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# The Effect of *Aeromonas spp.* on the Growth of *Legionella pneumophila in vitro*

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## Abstract

This study was designed to determining the ability of six *Aeromonas* spp. isolated from different water sources (sanitation plants, water distribution systems, and Reverse Osmosis water) to inhibit the growth of *Legionella pneumophila* serotype 1 -15, from the same sources, in vitro.

Eighteen isolates of 6 *Aeromonas* spp. were tested for their ability to inhibiting the growth of six isolates of *L. pneumophila* serotype1-15.The interactions between *L. pneumophila* and *Aeromonas* spp. were investigated by using cells from broth culture and as solid culture and cell-free supernatants (CFSs) of the latter on the growth of the former on the LAB medium. Results showed that *A. schubertii, A. encheleia* and *A. hydrophila* supernatant, cells and solid cultures have the ability to inhibiting the growth of *L. pneumophila* serotypes 1 and 2-15. While all tested *Aeromonas* spp. inhibited both *L. pneumophila* serotypes, when directly inoculated or transferred as ready grown solid culture. The results indicated that growth and multiplication of *L. pneumophila* could be inhibited by other bacteria sharing the same habitat and the level of this effect varies among the species.

The presence of *Aeromonas spp*. and *L. pneumophila* in drinking water can be an important threat to public health, thus greater awareness of these bacteria as potential enteropathogens is warranted.

## Introduction

Most species belonging to *Aeromonas* genus, particularly those associated with human infections, are widely distributed in the environment, especially in freshwater, sewage, marine environments, and drinking water, and are also found in a wide range of animal and plant food products [1,2]. The US Environmental Protection Agency proposed *Aeromonas hydrophila* as one of the contaminants of concern in waterborne diseases [3]. *Legionella pneumophila*, the causative organism of Legionnaires disease, can infect and kill specific species of amoebae in aquatic environments and can multiply as an intracellular parasite in human phagocytic cells [4]. *Legionella* spp. are commonly found in aquatic environments, and responsible for 1 to 5% of cases of community-acquired pneumonia (CAP) [5]. Approximately 70 to 90% of *Legionella* infections are caused by *L. pneumophila* serogroups 1 and 6 [6]. Scientists have realized that in the natural world, more than 99% of all bacteria exist as biofilms [7]. The most alarming results are the presence and multiplication of pathogenic and opportunistic pathogens such as *Escherichia coli, Pseudomonas, Aeromonas* and *Legionella* spp. occurring within biofilms [8,9]. The previous studies found that heterotrophic bacteria isolated from environmental water sources were capable of inhibiting the growth of *Legionella* species on solid media and included several *Aeromonas* strains [10,11]. The purpose of this investigation was to evaluate the ability of *Aeromonas* spp. isolated from aquatic sources (water sanitation plants, water distribution systems, and reverse osmosis water) in Basrah governorate Iraq, to inhibit the growth of *L. pneumophila* serogroup1-15, from the same sources, *in vitro*.

## Materials and Methods

### *Aeromonas* spp. and *L. pneumophila* growth conditions

A duplicate of 100 ml water samples were filtered by membrane filtration (MF) technique using 47 mm cellulose acetate filters with a nominal pore size of 0.45 µm (Sartorius, Germany). Filter papers were cultured aerobically at 37°C for 24 hours on Ampicillin Dextrin Agar with Vancomycin (ADA-V) supplemented with 5 ml of both ampicillin dextrin selective supplement and vancomycin (Himedia\India) [12]. The other filter papers were cultured aerobically at 35°C for 3 days on Legionella Agar Base (LAB) medium which consists of (g/l) yeast extract 10.0, charcoal activated 1.5, ACES buffer 6.0, α- ketoglutarate 1.0, potassium hydroxide 1.5 and agar 17.0 and supplemented with colistin sulphate, vancomycin, trimethoprim and amphotericin B (Himedia\ India) [13]. Identification of *Aeromonas spp*. was carried out by rapid identification system Hi Carbohydrate Kit (Himedia) and biochemical tests. Eighteen isolates have been tested for their inhibitory ability, while six isolates as *L. pneumophila* were identified by rapid Slide-Agglutination test system using Hi Legionella Latex Kit (Himedia) and biochemical tests, as serogroup 1 and serogroup 2- 15.

### Preparation of cell-free supernatants (CFSs)

Pure cultures of all the 18 *Aeromonas* isolates were suspended in 10 ml of nutrient broth each to obtain a concentration of $6\times10^8$ CFU/ml. and then centrifuged at 10000 rpm for 15 min. Supernatants were filtered through a filter papers with pore size of 0.22 µm(11) and stored at -20°C.

### Assay of ability of *Aeromonas spp.* to inhibit growth of *L. pneumophila*

Each of the *L. pneumophila* serogroup was suspended in 10 ml of

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nutrient broth to obtain a concentration of $3\times10^8$ CFU/ml, then 0.1 ml of the suspension was spread evenly over the complete surface of LAB plate and subjected to the following treatments

a. Cell-free supernatants (CFSs): by micro pipetting, 20 µl CFS of each of the six *Aeromonas* species on to the inoculated LAB medium plates. Sterile distilled water used as control.

b. Small pieces (6×6 mm) of each of the six *Aeromonas* species bacterial growth on ADA-V medium was placed onto the surface of inoculated LAB plates by using wire loop. A small part (6×6 mm) of sterile ADA-V medium was used as control.

c. A loopful of each of the six *Aeromonas* species bacterial growth on ADA-V medium (the bacterial growth without the medium) was placed onto the surface of inoculated LAB plates.

d. Inoculated plates were incubated aerobically at 37°C for 3 days, and then examined for zones of *L. pneumophila* growth inhibition surrounding each of the *Aeromonas* isolates. The inhibition zones were measured in millimeters, and then compared with control.

## Results

The results of the *Aeromonas* supernatants have revealed their ability to inhibiting the growth of *L. pneumophila* serogroup1 (Figure 1) as *A. encheleia* has the largest inhibitory diameter of 12 mm followed by *A. hydrophila* with an inhibitory diameter of 6.75 mm, while *A. schubertii* has inhibitory diameter of 5.75 mm.

The (CFSs) of *A. caviae, A. eucrenophila*, *A. veronii* bv. *veronii* showed no inhibitory effect on the growth of *L. pneumophila* of the first serogroup. The (CFSs) of *A. schubertii* had the largest inhibitory diameter of 18.5 mm on the growth of *L. pneumophila* (serogroup: 2-15) followed by *A. encheleia* (11.5 mm), *A. hydrophila* (9.5 mm) and

**Figure 1**: The inhibiting effect of Aeromonas spp. supernatants on the growth of *L. pneumophila.*

| Isolates | cell-free supernatants (CFSs) | |
|---|---|---|
| | *L. pneumophila* serogroup 1 | *L. pneumophila* serogroup 2-15 |
| ***A. hydrophila*** | 6.75 | 9.5 |
| ***A. caviae*** | - | - |
| ***A. eucrenophila*** | - | 6.5 |
| ***A. schubertii*** | 5.75 | 18.5 |
| ***A. veronii bv. Veronii*** | - | - |
| ***A. encheleia*** | 12 | 11.5 |

**Table 1:** Zone inhibition diameters (mm) of the cell-free supernatants (CFSs) of *Aeromonas* spp. on the growth of *L. pneumophila*_

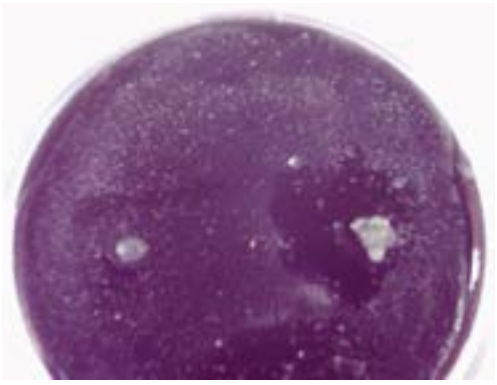

**Figure 2**: The inhibiting effect of Aeromonas solid culture on the growth of *L. pneumophila*.

| Isolates | *L. pneumophila* serogroup 1 | *L. pneumophila* serogroup2-15 |
|---|---|---|
| ***A. hydrophila*** | 11.5 | 9. 5 |
| ***A. caviae*** | 9.5 | 7.5 |
| ***A. eucrenophila*** | 9 | 7.5 |
| ***A. schubertii*** | 11.5 | 10.5 |
| ***A. veronii bv .veronii*** | 19 | 8.5 |
| ***A. encheleia*** | 12 | 11.5 |

**Table 2:** Zone inhibition diameters (mm) of *Aeromonas* spp. growth on the growth of *L. pneumophila.*

| Isolates | *L. pneumophila* serogroup 1 | *L. pneumophila* serogroup 2- 15 |
|---|---|---|
| ***A. hydrophila*** | 9.5 | 10.5 |
| ***A. caviae*** | 6 | 4 |
| ***A. eucrenophila*** | 10 | 6 |
| ***A. schubertii*** | 11 | 11.5 |
| ***A. veronii bv .veronii*** | 10 | 8 |
| ***A. encheleia*** | 8 | 7 |

**Table 3:** Zone inhibition diameters (mm) of Aeromonas spp. growth alone on the growth of *L. pneumophila.*

*A. eucrenophila* which had inhibitory diameter of 6.5 mm, while (CFSs) of *A. caviae* and *A. veronii* bv. *veronii* had not shown any inhibitory effect on the growth of *L. pneumophila* serogroup: 2-15 (Table 1).

It is noted that all in case of solid culture of *Aeromonas spp.* (Figure 2) have shown ability to inhibiting the growth of *L. pneumophila* serogroup 1 with different diameters from 19 mm for *A. veronii* bv. *veronii* to 9 mm for *A. eucrenophila*. While there was a lower inhibitory effect of *Aeromonas spp*. solid cultures on the growth of *L. pneumophila* serogroup 2-15 as *A. encheleia* and *A. schubertii* inhibitory zones were 11.5 mm and 10.5 mm respectively while the inhibitory zone of *A. caviae* and *A. eucrenophila* were 7.5 mm (Table 2).

Regarding the effect of *Aeromonas spp.* growth alone on growth of *L. pneumophila* serogroup 1, *A. schubertii* had the largest inhibitory diameter of 11 mm. This is followed by *A. eucrenophila* and *A. veronii* bv. *veronii* with an inhibitory diameter of 10 mm for each of them , and *A. encheleia* with an inhibitory diameter of 8 mm. While the ability of *Aeromonas spp*. growth alone in inhibiting the growth of *L. pneumophila* sero group: 2-15 has varied ranging from 11.5 mm by *A. schubertii* to 4 mm by *A. caviae* (Table 3).

As seen above, it is noted that all solid cultures and the bacterial

growth alone of the six *Aeromonas* spp. isolated from drinking water were able by different ranges in inhibiting the growth of *L. pneumophila* serogroup 2-15, and serogroup 1. In addition, these serogroups were more sensitive towards the solid culture and the *Aeromonas spp.* growth alone as compared with their CFSs.

## Discussion

*Aeromonas spp*. are found worldwide in aquatic environments, including ground water, surface waters, estuarine and marine waters, drinking water, and wastewater [14]. *Legionella* are also commonly found in aquatic environments, this bacterium has even been isolated from drinking water [15]. The presence of these two types and other bacterial types living in the same environment may affect the growth of each other [11,16]. Storey et al. [17] have indicated that the growth of *L. pneumophila* depends on other living microorganisms that are in the same environment and the reactions that occur between *L. pneumophila* and other living microorganisms are still unknown. These reactions may be limited by the production of bacteriocins and these compounds have a major role in dynamism of population in environmental systems [18]. The available information about the sensitivity of *L. pneumophila* for these compounds are limited as Héchard et al. [19] have indicated the role of the peptides secreted by *Staphylococcus warneri* in inhibiting the growth of *L. pneumophila*.

The supernatants (CFSs ) of *Aeromonas spp*. that are isolated from the drinking water have shown different sizes of inhibition zone diameter. It was also noted that there was no effect at all for the supernatants of *A. caviae and A. veronii bv. veronii* on the growth of *L. pneumophila*. The difference in the effect of the (CFSs) for the six types of *Aeromonas* that are isolated from the drinking water may be related to the difference of the extracellular compounds secreted by these types that in turn affect the growth of *L. pneumophila* as the secretion of these extracellular compounds varies according to the environment and this depends on the types of bacteria that are in the same environment and on the growth conditions [20]. The differences in the ability of the bacterial (CFSs) of the different types of *Aeromonas* on the growth of *L. pneumophila* can be also attributed to the differences in the bacterial strains belonging to different species of *Aeromonas* and the nature of the extracellular secretions for each strain [21].

It is noted in this study that *A. encheleia* has recorded the largest inhibitory rate for the growth of *L. pneumophila* of the first serogroup and no references were found about the presence of any studies indicating its effect on the growth of *L. pneumophila*. So, this study may be the first one of its type to isolate *A. encheleia* from the drinking water in Iraq and studying its effect on the growth of *L. pneumophila*. It is known that this species is one of the non-pathogenic species for human beings [22] and the presence of other non-pathogenic types of *Aeromonas species* that are able to inhibit the growth of *L. pneumophila* of the first serogroup causing 80% of pneumonia cases [23] is a sort of possible control as it works as limiting factors for the growth and spread of pathogenic *L. pneumophila* in water sources.

In the solid culture and bacterial growth experiments all *Aeromonas species* were able at different rates, in inhibiting the growth of *L. pneumophila* serogroup 1-15 especially *A. schubertii* which had the maximum inhibitory rate for the growth of *L. pneumophila*. This may be related to the incubation periods for *Aeromonas* species before being transferred to the growth medium for *L. pneumophila* which allowed the possibility for providing secondary metabolites when bacterial cells approached a static growth period and as *L. pneumophila* is relatively a slowly-growing bacteria. The difference in growth inhibitory rates may be related to the limited activity of these secondary metabolites for each type, besides, *Aeromonas species* produce some extra-cellular compounds including enzymes and toxins and probably one or more of these enzymes can have an anti-bacterial activity. The results of the current study are consistent with the results of [11,24] as *Aeromonas species* can affect the growth of *L. pneumophila* by different rates and it is obvious that environmental balance is a necessary factor for the growth of *L. pneumophila* in drinking water systems. The study showed that *L. pneumophila* occupies an environmental position in water distribution systems via their relationship with other bacteria like *Aeromonas*, and probably it is possible to control the growth of *L. pneumophila* through *Aeromonas* that are naturally present in the same environment.

The different effects of *Aeromonas* species on the growth of *L. pneumophila* are still unknown [25], and this requires further studies especially molecular studies to understand the causes behind the different effects in the growth of *L. pneumophila*.

To our knowledge, this is the first study in Iraq which determined the inhibitory effects of *Aeromonas spp*. against *L. pneumophila* isolated from different water sources in Basrah governorate. The results indicated that growth and multiplication of *L. pneumophila* could be affected by other bacteria sharing the same habitat and the level of this effect varies among the species. The presence of *Aeromonas* spp. and *L. pneumophila* in drinking water can be an important threat to public health, thus greater awareness of these bacteria as potential enteropathogens is warranted.

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