RESEARCH ARTICLE

Aeromonas hydrophila from Fish and Humans: Biofilm Formation and Genetic Relatedness

Mohamed E.M. Mohamed¹, Heba A. Ahmed¹, Maha M. Rezk¹,², Rasha M. A. Gharieb¹ and Shehata A. Abdel-Maksoud²

¹Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, 44511, Sharkia Governorate, Egypt

²Animal Health Research Institute, Damietta branch, Agriculture Researches Center, Egypt

Abstract

A comprehensive study was done to determine the genetic relatedness and the biofilm formation ability of Aeromonas hydrophila strains (n=25) isolated from fish (150 tilapia and 144 mugil) and human stool samples (n=102). The results revealed that Random Amplification of Polymorphic DNA (RAPD-PCR) classified A. hydrophila strains into seven distinct profiles (R1-R7), the amplicon sizes ranged from 183-2930 bp. The isolates were grouped into five main clusters, the presence of isolates from fish and humans in the same cluster indicates the possibility of cross contamination. Biofilm results showed that 96% of A. hydrophila isolates were biofilm producers. At 35°C, 16 (64%) and 8 (32%) showed strong and moderate biofilm production ability, respectively. At 25°C, 21 (84%) were biofilm producers, of which, 8 (32%), 7 (28%) and 6 (24%) were strong, moderate and weak, respectively. At 4°C, decreased biofilm production ability was noticed 13 (52%), where 8 (32%) and 5 (20%) were moderate and weak biofilm producers, respectively. Signiﬁcant correlation was showed between A. hydrophila isolates and different cultivation temperatures. This clariﬁed the potential virulence of A. hydrophila isolates from both ﬁsh and human sources and their public health hazard.

Keywords: A. hydrophila, Biofilm, Fish, RAPD, Humans.

Introduction

Fish production is one of the most important industrial activities in Egypt [1]. Damietta is a littoral Governorate with an important role in fish production. However, infection of ﬁsh with microbial pathogens is considered a risk factor in aquaculture industry resulting in a dramatic loss in economy [2]. Aeromonas infection in ﬁsh causes world economic problems because of high number of ﬁsh mortalities in different countries [2]. Fish can be contaminated with Aeromonas spp. either by polluted water or by handling, processing and bad storage conditions [3]. A. hydrophila is considered the most important zoonotic pathogen of concern. It may cause intestinal and extra-intestinal diseases in humans such as septic arthritis, diarrhea (traveler’s diarrhea), gastroenteritis, skin and wound infections, meningitis, and fulminating septicemia [4-8]. Numerous case reports have described the isolation of Aeromonas from patients with acute diarrhea, but the bacterium can also be isolated from stool of healthy persons [9]. Random Amplification of Polymorphic DNA technique (RAPD) is used to clarify the genetic relatedness among different strains and is considered an accurate method in classifying microorganisms for epidemiological studies [10, 11]. Moreover, RAPD could be utilized as species speciﬁc indicator and bacteriological diagnostic marker [3]. This technique has a role in differentiation among different subgenera, which helps in organizing the variance pattern of their genetics [12].

Some microorganisms have the ability of settlement on a biotical area; the first incriminated place is aquatic niches. When a single microorganism adheres to solid surface, it starts special colonization structure which is called a biofilm [13]. Gram-negative bacteria have the ability of biofilm formation more
than Gram-positive bacteria due to bacterial outer membrane structure [14]. The process of biofilm formation may take only 2-4 h and can help in bacterial cell communication [15, 16]. Microorganism in biofilms are 1000 times more resistant to antibiotics and biocides [17], moreover, the biofilm matrix is composed of polymeric substances and can contravene with the antibiotic diffusion ability, thus contributes in the microorganism virulence ability. *Aeromonas* have the ability to adhere forming biofilms on different surfaces [18] which is considered a public health hazard especially for those who inhabit the coastal area [19].

The aim of the study was to evaluate the genetic relatedness and biofilm formation abilities of *A. hydrophila* isolated from fish and human stool samples.

**Materials and Methods**

**Bacterial isolates and growth conditions**

Twenty-five *A. hydrophila* isolates identified by biochemical examination and confirmed by PCR were used to achieve the purpose of the current study. The isolates were obtained from our previous study [20]. The isolates were recovered from fish (tilapia and mugil) viscera and muscles samples (n=16) and human stool samples (n=9) from Damietta Governorate.

**RAPD-PCR**

*A. hydrophila* isolates were evaluated for genetic relatedness by RAPD-PCR using the primer TCG CGA GCT G [21]. The reaction conditions were primary denaturation at 94°C for 10 min, secondary denaturation at 94°C for 45 sec, then annealing was done at 37°C for 1 min, extension was at 72°C for 1.2 min and final extension was performed at 72°C for 12 min. After amplification, 1.5% agarose gel (Applichem, Germany, GmbH) was prepared in 1X TBE buffer and was stained with 5 μg/ml ethidium bromide (Sigma). The PCR products (15 μl) were loaded in each gel slot for analysis. A gene ruler 1 Kb plus DNA Ladder (Fermentas, Thermo Scientific, Germany) was used to determine the size of the fragments. The gel was run in 1X TBE and 5 μg/ml ethidium bromide for 45 min at 100 volts. The amplicons were visualized by a gel documentation system (Alpha Innotech, Biometra) and the data were analyzed. The reaction was repeated twice to ensure reproducibility. The RAPD-PCR data were transformed into binary code according to the presence or absence of each band. Dendrogram was generated by unweighted pair group method with arithmetic average (UPGMA) and Ward’s hierarchical cluster technique. The cluster analysis and the dendrogram construction were performed with SPSS, Inc. version 22 (IBM Corp. 2013, Armonk, NY). The measurement of the discriminatory power of RAPD-PCR was done by the Simpson’s index of diversity (D) which indicates the average probability that a typing system will assign a different type to two unrelated strains randomly sampled from a population [22]. D value of more than 0.9 indicates good differentiation.

**Biofilm formation**

The biofilm formation ability of *A. hydrophila* isolates was evaluated by microtiter plates of 96 wells (Falcon, BD Biosciences, NJ, U.S.A.) according to Odeyemi et al. [23] and Nagar et al. [24]. Bacterial cultures (200 μl) in Tryptic Soya Broth (TSB, Oxoid) were adjusted to a McFarland standard tube No. 0.5 (1.5×10⁸ CFU ml) by the addition of sterile saline in the microtiter plate wells in triplicate. The negative control wells with only TSB broth were used as negative controls. The plates were incubated at 4°C, 25°C and 35°C for 24 h. The plates were inverted to remove the media and then the wells were washed four times with 0.2 ml of phosphate buffer saline (PBS, pH 7.2) to remove the free-floating ‘planktonic’ cells. The remaining adhered bacteria were fix by gluteraldehyde 2.5% in PBS for 15 min. The staining step was carried out by 200 μl of crystal violet solution (0.2%) for 30 min with thorough washing by deionized water to remove excess stain. The microtiter plates were kept at 40°C for 15 min for drying. For biofilm quantification, 200 μl of 95% ethanol were added to each well. The Optical Density (OD) of the stained adherent bacteria was determined with an ELISA reader.
(model: sunrise R4, serial no: 610000079) at wavelength 620 nm (OD620 nm) after adjustment of the negative control to zero. This experiment was performed in triplicate and was repeated three times. The data are represented as mean and the standard deviation was calculated. The mean OD value was estimated by subtracting the control OD value from all OD obtained results (Biofilm OD = OD1 – ODc). The resulted OD was considered as an index of bacteria adhering to the surface forming biofilm. The strains were classified as non, weak, moderate and strong biofilm producers according to equations explained by Saxena et al. [25] as the following: Non-biofilm producer (0) OD ≤ ODc; Weak biofilm producer (+ or 1) = ODc < OD ≤ 2×ODc; Moderate biofilm producer (++ or 2) = 2×ODc < OD ≤ 4×ODc; Strong biofilm producer (+++ or 3), 4×ODc < OD.

Kruskal-Wallis H One-Way Analysis of Variance (ANOVA) and post hoc Bonferroni correction were performed to estimate the differences in biofilm formation degrees at the three different temperatures. Test results were calculated by SPSS version 22 (IBM Corp. 2013, Armonk, NY). Data are presented as mean ± SD and significance was considered at P < 0.05.

Results and Discussion

Molecular typing of Aeromonas spp. by RAPD-PCR is a rapid and time saving method for the identification of the bacteria [12]. It was recorded as an efficient method for A. hydrophila differentiation [26, 27]. RAPD-PCR aids in monitoring sources of infection, moreover, it has a role in explaining the genetic relationship among different Aeromonas isolates [28]. Several studies have used RAPD-PCR technique for epidemiological investigation of different microorganisms [10]. The usefulness of RAPD technique in the detection of the relationship between isolate has been reported [29]. Szczuka and Kaznowski [11] documented that both RAPD and ERIC techniques are of value in the differentiation between unrelated strains.

In the current work, RAPD-PCR analysis was performed to determine the relatedness between different A. hydrophila strains (n=25) isolated from fish (n=16) and human stool samples (n=9). The results revealed that A. hydrophila isolates were sub grouped into seven distinct profiles (R1-R7), the amplicon sizes ranged from 183-2930 bp (Figure 1). The discriminatory index was 0.84 and the isolates were classified into five main clusters at linkage distance 12.5; thus indicating heterogeneity (Table 1 and Figure 2).
Table 1: RAPD-PCR fingerprinting profiles and associated clusters for *A. hydrophila* recovered from fish tissues and human stool from Damietta Governorate

<table>
<thead>
<tr>
<th>Profile</th>
<th>No. of isolates</th>
<th>Source</th>
<th>Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>8</td>
<td>FND, FD, BVD, BVH, TVD</td>
<td>Ia</td>
</tr>
<tr>
<td>P2</td>
<td>3</td>
<td>TVD, TMD, BVH</td>
<td>Ib</td>
</tr>
<tr>
<td>P3</td>
<td>3</td>
<td>TVH, TMH, BVD</td>
<td>II</td>
</tr>
<tr>
<td>P4</td>
<td>5</td>
<td>BVH, BVD, TMH, TMD</td>
<td>III</td>
</tr>
<tr>
<td>P5</td>
<td>2</td>
<td>FD</td>
<td>IV</td>
</tr>
<tr>
<td>P6</td>
<td>3</td>
<td>FD</td>
<td>Va</td>
</tr>
<tr>
<td>P7</td>
<td>1</td>
<td>FND</td>
<td>Vb</td>
</tr>
</tbody>
</table>


Figure 2: Dendrogram showing the relatedness of *A. hydrophila* spp. isolated from fish and human from Damietta Governorate as determined by RAPD-PCR fingerprinting using the SPSS computer software program (based on the presence or absence of each band at 12.5 linkage distance. TVD: diseased tilapia viscera, TVH: healthy tilapia viscera, TMD: diseased tilapia muscle, TMH: healthy tilapia muscle, BVD: diseased mugil viscera, BVH: healthy mugil viscera, BMD: diseased mugil muscle, FD: diarrheic stool, FND: non-diarrheic stool.
Cluster I contained two sub-clusters; sub-cluster (Ia) included 8 isolates; 5 isolates were 100% similar and were originated from human (n=2) and mugil (n=3), while, the other 2 isolates (100% similarities) were from mugil samples and one isolate was from human samples. Sub-cluster (Ib) contained 3 isolates from fish origin, of which two were 100% similar. Cluster II included 3 isolates obtained from fish samples, two of them were 100% similar. Meanwhile, cluster III contained 5 isolates, all were originated from fish samples (tilapia and mugil) and two of them were 100% similar. In addition, cluster IV contained two isolates, both were from human stool. Cluster V consisted of two sub-cultures; Va and Vb. Sub-cluter Va included 3 isolates from human stool and sub-cluster (Vb) included one isolate from human stool. The presence of isolates from both human and fish in the same cluster indicates the possibility of cross contamination with A. hydrophila from different sources. Subashkumar et al. [21] reported that all A. hydrophila diarrheal isolates were genetic heterogeneous with a significant variation.

In India, Kamble et al. [12] reported that Aeromonas spp. isolated from fish and water samples were genetically diverse by RAPD-PCR, different RAPD profiles with 3 different clusters were observed. Moreover, Szczuka and Kaznowski [11] found a good discriminatory power between Aeromonas spp. (n=120) from environmental samples and gastroenteritis patients and showed 2-17 bands ranging from 100-3500 bp with similarity ranged from 98-100% between isolates. A. hydrophila strains (n= 40) from stool samples of human patients showed heterogeneity with environmental isolates with a clear clonal structure between the isolates Subashkumar et al. [21], however, no identical profiles were reported illustrating the wide genetic diversity, while the dendogram analysis showed good discrimination between different isolates from milk, diarrhea and fish forming at least 12 groups. Moreover, Ramalivhana et al. [30] revealed 12 different RAPD patterns of 32 A. hydrophila isolates from gastroenteritis human stool and their drinking water, the RAPD profiles were clustered according to origin. This explained the importance of dendogram RAPD analysis in discriminating the origin of samples.

A. hydrophila strains were isolated from fish samples in China and were analyzed by RAPD-PCR, the results revealed the clustering of the isolates in 14 clusters, the D value was 0.958 indicating good discrimination among isolates [31]. Furthermore, Aeromonas spp. isolates were grouped in 5 clusters and 9 single isolates with D value of 0.995 in a study reported by Tahoun et al. [32] in Egypt to discriminate between A. hydrophila isolates originated from milk, dairy products and diarrheic human stool samples. They recommended RAPD technique for Aeromonas spp. epidemiological studies.

The current results showed the presence of A. hydrophila from different sources under the same cluster group, this might indicate cross contamination. Oladele and Temitope [33] performed genotyping of 32 A. hydrophila from different sources (cabbage, tap water and diarrheic human stool) and the results revealed different profiles, and the isolates were grouped in 10 main clusters with a genetic similarity of 60-100%.

Several studies reported that RAPD-PCR is highly discriminative for A. hydrophila isolates; this is in accord with Sarkar et al. [26] who recorded polymorphism of RAPD-PCR profiles between A. hydrophila isolates from different sources.

The findings of the current study revealed that RAPD-PCR is a simple, rapid and reproducible fingerprinting tool for detecting the relatedness among A. hydrophila isolates originated from different sources.

Biofilm is a group of sessile bacteria that settle on biotic and abiotic surfaces [34]. It is a common trait in both gram positive and gram-negative bacteria, which is formed by extracellular polymeric substance that enabled the bacteria to withstand adverse environmental conditions [19, 35, 36]. Biofilm is a complicated process that needs multifactorial aspects, composing of viable and non-viable organisms and surrounded by hydrated polyanionic extracellular or polymeric substances. Biological films can harbor different bacterial types with specific interaction [37-39]. The communication of biofilm colonizers is achieved by production of signal molecules called autoinducers, this is what is known as quorum sensing [40]. Biofilm forming bacteria are incriminated in 80% of chronic inflammation of bacterial type [41]. Moreover, biofilm process is considered one of the virulence factors of the bacteria enabling infection transmission and disease establishment [42, 43]. In addition, it increases
the bacterial resistance to chlorinated water and antibiotics [40]. Bacteria forming biofilm could attach to the host intestinal epithelium resulting in disease establishment [35, 44]. A. hydrophila was incriminated in biofilm formation [45]. The ability of Aeromonas species to form biofilm is considered a kind of challenge during bacterial treatment because this alleviates the antimicrobial resistance [46]. This is illustrated by the slow rate of antibiotic diffusion in the biofilm matrix [13]. Moreover, biofilm hinders the accessibility of toxic agents to deep layers [47]. The bacterial biofilm community can be 1000 times more resistant to antibiotics [48, 49]. Biofilm formation microorganisms are accused for changes in food, resulted in low quality food products, this is called as SSÖ (specific spoilage organisms) Wang et al. [50].

Table 2: Biofilm production in Aeromonas hydrophila species isolated from fish tissues and human stool at 4°C, 25°C and 35°C

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Non-producer</th>
<th>Weak</th>
<th>Moderate</th>
<th>Strong</th>
<th>Overall biofilm producers</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>12 (48%, 0.029± 0.008)</td>
<td>5 (20%, 0.130± 0.011)</td>
<td>8 (32%, 0.279± 0.014)</td>
<td>0</td>
<td>13 (52%, 0.222± 0.013)</td>
</tr>
<tr>
<td>25°C</td>
<td>4 (16%, 0.051± 0.009)</td>
<td>6 (24%, 0.171± 0.010)</td>
<td>7 (28%, 0.334± 0.020)</td>
<td>8 (32%, 0.785± 0.009)</td>
<td>21 (84%, 0.459± 0.013)</td>
</tr>
<tr>
<td>35°C</td>
<td>1 (4%, 0.072± 0.002)</td>
<td>0</td>
<td>8 (32%, 0.297± 0.020)</td>
<td>16 (64%, 0.714± 0.018)</td>
<td>24 (96%, 0.575± 0.019)</td>
</tr>
</tbody>
</table>

OD: Optical Density
SD: Standard Deviation

The results in Table (2) showed that 96% of the examined A. hydrophila strains (24/25) were biofilm producers at 35°C. Interestingly, 8 (32%) and 16 (64%) were moderate and strong biofilm producers, respectively, while one isolate was non-biofilm producer. In addition, at 25°C showed that 21 (84%) were biofilm producers; 6 (24%), 7 (28%) and 8 (32%) were weak, moderate and strong producers, respectively, while four isolates (16%) were non-biofilm producers. On the other hand, 52% of the isolates (n=13) had the ability to form biofilm at 4°C, of which, 5 (20%) and 8 (32%) were weak and moderate biofilm producers, respectively, while 12 (48%) showed no biofilm formation.

In accordance with the current observations, 90.9% of Aeromonas spp. isolates were biofilm producers on polystyrene microtiter plates at 30°C, of which, 81.8 and 9.1% were weak and moderate producers [24]. In South Africa, out of 45 Aeromonas spp. strains isolated from water samples, 53.3% were weak biofilm producers, while 28.9% and 15.6% had strong and moderate biofilm formation ability, respectively [51]. In addition, out of 28 Aeromonas spp. isolates collected from different sources in Brazil, 17.9% were weak, 32.1% were moderate and 50% were strong biofilm producers [52]. Another study tested the ability of A. caviae strains from Brazil to produce biofilm, 72% were biofilm producers, of which, 60% were weak to moderate biofilm producers, while 12% were strong biofilm producers [53]. Moreover, Odeyemi et al. [23] reported that all tested A. hydrophila strains isolated from water and sediment samples in Malaysia were biofilm producers, 53.3, 20 and 26.6% were weak, moderate and strong biofilm producers, respectively. The differences in the results of the previous studies could be related to the origin and nature of the strains and differences in cultivation methods, media and incubation conditions.

The present study revealed a significant difference between biofilm formation ability of A. hydrophila isolates in different temperatures (p ≤ 0.05). Between temperature groups, the ability of A. hydrophila to form biofilm at 25°C was significantly higher than at 4°C. Moreover, the A. hydrophila ability to produce biofilm at 35°C was significantly higher than at 4°C (Figure 3). Stress conditions such as change in temperatures affect A. hydrophila by altering surface structure...
leading to morphological changes, which enhance the bacterial ability to adhere on sessile niches [54, 55]. Temperature is an important factor that affects the biofilm ability of the aquatic organism [56]. This supports the results of our study; the increase of temperature has an effect on biofilm formation, as the number of non-biofilm producers was higher at 4°C than at 25 and 35°C. Moreover, strong biofilm producers were only observed at 35 and 25°C. Rachid et al. [57] recorded that the increase of temperature resulted in increased bacterial ability to produce biofilm.

Figure 3: Boxplot showing the median optical density of biofilm formation by A. hydrophila spp. isolated from fish and human samples from Damietta Governorate at different temperatures (4°C, 25°C and 35°C). Different letters indicate significant difference at p ≤ 0.05.

In contrary, Nagar et al. [58] reported that most of Aeromonas spp. produced biofilm at 10°C, while cultivation at 30 and 37°C revealed lower biofilm production. Previous studies reported that the biofilm production ability of Aeromonas spp. at temperature range 25-23°C is an evidence of bacterial survival in ectothermic hosts and seafood, which resulted in food borne infection [59]. Moreover, Mizan et al. [34] reported that temperature range of 20-25°C was the most favorable condition for biofilm formation ability of Aeromonas isolates, while decrease in biofilm was observed by cultivation at temperatures below 20°C (4, 10, 15°C) and over 25°C (30, 35, 37°C). Another study found difference in biofilm formation of Aeromonas spp. at different temperatures, a significant difference was noticed (P< 0.05) up on cultivation of isolates under different temperatures (~21, 30 and 37°C) [37].

The present study revealed that A. hydrophila isolates from fish samples and clinical human stool samples origin produced biofilm with varied degree. Mizan et al. [34] and Nagar et al. [24] recorded no relation between isolates origin and the ability of biofilm production. However, Chenia and Duma [37] reported that the difference in biofilm formation ability of the isolates might
attributed to the origin, isolates from fish need nutrient rich environment, while isolates from water might be adopted to lack of nutrients.

**Conclusion**

RAPD-PCR technique is a useful tool for monitoring the fingerprinting of *A. hydrophila* isolates from different origins. Tilapia and mugil fish sold in Damietta, Egypt are considered reservoirs for *Aeromonas* spp. with biofilm formation ability. Temperature is an important factor for *A. hydrophila* biofilm production ability with increasing the cultivation temperature.

**Conflict of interest**

The authors declare no conflict of interest.

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الملخص العربي

ميكروب الأيزوريوماناس هيدروفيلا من الأسماك والإنسان: القدرة على إنتاج البيوفيلم والعلاقة الجينية

محمد السيد، يحيى أحمد عبد الله، هناء محمود رزق، راشد عبد الحكيم عبد المصطفى

قسم الأمراض المشتركة، كلية الطب البيطري، جامعة الزقازيق، مصر

تم إجراء هذه الدراسة لعمر نماذج الجيني والقدرة على إنتاج البيوفيلم لعدد 23 من بيئة الأيزوريوماناس هيدروفيلا المعزول من أسماك البلطي (عدد 150) والبري (عدد 44) وبراز الإنسان (عدد 22) في محافظة دمياط، وقد أوضحت نتائج اختبار التضافع العشوائي المتعدد الأشكال للحصص التجريبي المستخدم أن تقييم القرابة الجينية تصنف عزلات الأيزوريوماناس هيدروفيلا إلى عدد 7 أنماط جينية مع معامل تقاومية قدره 84.8. كمؤشر للتباين، تم التأكيد من وجود خمسة أفرع رئيسية باستخدام الرسم الشجري. ولد تواجد عجزات من الإنسان والأسماك في ذات المجموعة على احتمالية انتقائية العجزات بينهما. بينما أظهرت نتائج اختبار الشريط الحيوي اختلاف قدرة العزلات مع اختلاف درجة الحرارة حيث أنه في درجة حرارة 35 سليزيوس وجد أن 24 عزلة (92%) كانت لها القدرة على إنتاج البيوفيلم، 8 عزلة (33%) قدرتهم متوسطة بينما 16 عزلة (64%) لم تكن قادرة على إنتاج البيوفيلم، بينما في درجة حرارة 40 سليزيوس أوضحت النتائج أن 21 عزلة (81%) كانت لها القدرة على إنتاج البيوفيلم، حيث كان 16 عزلة (64%) من تلك العزلات لهم قدرة ضعيفة على إنتاج البيوفيلم، 7 عزلة (28%) قدرتهم متوسطة و 8 عزلة (32%) قدرتهم قوية على إنتاج البيوفيلم، وفي درجة حرارة 45 سليزيوس وجد أن 13 عزلة (52%) كان لها القدرة على إنتاج البيوفيلم، حيث كان 8 عزلة (32%) و 5 عزلة (20%) من العزلات لهم قدرة متوسطة وضعيفة في إنتاج البيوفيلم، على التوالي. قد وجدت علاقة معينة بين قدرة عزلات الأيزوريوماناس هيدروفيلا على إنتاج البيوفيلم في درجات الحرارة المختلفة، وقد أوضحت هذه الدراسة أهمية أسماك البلطي في نقل مرض الأيزوريوماناس في الإنسان مع قدرة المعزولات على إنتاج البيوفيلم مما يشكل خطر على صحة الإنسان.