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. enchelea 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 amoeboae 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 serogroup 1-15, 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 6×10⁸ 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
nutrient broth to obtain a concentration of $3 \times 10^8$ 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 serogroup 1 (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 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 serogroup 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

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

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

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

<table>
<thead>
<tr>
<th>Isolates</th>
<th>L. pneumophila serogroup 1</th>
<th>L. pneumophila serogroup 2-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. hydrophila</td>
<td>6.75</td>
<td>9.5</td>
</tr>
<tr>
<td>A. caviae</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A. eucrenophila</td>
<td>-</td>
<td>6.5</td>
</tr>
<tr>
<td>A. schubertii</td>
<td>5.75</td>
<td>18.5</td>
</tr>
<tr>
<td>A. veronii bv. Veronii</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A. encheleia</td>
<td>12</td>
<td>11.5</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Isolates</th>
<th>L. pneumophila serogroup 1</th>
<th>L. pneumophila serogroup 2-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. hydrophila</td>
<td>9.5</td>
<td>10.5</td>
</tr>
<tr>
<td>A. caviae</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>A. eucrenophila</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>A. schubertii</td>
<td>11</td>
<td>11.5</td>
</tr>
<tr>
<td>A. veronii bv. Veronii</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>A. encheleia</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
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<th>L. pneumophila serogroup 2-15</th>
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</thead>
<tbody>
<tr>
<td>A. hydrophila</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A. caviae</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A. eucrenophila</td>
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<tr>
<td>A. schubertii</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A. veronii bv. Veronii</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A. encheleia</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

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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 CFs.

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 (CFs) 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 (CFs) 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 (CFs) 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. schuberti 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.

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.

References


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