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ORIGINAL ARTICLE

Role of biofilms in the survival of *Legionella pneumophila* to sodium chloride treatment

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ABSTRACT

Background and Objectives: Legionnaires' disease continues to be a public health concern. Colonized water distribution systems are often implicated in *Legionella* transmission, despite the use of various disinfection strategies, the bacterium is capable to persist and survive in water systems. The aim of this study was to investigate the persistence of *Legionella pneumophila* to sodium chloride over time at different temperatures and analysing the role of biofilms in the survival of this bacteria.

Materials and Methods: *L. pneumophila* serogroup 1 and *L. pneumophila* serogroup 2-15 were used to study the effect of sodium chloride on planktonic and sessile cells. The tested concentrations were: 0.5%, 1%, 2%, 3%, 4%, 6% and 8% (W/V) NaCl. Biofilms were grown on 24-well microplates.

Results: At 20°C, *L. pneumophila* planktonic cells were able to survive in sodium chloride concentrations up to 2%. However, at 37°C, a sodium chloride concentration over 1.5%, reduced systematically the numbers of bacterial cells. Biofilms were grown for 20 days in the absence and presence of sodium chloride. The results show that bacterial strains were able to survive and regrow after the sodium chloride shock (2-3%). Moreover, it seems that this effect is less expressed with the age of the biofilm; old biofilms were more persistent than the young ones.

Conclusion: Results from this study demonstrate that the sodium chloride disinfection strategy was effective on *Legionella pneumophila* planktonic cells but not on biofilms, which demonstrate the role of biofilms in the persistence and recolonization of *L. pneumophila* in water distribution systems.

Keywords: Biofilms; *Legionella pneumophila*; Disinfection; Sodium chloride; Water-borne diseases

INTRODUCTION

Legionella pneumophila is a waterborne pathogen and the causative agent of Legionnaires' disease, an atypical pneumonia (1) and Pontiac fever, a self-limited flu-like illness (2). Currently, the genus *Legionella* comprises 59 species and more than 70 distinct serogroups (3). *L. pneumophila* causes approximately 90% of all reported Legionnaires' disease cases, 84% of which are associated to *L. pneumophila* serogroup 1 (3, 4). The infection is normally transmitted through the inhalation of aerosols containing the bacteria generated from contaminated water sources such as cooling towers, water distribution networks,

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spa pools, fountains, etc. *L. pneumophila* is an inhabitant of natural and manmade aquatic environments, which can be involved in biofilm-associated contamination of plumbing systems (5, 6) surviving free and as an intracellular parasite of protozoa (7). Various factors can influence the colonization and proliferation of bacteria on pipeline surfaces and biofilm formation including temperature, type of pipe materials, chemical element concentration of the water and hardness and, flow rate, presence of nutrients and disinfectants (8).

Contamination of water networks occurs when opportunistic pathogens are released from a biofilm as a consequence of physical disturbance or active detachment of infectious cells, which then pose a potential threat to human health (9, 10). These pathogens have the potential to detach from the biofilm to return to the planktonic state or colonize other surfaces which increases the risk of water contamination (11, 12). In water distribution systems, *L. pneumophila* can colonise and adhere to solid surfaces of pipe materials, hot water tanks, taps, shower, cooling towers. Biofilm, as being a microbial community which may include bacteria, yeasts, fungi, and protozoa adherent to a surface and surrounded by matrix composed of organic polymers (13), may play an important role as a niche protecting bacteria from external stresses such as the action of disinfectants (14).

Previous studies have been conducted in order to compare the susceptibility of *L. pneumophila* to various concentrations of free residual chlorine, temperature and pH values (15-17) and *L. pneumophila* has been found to be much more resistant and persistent to chlorine, and also able to surviveand recolonize water networks after repeated cycles of treatment (15, 18). It has been generally concluded that higher pH values, lower temperatures and lower chlorine content increase the survival rate of *L. pneumophila* (16, 18). Temperatures between 20 and 50°C are suitable for the growth of *L. pneumophila*. Accelerated growth occurs at temperatures between 37 and 42°C, while measurable slow growth commences at temperatures above 50°C (14, 18). Despite the increased monitoring and advances in detection methods, there is still a lack of knowledge about the microbial ecology of *Legionella* and its response to treatment. Therefore, contamination of hot water systems with *Legionella* remains a persistent major concern and a threat to public health. In order to improve the efficiency of water treatment, we aimed to investigate the persistence of *Legionella pneumophila* to sodium chloride over time at different temperatures (20 and 37°C) and analysing the role of biofilms in the survival of this bacterium.

MATERIALS AND METHODS

Bacterial strains, growth conditions and preparation of bacterial suspension. Strains used in this study were *L. pneumophila* serogroup 1 and *L. pneumophila* serogroup 2-15 obtained as previously described (14). *L. pneumophila* strains were cultured in Glycine-Vancomycin-Polymyxin-Cycloheximide (GVPC) at 37°C ± CO_2 (2.5%) for 72 h. After culture, the cells were harvested by centrifugation for 15 min at 8400 g and were washed twice and resuspended in KNO_3 solution with ionic strength 0.1M.

Survival experiments of planktonic cells. The survival of *L. pneumophila* in planktonic culture was investigated as follow. Sodium chloride was dissolved in distilled water, autoclaved, and distributed in test tubes (10 ml). Five different solutions were prepared to investigate the sodium chloride tolerance of *L. pneumophila*. The tested concentrations were: 0.5%, 1%, 2%, 3%, 4%, 6% and 8% (W/V) NaCl and sterile distilled water was used as control.

The effect of two different temperature on the survival of *L. pneumophila* in NaCl solutions was tested. Each concentration of NaCl was examined at 20 and 37°C. All test liquids were inoculated with 100 µL of the test suspension (10^6 cfu/mL) and incubated at 37°C for 72 h. Cell numbers were determined by plate count immediately after inoculation of the tubes and after 6, 24, 48, 72 and 96 h. The plates were incubated at 37°C for 72 h. Analysis was performed in triplicate on each sample.

Survival experiments of *L. pneumophila* biofilm. This study was conducted using the 24-well microplates. 3 mL of broth medium supplemented with L-Cysteine (BYE) was added to each well. A total volume of 100 µl of the test inoculum was added to each well. The plates were sealed with parafilm and incubated at 37°C, with the media being replaced at two or three days. Biofilm development was followed over a period of 20 days, based on the determination of total cell counts. CFUs were counted by using the serial dilution technique of the bacterial suspension

obtained after sonication. Counts were determined on GVPC after incubation at 37°C ± 2.5% CO_2 for 72 h.

Sodium chloride shock was performed on day 6 and 15 for the biofilm previously formed on each well. Therefore, NaCl was prepared into sterile distilled water to provide concentrations of 1%, 2%, 4%, 6% et 8%. The 24-well microplates were rinsed twice with 3 ml of sterile distilled water and agitated gently to remove any non-adherent cells, then 3 ml of each salt concentration were added to each well. The microplates are covered and incubated at room temperature for 6 hours. Subsequently, the microplates were rinsed twice with 3 ml of sterile distilled water to remove any residual sodium chloride. The adhered cells were detached by sonication (2 min/60 kHz). The number of colonies forming units (CFU) was determined immediately after shock and after 6, 10, 15, 18 and 20 h. The plates were incubated at 37°C ± 2.5% CO_2 for 72 h.

Data analysis. Cell counts were log-transformed to meet the assumption of homoscedasticity. Statistical analyses of the NaCl effects on sessile vs. planktonic cells and time in culture were performed by two-way analysis of variance (ANOVA). Tests were two tailed, with a = 0.05. All analyses were performed using SPSS 13.0.

Ethical approval. This article does not contain any studies with human participants or animals performed by any of the authors.

RESULTS

Effect of sodium chloride on planktonic cells. We investigated the ability of planktonic cultures of *L. pneumophila* to survive to sodium chloride disinfection at 20 and 37°C. The NaCl concentrations ranging from 1% to 4%. The results of *L. pneumophila* survival after incubation at 20 and 37°C in NaCl solution with different concentrations are shown in Figs. 1 and 2.

A slight decrease was observed during the first hours at 20 and 37°C for concentrations up to 2%. At 20°C, the reduction occurred mainly during the first hours of incubation, after which cell numbers remained relatively unchanged between day one and day 3 for the concentrations up to 3%. The salt concentrations had only a minimal effect on the reduction of cell numbers at concentrations below 3%. The reduction in all assays was about 1 log units, showing that the level of salt concentration had no effect on the survival rate of *L. pneumophila*. However, at 37°C, higher sodium chloride concentrations had a great influence on the survival of *L. pneumophila*. As seen before at lower incubation temperatures, NaCl concentrations up to 2% and 3% had a minimal effect on the reduction rates, but when the concentrations were increased further, cell numbers declined rapidly.

At 37°C, 96 h after inoculation into 1% and 1.5% NaCl solutions, the reduction was about 2 log units. The reduction at 2% and 3% were 2.5 and 3 log units, respectively. At 4%, the log reduction exceeded 5 log units. However, low concentrations of 0.5% clearly improve the survival rate of *L. pneumophila* in comparison with the control without NaCl. Comparing the survival of positive control, no noticeable differences were seen over the whole experimental period.

The comparison of the survival curves obtained at 20 and 37°C showed that the decrease of cell numbers was more important at 37°C and the reaction was more pronounced.

These results indicate that the combined deleterious effects of sodium chloride and temperature were most pronounced when stressing bacteria with 4% NaCl and incubation temperature of 37°C. The reduction of the cells is greater at 37 than at 20°C for the tested concentrations (the efficiency of sodium chloride is better at 37°C). We have found that the combination of high temperature (37°C) with concentrations above 4% showed a significant reduction in bacterial cell numbers. These finding suggest that the efficiency of these concentrations was not sufficient to reduce the numbers of *Legionella* may occasionally survive in waters that have been judged to be microbiologically acceptable.

Effect of sodium chloride on biofilm. Biofilm development was followed over a period of 20 days, based on the determination of total cell counts. Biofilm was grown on 24-well microplates. Sodium chloride shock took place on day 6 and 15 for the biofilm previously formed on each well. Therefore, sodium chloride was prepared into sterile distilled water to provide concentrations of 1%, 2%, 4%, 6% et 8%. The number of colonies forming units (CFU) was determined immediately after shock and after 6, 10, 15, 18 and 20 h. The plates were incubated at 37°C ± 2.5%

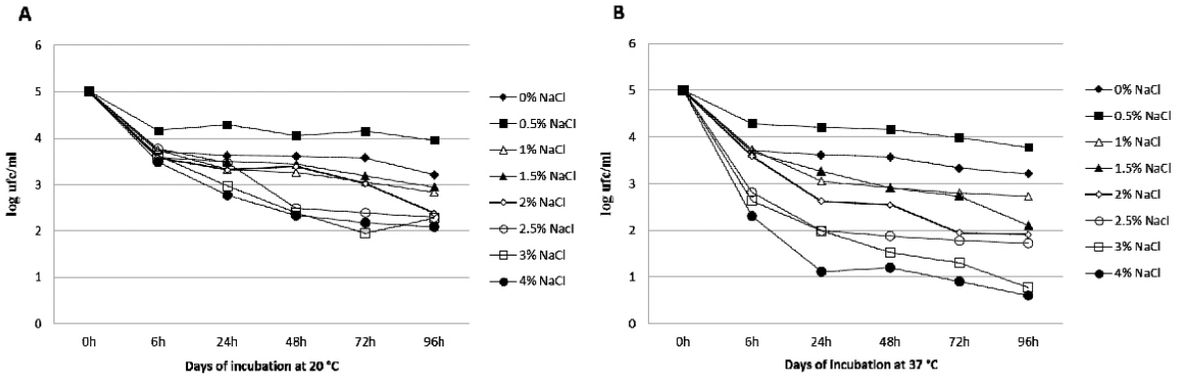

Fig. 1. Effect of NaCl concentrations on the survival of *L. pneumophila* sg1 at 20°C (A) and 37°C (B).

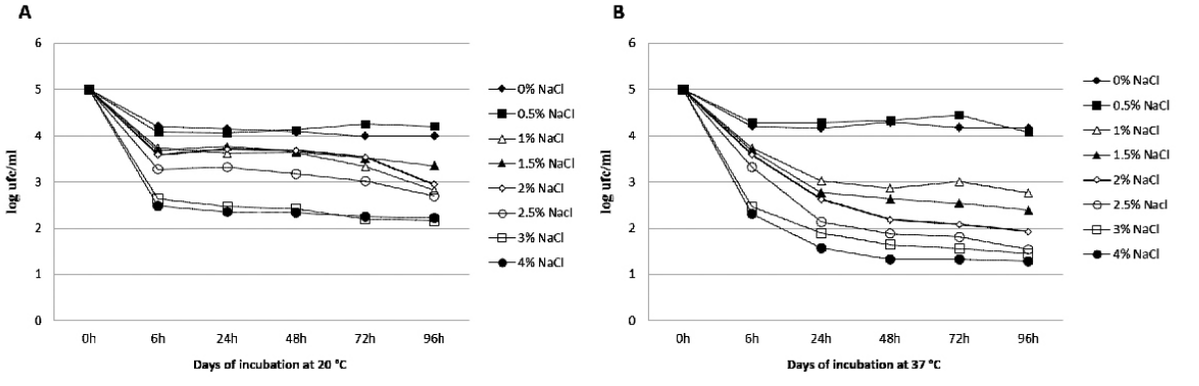

Fig. 2. Effect of NaCl concentrations on the survival of *L. pneumophila* sg2-15 at 20°C (A) and 37°C (B).

CO_2 for 72 h.

Fig. 3. shows the growth of *L. pneumophila* sg1 and 2-15 biofilm challenged with a range of sodium chloride concentrations at day 6 and 15. The total number of cells increased rapidly on the surface of each well. However, neither of the two *L. pneumophila* strains tested was detectable immediately after sodium chloride shock.

Biofilms of the *L. pneumophila* sg1 and sg2-15 strains both decreased in viable count after challenge with sodium chloride at various tested concentrations.

Comparing biofilm growth curves after NaCl shock on 6^{th} and 15^{th} day, a minimal reduction of cells forming the biofilm was observed after treatmentat concentrations up to 1 and 2%. This allows a re-increase of the biofilm biomass the days after treatment. However, solutions exceeding 4% have allowed an important and continuous reduction of biofilm.

Immediately after treatment, no colonies were recovered, but detectable cells being observed six days after. The number of cells forming biofilms recovered from the treated biofilms re-increase in a stable progression, however, did not reach the rate of the untreated biofilms. The biofilms re-increase to become about 100-fold smaller than the positive control.

For 15-day biofilms of *L. pneumophila*, the greatest log reduction rate was observed after exposure to 4% (4 log units). The sodium chloride did not eradicate all the biofilm cells, rather they continued to grow, but at levels lower than the untreated biofilms.

The majority of biofilms exposed to sodium chloride reached 5.79 log for *L. pneumophila* sg2-15 and 5.6 log for *L. pneumophila* sg1, compared to 9.81×10^6 CFU per ml for *L. pneumophila* sg1 and 1.25×10^7 CFU for *L. pneumophila* sg2-15 for the positive control, which indicate a measurable reduction of cells forming biofilms resulting from the effect of sodium chloride (Fig. 3).

The reduction in number of cells forming the six-day biofilm is greater than that of the 15-day biofilm, suggesting that the old biofilm is more resistant to treatment compared to the young biofilm.

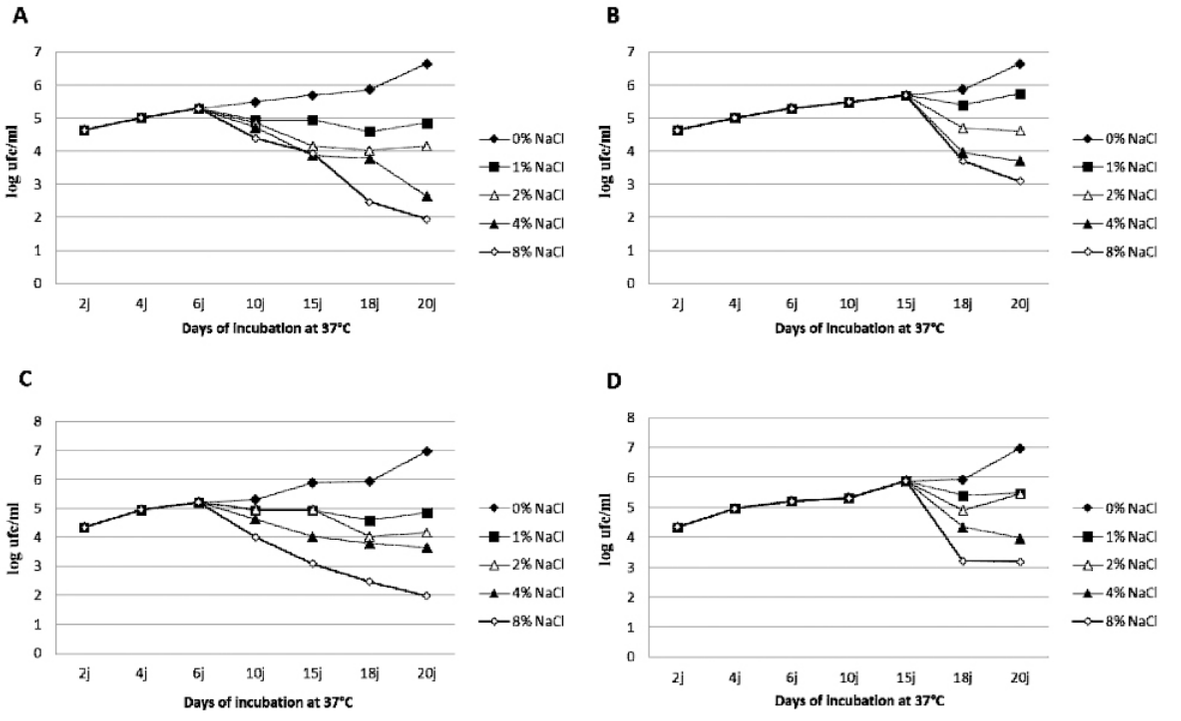

Fig. 3. The growth of *L. pneumophila* sg1 (A and B) and *L. pneumophila* sg2-15 (C&D) when challenged with a range of NaCl concentrations at day 6 and 15.

DISCUSSION

The colonization of various water systems by bacteria is increasingly identified as a recurring source of problems for industry and society. It has been estimated that about 95% of all microbial cells present in water distribution systems exist as biofilms on pipe surfaces and only 5% occur in the water phase (9); similarly, in a domestic hot water system, most of the culturable bacteria (72%) were found to be surface-associated (19). During the past several years, *Legionella* has been isolated from shower heads, taps, hot water systems of hotels, hospitals and homes. In a number of cases, the occurrence of *Legionella* in the plumbing systems was associated with diseases. The contamination of hot water systems with *Legionella* remains a persistent environmental challenge and a threat to public health.

The fact that a large number of residentials facilities and hotels are in coastal Moroccan cities, we thought to use seawater as an alternative approach to disinfect the water systems. Therefore, to improve the efficiency of water treatment and validate our assumption, we examined in laboratory conditions the effect of sodium chloride against *L. pneumophila* planktonic cells and biofilm; we have tested the NaCl sock to reduce and minimize the colonisation and proliferation of *L. pneumophila* in water distribution systems. It is known that the chlorination and thermal disinfection were the two-disinfection method usually used worldwide, but despite the advantages they present, they are expensive, and they can generate indisputable effects relating to the deterioration of the pipe materials and canalisation.

Many disinfection procedures exist, such as hyperchlorination, monochloramine, copper–silver ionization, temperature, point-of-use-filtration, and UV light; however, each of these methods has benefits and shortfalls (20, 21). The effect of sodium chloride on growth and survival of *Legionella* in water distribution systems has been exanimated by a few studies. Results on the effectiveness of salt shock, chlorine and other methods for the eradication of these bacteria differ (22). Several studies have tested the response of the *Legionella* planktonic cells in the presence of free chlorine and the effect of hyperchlorination on biofilms but have not observed a significant reduction in the number of the cells (11, 23-25). In other hand, previous studies have reported the success of different procedures for removing bacterial

cells from premise plumbing systems (20, 21). Most of the disinfection methods are often unsuccessful against *Legionella* biofilms, for this reason, the determination of the persistence of these pathogens is primordial. In the present study, *L. pneumophila* was found to persist in salt solutions up to 2% NaCl at temperature of 20 and 37°C. These finding indicate that these bacteria can survive also in sea water as the NaCl level in Moroccan seawater does not exceed 2.5% and seawater temperature is about 20°C. At 37°C, a relationship was observed between increased salt concentrations and the higher reduction rate of cell numbers.

The specific role played by sodium chloride in the metabolism of *L. pneumophila* has not been examined, but it is well known that sodium is involved in metabolic carrier systems and is also important co-factor in enzymes. For chloride ions, a mild association with growth of *L. pneumophila* has been described with earlier studies which mentioned the fact that *L. pneumophila* can resist to NaCl effect. Moreover, Żbikowska (26) demonstrated that *L. pneumophila* organism are more resistant to the combined bactericidal effect of sea water and sunlight than normal sewage bacteria. Furthermore, the present results also agree with the investigations of Gast (27) who showed that *Legionella* were capable of growing in broth containing low concentrations of NaCl.

On the other hand, several authors have reported an inhibitory effect of NaCl on growth and isolation of *L. pneumophila* (28). To some extent, our findings confirm these toxic effects of NaCl. Small amounts of 0.5% NaCl promote bacterial growth. Salt concentrations up to 0.5% had no toxic effects, but association of concentrations over 2% and high temperature clearly decreased the cell numbers.

CONCLUSION

Resistance among biofilms is higher, this finding showed an overview of the ability offered by biofilms to *L. pneumophila* to survive sodium chloride disinfection and continue to grow even after treatment. Sodium chloride concentrations up to 2% did not have effective effects on planktonic cells, but concentrations over 2% clearly decreased the number of cells. Results from this study demonstrate that the sodium chloride disinfection strategy was effective on *Legionella pneumophila* planktonic cells but not on biofilms, which demonstrate the role of biofilms in the persistence and recolonization of *L. pneumophila* in water distribution systems. Therefore, new preventive and eradication strategies should be developed to reduce *L. pneumophila* infections.

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