the survival of *L. longbeachae* in stagnant tap water were tested [16].

pH. Four different pH values were tested: pH2, pH 5, pH7 and pH8. Each value was tested at 25°C and 37°C [13].

NaCl solutions. Five different salt solutions were used to study the salt tolerance of *L. longbeachae*. The following concentrations were tested: 0.8%, 1%, 2%, and 3% (w/v) NaCl. Sodium chloride was dissolved in distilled water, autoclaved, and distributed in plastic test tubes (TPP, Switzerland). Sterile, distilled water was used as a control. Each concentration was tested at 25 and 37°C (6).

Survival studies. Viable *L. longbeachae* counts were determined by cultivating the samples immediately after inoculation and at different time points up to 50 or 100 days. For each time point, 3 plastic test tubes (TPP, Switzerland) were inoculated in parallel. Cultures were vortexed and the number of viable bacteria in the samples was determined by enumeration of the bacteria on BCYE α -agar.

RESULTS AND DISCUSSION

A water environment presents a unique challenge for bacterial survival, mainly because of the lack of nutrients. Recently, the global incidence of reported *L. longbeachae* infections increased, but the factors explaining this emergence of infections are still unknown. The major source of human infection is considered to be commercial potting mixes and other decomposing materials, such as bark and sawdust [4, 6, 17]. *L. longbeachae* is not often detected in water samples and man-made water systems [4]. The assumption is that the primary transmission mode of this microorganism is inhalation of dust or water aerosol created during watering from contaminated compost or soil. Steele *et al.* suggested that *L. longbeachae* leaches out of potting mix after watering, and may be present in any aerosols formed during the watering process, which could be inhaled by the gardener [6]. It is very common for people to collect rain and tap water to use for watering their gardens; however, this creates aerosols which could present potential health risks. Although laboratory

microcosms are not exact replicates for condition in nature, they can provide baseline ecological information. Therefore, we explored how different environmental conditions affect the survival of *L. longbeachae* in stagnant tap water.

***L. longbeachae* is viable in stagnant tap water for over 100 days.** We have shown that stagnant tap water is a suitable medium for the survival of *L. longbeachae*, and after analyzing all tested conditions the survival of bacteria was longer when the inoculum was higher. The presented results show that higher temperature, as well as a lower number of bacteria, had a negative impact on the survival of *L. longbeachae* in stagnant tap water (Fig. 1). After inoculating 10^6 CFU/ml bacteria in the tap water a loss of cultivability on temperatures 42°C , 37°C and 25°C , at day 5, 15 and 90, were detected, respectively. These results are consistent with the results of Arago-Cervero *et al.* who recently showed the sensitivity of *L. longbeachae* to thermal treatment in tap water at temperatures above 50°C [14]. In the same study, *L. longbeachae* was extremely sensitive to chlorine concentrations of 0.2 mg/ml and 0.5 mg/ml. In the presented experiments we used stagnant tap water in which the chlorine level was reduced during the testing period which led to increased bacterial survival. This model mimics the conditions during gardening. At 4°C the number of bacteria declined for 2 log units by the end of the experiments (100th day after inoculation). When higher inoculums was used, *L. longbeachae* was cultivable at 4°C and 25°C during the whole experiment period, while at 37°C and 42°C , for 25 and 5 days, respectively.

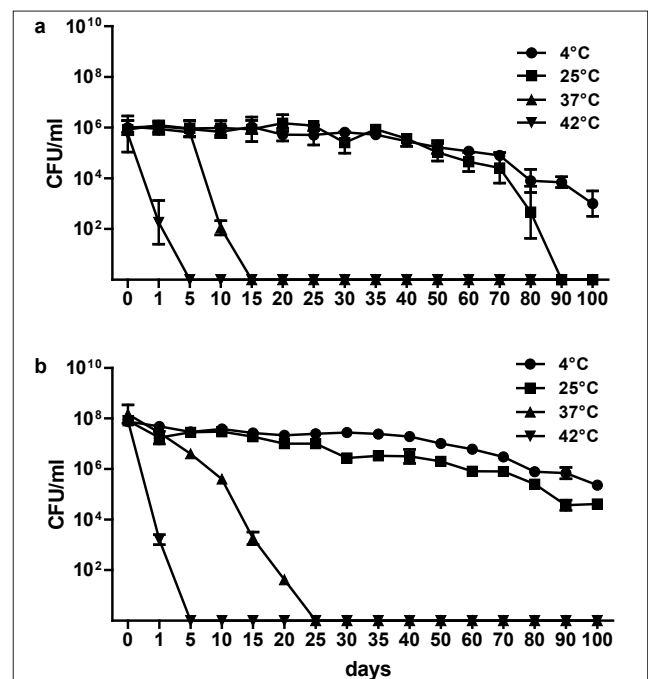


Figure 1. Effect of inoculum sizes 10^6 CFU/mL (a) and 10^8 CFU/mL (b) and temperature on survival of *L. longbeachae* in tap water. Plate counts were performed at various time points to determine the number of viable cells

Diverse temperature optimum between two *Legionella* species. The ideal temperature range for *L. pneumophila* propagation is between 25°C – 42°C [18], whereas *L. longbeachae* favours a lower temperature range of between 4°C – 25°C . Also, *L. longbeachae* is more sensitive to pH under

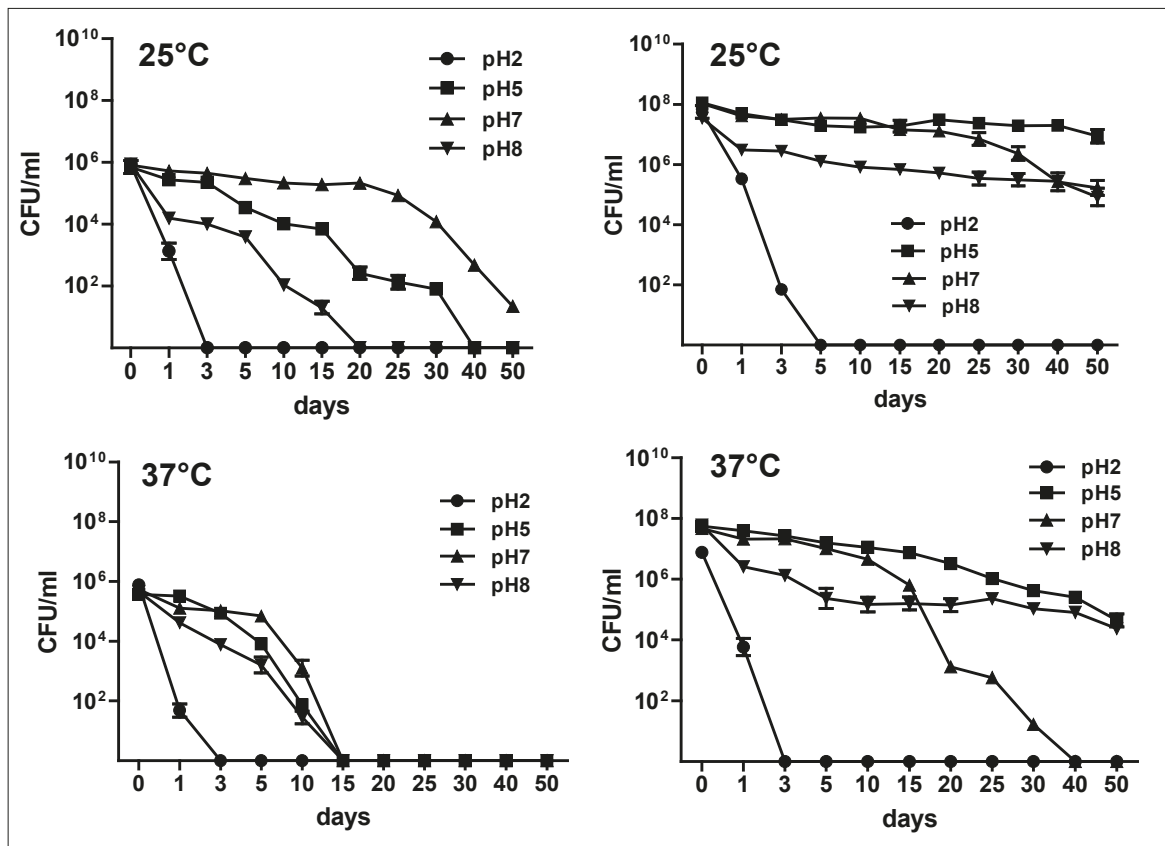


Figure 2. Effect of inoculum sizes (10^6 CFU/mL (left) and 10^8 CFU/mL (right)), pH (pH2, pH5, pH7 and pH8) and temperature (25°C and 37°C) on the survival of *L. longbeachae* in tap water. Plate counts were performed at various time points to determine the number of viable cells

incubation at higher temperature of 37°C , and loss of cultivability was detected after exposure to pH2 and pH7 after 3 and 40 days, respectively (Fig. 2). In the current study, the presence of viable but non-cultivable (VBNC) were not investigated.

Resistance of *L. longbeachae* to extremely low pH. A larger inoculum size (10^8 CFU/ml), and incubation at 25°C extended survival time when exposed to altered pH values; with the exception of pH 2 when bacteria were cultivable after 50 days (Fig. 2). Katz and Hammel demonstrated that *L. pneumophila* was cultivable for 1 month in tap water varying in pH from 4.0–8.0 [19]. With extremely low pH2 where *L. pneumophila* is rapidly killed and most of bacteria survived for only a few hours, *L. longbeachae* survived up to 3 days, suggesting the resistance of this bacteria to low pH [18].

Long survival in an unfavourable water environment.

The survival of *L. longbeachae* in distilled water, as well as in distilled water with different NaCl concentrations was examined. Significantly better survival was detected at 25°C where bacteria were cultivable more than 50 days. The present results are in accordance with earlier studies showing that a small amount of NaCl enhanced the survival of *Legionella* at lower temperatures [16, 20]. *L. longbeachae* were cultivable for 50 days in pure sterile distilled water (Fig. 3). One explanation is a recent discovery which suggests that this bacterium possesses a capsule which enables its longer survival in unfavorable environmental conditions [10]. However, we wish to point out that the structure and function of a *L. longbeachae* capsule has not yet been

characterized, nor whether the capsule affects the survival of this bacterium in different environmental conditions. Further research is necessary.

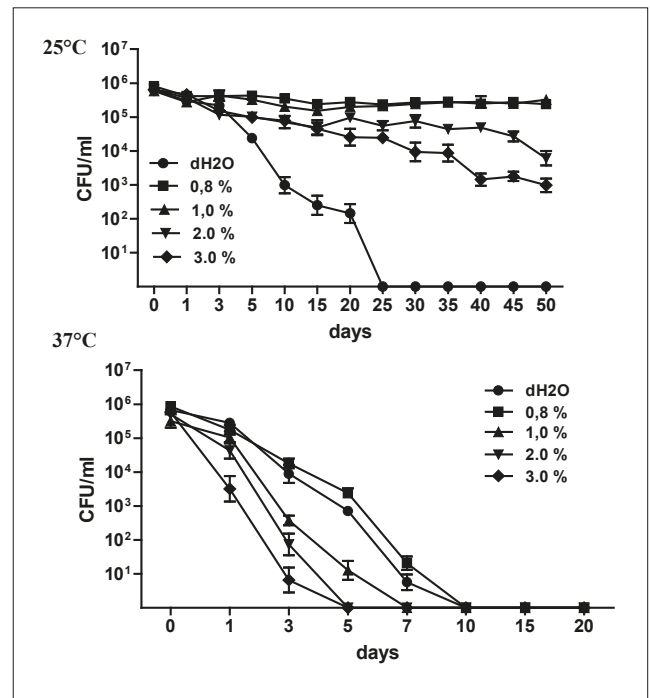


Figure 3. Effect of incubation temperature (25°C and 37°C) and different concentrations of NaCl on the survival of *Legionella longbeachae* in distilled water. Plate counts were performed at various time points to determine the number of viable cells

CONCLUSION

The soil dwelling *Legionella* is able to survive in stagnant tap water under lower temperatures for a long period of time and is relatively resistant to changes of pH and the concentrations of NaCl. These conditions are possible to find in stagnant tap water in gardens, making it a potential reservoir of *L. longbeachae* infection. The presented study contributes to the incomplete knowledge about the impact of fundamental environmental factors on the survival of *L. longbeachae* in a water environment, but further research of this bacterium is required, e.g. its natural habitat, propagation abilities, and life cycles.

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