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The survival of *Legionella* in rainwater

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ABSTRACT

Legionella is an environmental bacteria and a significant worldwide public health problem. These bacteria can survive a long period of time in distilled water, and more than a year in tap water. Due to these characteristics we were interested to explore how long it will survive in rainwater and if heavy bacterial contamination or bacterial microbiota would affect it's survival. Rainwater is a good alternative source of water in households and is used for watering gardens, washing yards and as bathing water. The rainwater from ten different tanks was analyzed and the results showed that in 90 % of samples the number of microorganisms is elevated. Two rainwater samples were chosen according to the amount of microbiota and survival of *L. pneumophila* and *L. longbeachae* was monitored. The results showed that both types of *Legionella* have been able to survive for 30 days in the rainwater samples but haven't been able to replicate. The rainwater microbiota restricted the survival of both *Legionella* strains although the effect was more pronounced in a case of *L. longbeachae.* These results indicate that both *Legionella* species can survive in the rainwater environment which means the rainwater could represent a reservoir for *Legionella*.

Key words: *Legionella*, rainwater, survival

INTRODUCTION

The genus *Legionella* belonging to the family *Legionellaceae* currently has at least 52 species comprising more than 70 distinct serogroups. Most *Legionella* species are found in aquatic environments, the exception is *L. longbeachae*, the only soil-dwelling pathogenic *Legionella* species [1]. *Legionella* are ubiquitous in natural aquatic environments and are capable to survive in waters with varied temperatures, pH levels, nutrient and oxygen contents. Also, *Legionella* species have the ability to successfully colonize man-made water handling and storage systems, which often provide ideal conditions of nutrition and temperature for their proliferation. Their widespread survival in water can be attributed to their relationships with other microorganisms. Symbiotic existence with algae and other bacteria, particularly biofilms, increases the availability of nutrients. They are also able to infect protozoas and subsequently reproduce within these organisms.

Although *L. pneumophila* is responsible for the vast majority of legionellosis worldwide some of non-pneumophila species like *L. longbeachae* are also capable of causing disease [1]. *L. longbeachae* is responsible for up to half of legionellosis in Australia and Thailand while in Europe and USA it causes sporadic cases [2].

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Rainwater is traditionally collected in Primorsko-goranska County in water tanks, which are, by the appearance of water supply, increasingly used as an alternative source rather than primary sources of drinking water [3]. Previous study of microbiological quality of rainwater showed that 66 % of samples have heavy bacterial contamination while 44 % of samples had fecal contamination. The lack of cleaning and maintenance were the main reasons for that condition.

The objective of the present study was to test if the presence of heavy bacterial contamination in rainwater could create ecosystem favorable to *Legionella* survival and multiplication. The survival of two types of *Legionella*, *L. pneumophila* and *L. longbeachae* in two samples of rainwater were examined.

MATERIALS AND METHODS

Bacterial inoculum

Two types of *Legionella* were used in the experiment: *Legionella longbeache* NSW150, *Legionella pneumophila* strains Corby and 130b. Bacteria were stored at -80°C in glycerol broth (10 % glycerol). *Legionella* were used directly from the buffered charcoal yeast extract agar plates (BCYE-α, Oxoid). The number of bacteria in the inoculum was measured spectrophotometically (600 nm), OD (Optical density) was set to 1 what corresponded to a concentration of 1.0 x 10^9CFU (Colony Forming Units). The number of viable bacteria in the inoculums was confirmed by enumeration of the bacteria on BCYE agar. Bacteria cells were transferred into 10 mL of filter-sterilized and non-sterilized rainwater samples to a final concentration of approximately 1.0 x 10^6 CFU.

Rainwater samples

Ten samples of rain water (**Table 1**) were collected from water tanks from different region of Primorsko-goranska County. To test the presence of bacterial microbiota in rainwater samples the total aerobic bacteria at 22 °C, 37 °C and 44 °C on Peptone yeast extract glucose agar were determined. To test the presence of indicators of fecal contaminations, total coliform bacteria on Les Endo agar; fecal coliform bacteria on mFC agar; intestinal enterococci on KEA agar; sulphite-reducing clostridia on Sulfite agar were detected by membrane filtration. For survival studies two rainwater samples were selected according to the presence of bacterial contamination and analysis were conducted in non-sterile and sterile samples. The rainwater samples were sterilized by repeated filtration through a 0.2-μm-pore-size filter.

Table 1.
Microbiological quality of 10 rainwater samples.

Samples	Fecal coliform	Fecal enterococci	Total coliform	Total aerobic bacteria on 22 °C	Total aerobic bacteria on 37 °C	Total aerobic bacteria on 44 °C
	cfu/100 mL			cfu/mL		
1	0	0	57	96	164	6
2	0	0	69	100	40	2
3	5	11	12	259	140	0
4	11	17	Uncountable	1200	992	27
5	0	0	1	23	55	0
6	0	1	0	106	260	0
7	0	0	0	0	0	0
8	0	0	14	0	53	0
9	0	0	13	6	1	0
10	0	0	12	1	5	0

The limit for FC, FE, TC were 0 CFU/100 mL; TAB on 22 °C 100 cfu/mL; TAB on 37 °C 20 cfu/mL

Quantification of viable *Legionella* in water samples

At various time points after inoculation, a 100-μL aliquot was aseptically removed from each of the 10-mL experimental cultures. All plate counts were done in duplicate using BCYE agar plates with antibiotic (Vancomycin (0.001 g/L) and Colistin (0.0015 g/L). Results were expressed as the number of CFU per mL.

Statistical analysis of data

All data points in each experiment were obtained in triplicate, and statistical calculations were made with GraphPad Prism version 4.00 for Windows. P values were calculated using the unpaired t test, and results were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Rainwater is collected all around the world as good alternative source of water. It is collected by gutters on the roof and stored in appropriate water tanks [4,5]. Factors that contribute to the bacterial contamination of rainwater are inadequate cleaning and maintenance of tanks, the stagnation of water in the tanks and the water temperature in the hot parts of the year [5].

In this study the microbiological quality of rainwater from 10 different rainwater tanks in Primorsko-goranska County were analyzed (Table 1). Our results showed that in 70 % rainwater samples the number of bacteria cultivated at 37 °C was elevated. In three samples the fecal contamination was detected while in 8 samples total coliform bacteria were detected.

This result is consistent with previous results which showed that due to inadequate cleaning and maintenance of rainwater tanks the heavy bacterial contaminations of rainwater is common. For example, 41 % of rainwater samples tested by Sazakli *et al.* in Kefalonia, Greece, were positive for *E. coli* [6]. In studies from 2006, in Brazil 50 % of the samples fecal contamination was detected and 98 % of samples had elevated fecal enterococci. The recent study in Primorsko-goranska County also showed that 73 % of the rainwater samples had heavy bacterial contamination [10].

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As for many other purposes rainwater is often used for watering gardens during which aerosols could be created and that presents potential health risks. It is well known that human inhalation of contaminated aerosols leads to *Legionella* infections and disease outbreaks. *L. pneumophila* was linked to Legionnaires' disease, respectively, amongst users of the rainwater as a potable water supply in the US Virgin Islands [7]. The investigation from New Zealand showed that roof-collected rainwater systems in a temperate climate can provide a suitable reservoir for the survival and proliferation of *Legionella* and may have subsequently caused cases of Legionnaires' disease [5].

So, the purpose of this study was to determine whether *L. pneumophila* and *L. longbeachae* are able to survive and multiply in rainwater and if the presence of bacterial contamination has an impact on their survival.

Two rainwater samples were chosen for survival study according to the presence of bacterial contamination (Table 1). Rainwater from sample 4 showed heavy bacterial contamination with high number of fecal coliforms and fecal enterococci, while in sample 5 small amount of bacterial microbiota were present and indicator of fecal contamination were not detected.

Since the water is a natural habitat of *L. pneumophila* two different strains of this species: *L. pneumophila* strain 130B and strain Corby were tested.

Since the water is a natural habitat of *L. pneumophila* two different strains of this species: *L. pneumophila* strain 130B and strain Corby were tested. The starting dose of bacteria was 10^6 CFU/ml, and the samples were incubated at room temperature. The results showed that after the inoculation the number of bacteria was constant for 5 days. After that the decline in the number of bacteria occurs in unfiltered samples and after 30 day of incubation number of bacteria decreased for 3 logarithmic units. In filtered samples there were no significant changes in bacterial number during 30 days. There were no differences in survival between two *L. pneumophila* strains and comparison between unfiltered samples showed no difference in the survival of *L. pneumophila* strain 130b (Figure 1 A), while Corby strains survived better in the 4th sample (Figure 1 B).

Although the natural environment of *L. longbeachae* is moist soil, it is not yet known exactly how these bacteria come into the ground and what is the exact route of transmission [8,9]. Therefore, the survival of *L. longbeachae* was tested in the filtered and unfiltered samples of rainwater. The results showed that *Legionella* also survived all 30 days in rainwater samples. The number of bacteria in filtered water samples is constant throughout the study period, while in the unfiltered samples the number of bacteria was significantly reduced after the second day of incubation. On the 30th day of incubation 10^2 cfu/ mL *L. longbeachae*

The results showed that *Legionella* also survived all 30 days in rainwater samples.

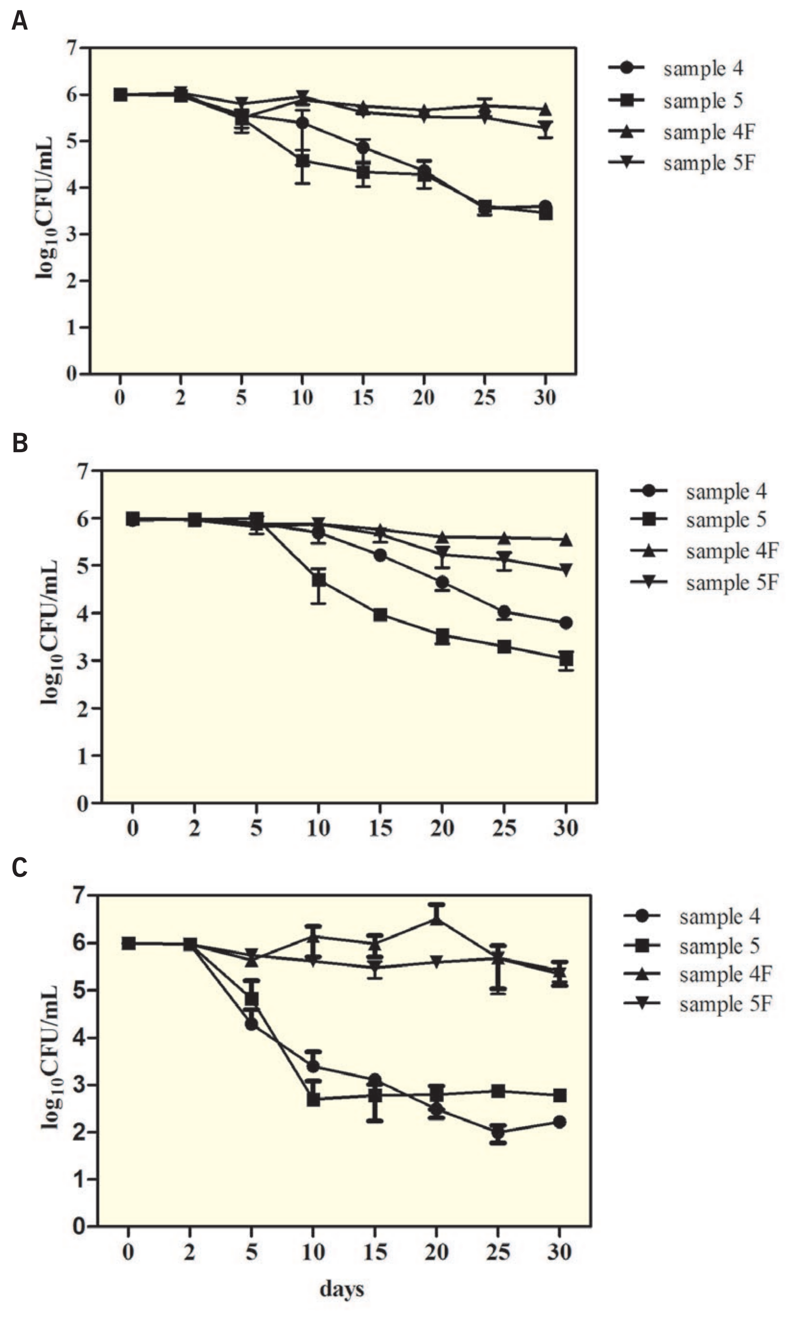

Figure 1.
Survival of *L. pneumophila* strain 130b (A), strain Corby (B) and *L. longbeachae* strain NSW150 (C) in two rainwater samples. Plate counts were done at various time points to determine viable cells remaining. The data are the means of duplicate experiments and the error bars indicate standard deviations.

To conclude, both *Legionella* strains were not able to multiply but they did survive in rainwater for 30 day.

was detected (Figure 1 C). The results showed that heavy bacterial contamination of rainwater restricted the survival of *L. longbeachae.* It seems that this *Legionella* species is not adapted to the rainwater microbiota. This hypothesis was recently confirmed and analysis of the *L. longbeachae* genome has demonstrated that *L. longbeachae* is highly adapted to the soil environment [11, 12].

To conclude, both *Legionella* strains were not able to multiply, but they did survive in rainwater for 30 day. *L. pneumophila* survives better in rainwater with a complex bacterial flora in comparison to *L. longbeachae*. All tested bacteria survive better in filtered samples so the presence of bacterial contamination affects their survival in rainwater. Further research into the ecology of *Legionella* species in rainwater systems is needed.

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