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Comparative Study of Some Virulence Factors and Analysis of Phylogenetic Tree by *16S rDNA* Sequencing of *Aeromonas hydrophila* Isolated from Clinical and Environmental Samples

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Abstract

Aeromonas hydrophila is widely distributed throughout the world and causes diseases to animals and human exposed to contaminated environments such as water and soil. This study aimed to compare between isolates of *A. hydrophila* collected from clinical and environmental samples, through investigating the phenotype of some virulence factors *in vitro,* including hemolysin, protease, lipase, nuclease and biofilm formation ability. Also, the antimicrobial susceptibility for different antibiotics was determined using disc diffusion method. For genotypic identification of isolates and phylogenetic tree construction, *16S rDNA* target gene was amplified and sequenced. The phenotypic results showed some differences between the isolates (clinical and environmental). All isolates were resistance to clindamycin, amoxicillin and erythromycin while susceptible to gentamicin, amikacin and vancomycin. Sequences of *16S rDNA* confirmed the identification of the studied bacteria as *A. hydrophila* with 99-100% , and identity and phylogenetic tree by neighbor-joining clearly separated the isolates in a branching pattern which displayed similarity to the GenBank isolates obtained from Asian regions. The clinical isolates showed less polymorphism than the environmental isolates.

Keywords: *A. hydrophila*, *16S rDNA* phylogenetic tree, plants rhizospheres soil, virulence factors.

دراسة مقارنةفي بعض عوامل الضراوة وتحليل الشجرة الجينية التطورية بواسطة تسلسل قواعد *16S rDNA* لبكتريا *Aeromonas hydrophila* المعزولة من العينات السريرية والبيئية

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الخلاصة

تنتشر بكتريا *Aeromonas hydrophila* على نطاق واسع في جميع أنحاء العالم وتسبب الأمراض للحيوانات والبشر المتعرضة للبيئات الملوثة مثل الماء والتربة. هدفت هذه الدراسة إلى مقارنة بين بكتريا *A. hydrophila* المعزولة من عينات سريرية وبيئية (تربة منطقة جذور النباتات) من خلال دراسة الخصائص المظهرية لبعض عوامل الفوعة في المختبر والتي شملت تحلل الدم، افرازها للأنزيم الحال للبروتين، الانزيم الحال للدهون، الانزيم الحال للحامض النووي والقدرة على تشكيل الغشاء الحياتي. أيضا ، تم تحديد حساسية العزلات لمضادات حيوية مختلفة باستخدام طريقة نشر القرص. من أجل التحديد الوراثي للعزلات وبناء الشجرة

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الجينية ، تم تضخيم وتتابع تسلسل الجين *16S rDNA*. وقد أظهرت النتائج المظهرية بعض الاختلافات بين العزلات السريرية والبيئية. كانت جميع العزلات مقاومة للكينداميسين وأموكسيسيلين وإريثروميسين في حين أنها حساسة للجنتاميسين والأميكاسين والفانكوميسين. اكد تسلسل الحمض النووي *16S rDNA* للبكتريا المدروسة على انها بكتريا *A. hydrophila* بنسبة تشابه ٩٩-١٠٠٪ وتم فصل العزلات المدروسة بانماط متفرعة عن طريق شجرة النشوء والتطور وبأستخدام طريقة neighbor-joining والتي اظهرت تشابه مع عزلات بنك الجينات المعزولة من المناطق الآسيوية. اظهرت العزلات السريرية تباينًا أقل من العزلا ت البيئية.

Introduction

Aeromonas hydrophila is an ubiquitous, aerobic, Gram negative, mesophilic and motile bacteria. It is isolated from aquatic environments, fish, various food products [1] and soil[2]. It is usually involved in human infections such as septicemia, gastroenteritis, cellulitis, wound sepsis with necrosis, gangrene, pneumonia and traveler's diarrhea, resulting from improper handling or consumption of contaminated food [3].

The pathogenesis of *A. hydrophila* is complex and involves many virulence factors [4]. *A. hydrophila* produces several extracellular products such as proteases, haemolysins, aerolysin, cytolytic enterotoxins that are related with virulence [5]. Hemolysin and cytolytic enterotoxin secreted by bacteria are important for lytic activities in host cells [6]. Protein layers, O-antigens, fimbriae and outer membrane proteins of *A. hydrophila* play essential roles in adherence mechanisms and contribute to colonization of fish tissue [7]. The genes coding for these virulence factors might be differentially expressed in *Aeromonas* species depending on the environmental conditions of water or the host [8].

Antimicrobial resistance in *Aeromonas* is mediated usually by genes mapped to bacterial chromosome, plasmids or integrons that confer resistance to most beta lactam antimicrobial agents [9].

Identification and classification of bacterial species by molecular methods is widely used. The sequences of several housekeeping genes such as *gyrB, rpoB, rpoD* and *cpn60 UT* were shown to be an effective approach for the classification of *Aeromonas* species [10]. 16S ribosomal RNA represents a powerful molecular tool for the identification of bacterial species [11]. *16S rDNA* consists of extremely conserved regions interspersed with moderate to low homology regions in related species [12]. Direct sequencing of this gene is well accepted as a marker for bacterial identification due to its stability and specificity [13]. Bacterial classification based on sequencing proved to be efficiently useful for phylogenetic identification at different levels [14].

The present study aims to compare between *A. hydrophila* isolated from clinical and plant rhizosphers soil samples at the phenotypic level for some virulence factors and at the genotypic level using *16S rDNA* gene.

Materials and methods

1-Bacterial isolates

Isolates of *A. hydrophila* used in this study were collected from clinical and environmental sources that included 22 clinical specimens and 35 soil samples of different plants rhizospheres. They were identified using VITEK2 system (Biomerienx, USA). The maintenance of pure isolates was performed in nutrient agar (HiMedia, India) for subsequent tests.

2- Identification of the phenotype of virulence factors

The isolates were grown in nutrient broth (HiMedia, India) and incubated overnight at 37°C. The cell density was adjusted to 1.5×10^{8} cells/mL. For characterization of the virulence factors phenotype, 10 µl of this suspension were used. All experiments were performed in duplicate [1].

2.1- Hemolytic activity

Blood agar base (HiMedia, India) with 5% of human blood was used for testing the production of hemolysin. A loop full of bacteria was streaked on the surface of blood agar and incubated overnight at 37°C . The clear colourless zone around the bacterial colonies indicated β-hemolytic activity [1].

2.2-Casein hydrolysis

To detect the proteolytic activity of bacteria, nutrient agar with 10% (w/v) skimmed milk was used. A loop full of bacteria was streaked and incubated at 37°C for 24 hr. Caseinase activity was indicated by presence of clear zones around the colonies [15].

2.3-Lipolytic activity

Tween 20 agar was used to detect the Lipase activity. A loop full of bacteria was streaked and incubated at 37°C for 48 hr. A transparent zone surrounding the colonies indicated lipase [16].

2.4-Nuclease activity

DNase agar media with 0.005% methyl green was used to detect DNase activity. Five microliters of bacterial suspension was placed onto the plates and incubated overnight at 37°C. Positive nuclease activity was indicated by a pink color hallowsurrounding the colonies [1].

3- Biofilm formation assay

The isolated bacteria were tested for biofilm formation as described by Wojnicz *et al*. [17] with some modification. Briefly, the bacteria were grown overnight at 37°C in nutrient broth supplemented with 1% glucose. The 96 well microtitere plate was filled with 180 µl from the same medium and 20 µl of the bacterial suspension (0.5 McFarland) which were transferred to each well in triplicate then incubated at 37ºC for 24 hr, whereas negative control contained media only. The media was removed and washed three times with phosphate puffer saline, then 200µl of 0.1% crystal violate was added for 15 min, removed, and washed with PBS three times. The crystal violate inside the cells was dissolved by absolute ethanol and the absorbance was measured by an ELISA reader. After comparing the optical density (O.D) of the biofilm to that of the control, and according to the readings, the isolates were classified as follows: O.D ≤ O.Dc no biofilm producer, O.Dc <O.D ≤ 2 ×O.Dc weak biofilm, 2 ×O.Dc <O.D ≤ 4 ×O.Dc moderate and 4 ×O.Dc <O.D strong biofilm.

4- Antibiotic susceptibility test

For the antibiotic susceptibility test, the bacterial isolates were screened versus eight commercial antibiotics on Mueller-Hinton agar using disc diffusion method [18]. The antibiotics and their concentrations used for this study were as follows: amikacin (30mg), amoxicillin (30mg), clindamycin (10mg), gentamicin(10mg), cefoxitin (30mg), erythromycin (15mg), tetracycline (30mg), vancomycin (30mg). The zone of inhibition was recorded after 24h of incubation at 37ºC.

5- Molecular assays

5.1-DNA extraction

G-spin DNA extraction kit (iNtRon biotechnology,/Korea) was used for DNA extraction from the bacterial isolates. Electrophoresis was performed on 1% agarose gel to determine the quality of DNA.

5.2- Primers selection

The set of forward primer 27F (5' AGAGTTTGATCCTGGCTCAG 3') and reverse primer 1492R (5' TACCTTGTTACGACTT 3') was used for amplification of *16S rDNA* for the detection of bacterial isolates at the gene level [19].

5.3- Polymerase chain reaction

In a total volume of 25 µl, the PCR mixture was prepared from 5µL of Taq PCR PreMix (Intron, Korea), 1 µM of each primer and 2 ng/µL of template DNA, then the remaining volume was completed with nuclease-free water. The PCR protocol involved an initial denaturation for 3 min at 95ºC; 35 cycles of denaturation for 45 sec at 95ºC, annealing for 1min at 52ºC, extension for 1min at 72ºC then final extension for 7 min at 72ºC. The PCR products were run on 2 % agarose gel stained with Red safe in Tris Acetate EDTA buffer (TAE, pH 8.4) and observed under UV Transilluminator. The 100 pb DNA ladder (iNtRon, Korea) was used to determine the size of PCR products.

5.4- DNA sequencing

After amplification, PCR products were transmitted to the National Instrumentation Center for Environmental Management (NICEM) for Sanger sequencing of *16S rDNA* using DNA sequencer 3730XL (Applied Biosystem, USA). BLAST was used for homology search which is available at the NCBI online at (http:// www.ncbi.nlm.nih.gov). Bioedit program was used for estimating the similarity matrix. MEGA7 sequence analyzing software with 1000 bootstrap value was utilized for constructing the phylogenetic tree.

6- Statistical analysis

SPSS, version 20, was used for data analysis. Antibiotic susceptibility data were compared using independent sample T-test for comparison between antibiotics inhibition zone diameter of the tested isolates and for biofilm formation. Differences at $p \leq 0.05$ were considered statistically significant. Susceptibility, intermediate responses, and resistance of isolates to antibiotics were calculated as percentages.

3. Results

Out of eight isolates of *A. hydrophila*, 4 (18.2%) isolates were collected from clinical specimens and from environmental samples, and 4 (11.4%) isolates were obtained from different soil samples of plant rhizospheres.

In vitro, the experimental results of some virulence factors of *A. hydrophila* showed that all clinical isolates had β-hemolytic activity on blood agar media while the environmental ones had γ- hemolytic activity. Furthermore, the clinical isolates showed 100% lipase, protease and DNase activities, whereas the environmental isolates had 50%, 100% and 100% for the aforementioned enzymes, respectively.

The biofilm formation assay using microtitere plates revealed that all studied isolates (100%) were weak biofilm producers with no statistical differences among them (P>0.05).

The results of antimicrobial susceptibility of *A. hydrophila* isolates demonstrated close similarity in antibiotic susceptibility profiles (Figure. 1). They were susceptible (100%) to gentamicin, amikacin and vancomycin, while resistant (100%) to clindamycin, amoxicillin and erythromycin.

to gentamicin, amikacin and vancomycin, while resistant (100%) to clindamycin, amoxicillin and erythromycin.

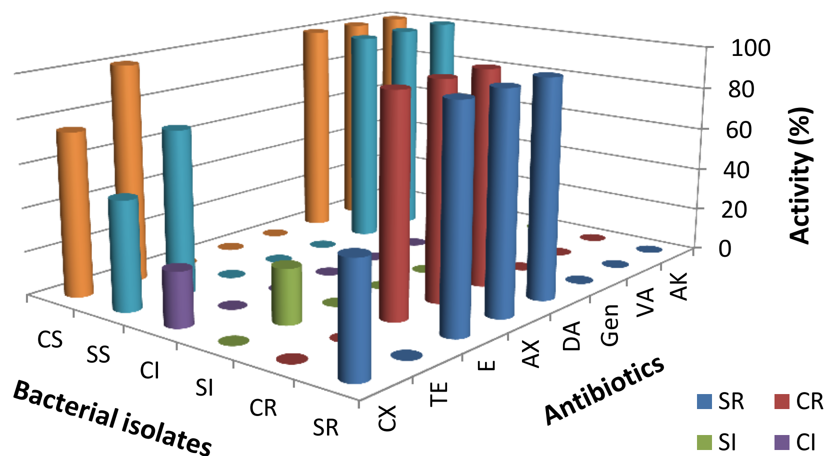

Figure 1- Antibiotic Susceptibility Profile of Clinical and Environmental *A. hydrophila* Isolates. Amikacin (AK), Amoxicillin (AX), Clindamycin (DA), Gentamicin(Gen), Cefoxitin (CX), Erythromycin (E), Tetracycline (TE), Vancomycin (VA). Soil Resistant (SR), Clinical Resistant (CR), Soil Intermediate (IR), Clinical Intermediate (CR), Soil Sensitive (SS) and Clinical Sensitive (CS).

All the clinical (100%) and 75% of the environmental isolates were sensitive to tetracycline. Furthermore, 75% of the clinical isolates were sensitive to cefoxitin whereas 50% of the environmental ones were resistant to it. According to the inhibition zone diameter, significant differences were shown (P≤0.05) between clinical and environmental results in response to amikacin, tetracycline and cefoxitin.

Polymerase chain reaction was used for *16S rDNA* gene amplification from *A. hydrophila* isolates. The results revealed distinct amplicon size (1400 bp) from all DNA isolates (Figure. 2).

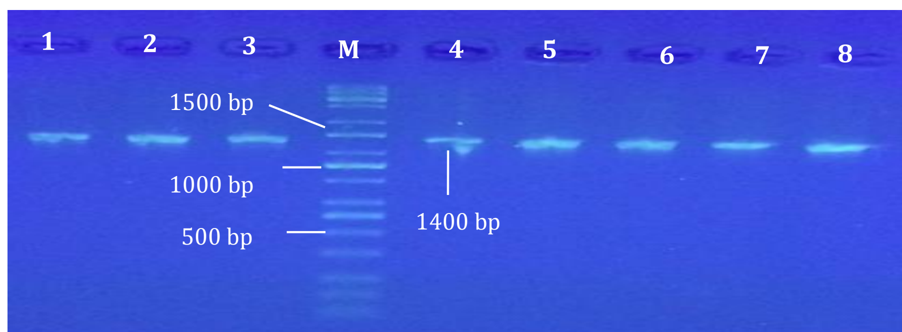

Figure 2- Gel Electrophoresis of Amplified *16SrDNA* (1400bp) in *A. hydrophila* Isolates. Agarose (1.5%), 5 V/cm for 2 hrs, stained with Red safe and visualized under UV transilluminator. M. 100 bp DNA ladder. Lane 1-8: bacterial isolates.

Sequences of *16S rDNA* of seven *A. hydrophila* isolates (one clinical isolate was failed in sequence reading) were compared with GenBank sequences through BLAST program. The results confirmed identification of the studied bacteria as *A. hydrophila* with 99-100% identity with Accession No. KC252600, KU570318, MF079288, MF445123, KR819398 and AB032088.

ClustalW for Multiple Sequence Alignment was used for *16S rDNA* sequences [20]. Eighteen sequence positions difference (1.97 % divergence), including 14 transition, 3 transversion and one insertion, were observed between the clinical and environmental isolates of *A. hydrophila*. Among the clinical isolates, only one polymorphism (transversion) was observed, while ten polymorphisms (7 transition, 2 transversion and one insertion) were observed among the environmental isolates. Furthermore, the sequence of one environmental isolate (AH-E4-16S Iraq) showed the lowest variation when compared to clinical isolates sequences. Five nucleotide positions (1.29 % divergence) were identical to clinical isolates and differ from other environmental isolates.

The sequence similarity matrix for *16S rDNA* of *A. hydrophila* isolates was estimated by Bioedit program and the results showed 98% sequence similarities between clinical and environmental isolates, whereas intra-isolates results showed 99% sequence similarities.

A phylogenetic tree constructed by neighbor-joining method (1000 bootstraps) for the *16S rDNA* region of *A. hydrophila* was used to study the isolates by the MEGA7 program [21]. The percentages of bootstrap values are shown at the internal nodes. Figure 3 shows that the studied isolates were distributed in two clusters; the first cluster represents the environmental isolates (AH-E-16S Iraq) and the second one represents the clinical isolates (AH-C-16S Iraq).

The results illustrated that AH-E1-16S Iraq and AH-E2-16S Iraq as well as AH-C2-16S Iraq and AH-C3-16S Iraq isolates were sister groups. All studied isolates displayed similarity to GenBank isolates obtained from Asian regions.

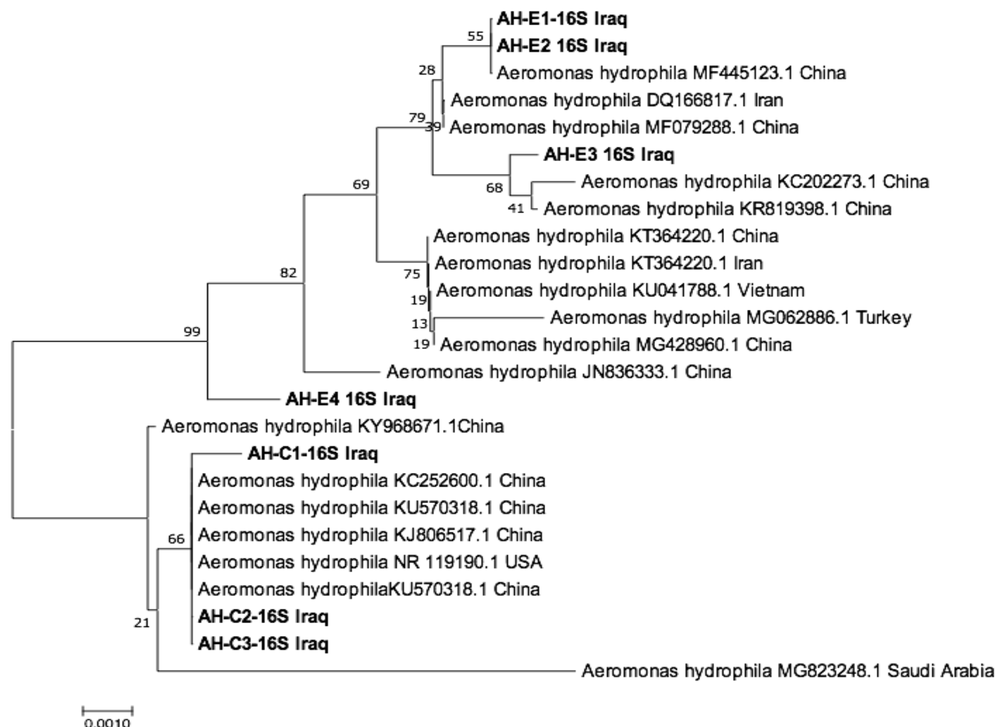

Figure 3- Neighbor-Joining Phylogenetic Analysis of *16S rDNA* of *A. hydrophila* Local Clinical (AH-C-16S) and Environmental Isolates (AH-E-16S). The analysis performed using MEGA7.

Discussion

A. hydrophila is an important pathogen that causes various infections to humans as well as animals such as fish, amphibians, and mammals. The bacterium is present normally in the aquatic environment but may be found in soil which have an important role as reservoir and in the epidemiology of Aeromonas-associated human infections [2].

Several virulence factors participate in the infection of *A. hydrophila*, including hemolysins, enterotoxins, leucocidins proteases and lipases. Some of these factors, including hemolysin, lipase, protease and DNase activities, were studied *in vitro*. The results were similar to those from a previous local study on *A. hydrophila* virulence factors isolated from clinical specimens [22]. Another study by Das and colleagues [1] on *A. hydrophila* from environmental sources found that 24% of the isolates were β-hemolytic, whereas 36%, 24% and 15% had protease, lipase and nuclease activities, respectively. They also found that virulence factors of *Aeromonas* isolated from environmental samples were less effective than those isolated from commercial food samples.

It was indicated that high pathogenicity of clinical isolates results from secretion of different toxins that lead to diseases in human [23], such as the production of hemolytic toxins that cause particular lytic activities in the host cell [24]. Extracellular proteases allow persistence in various habitats and facilitate ecological interactions with other organism. In general, proteases can confer pathogenicity by directly damaging the host tissues or by proteolytic activation of toxins, as well as contributing to the establishment of infection due to overcome of the initial host defenses [25]. It is believed that lipolytic activity of lipases may affect several immune system functions through the generation of free fatty acids[26].

Biofilm capacity represents an important mechanism of bacterial escape, as *Aeromonas* in biofilms could resist disinfection and persist for long periods of time [27]. The results of this study showed that all studied isolates had a low ability for biofilm formation. This may attributed to the cultivation of the isolates in media supplemented with 1% glucose. Also, the incubation temperature might influenced the biofilm formation by *A. hydrophila* which were previously shown to decline at temperatures over 25°C (i.e., 30-37°C) or below 20°C [28]. Jahid et al. [29] reported that low salinity (0.25%) enhances biofilm formation by *A. hydrophila*, whereas glucose concentration above 0.05% impairs its formation.

Increased antibiotic resistance has a great risk to human health. In general, the available susceptibility data indicated variable antibiotics resistance patterns, relying on the *Aeromonas* species, country and source of isolation [29].

Antibiotic susceptibility profiles of eight clinical and environmental *A. hydrophila* isolates showed high resistance to clindamycin, amoxicillin and erythromycin, while they were susceptible to gentamicin, amikacin and vancomycin. Similar results were reported in a study by Dahdouh and colleagues [30] on *A. hydrophila* isolated from fresh, brackish and marine fish. Also, significant differences were shown between the clinical and environmental isolates as related to their susceptibility to amikacin, tetracycline and cefoxitin. *A. hydrophila* from different sources were shown to display multiple antibiotic resistance [31]. Differences in antibiotic susceptibility between clinical and environmental isolates of *Aeromonas* were reported in previous works, which suggested the heavily polluted environment as a source for multiple plasmid resistance, that might be also acquired from clinical isolates [9].

DNA sequencing and constructing the phylogenetic tree have a greater role in enhancement of bacterial identification, structure and epidemiology [32]. Amplifying and sequencing of *16S rDNA* target gene confirmed the identification of the studied bacteria, *A. hydrophila*. Numerous of studies used *16S rDNA* for identification and classification of *A. hydrophila* [22, 33]. *16S rDNA* of the studied isolates showed high sequence similarities between clinical and environmental isolates, which suggests a relationship between them, with the possibility that the environmental isolates might be a source for the clinical ones.

The divergence (1.97 %) that observed between the clinical and environmental *A. hydrophila* isolates might be due to a mutation that caused variation in nucleotides content, where environmental factors might be responsible for creating this variation. It was demonstrated that the environmental factors may influence the nucleotide content, not only among different environments such as soil, water and the host microbiome, but also within a single type of environment [34].

Finally, the phylogenetic tree by neighbor-joining of *16S rDNA* sequence showed that all of the studied *A. hydrophila* isolates were clearly separated in branching patterns and these isolates branched with various isolates from different countries of Asian origin. The 16S ribosomal gene was also previously used to estimate the phylogenetic relationships among *Aeromonas* isolates[25].

Conclusion

From the obtained data, the phenotypic and genetic study identified relationships between the clinical and the environmental isolates of *A. hydrophila,* and the later may be the source for the clinical isolates. This might imply that isolates from the soils of plant rhizospheres have the risk of transmission into humans.

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