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RESEARCH ARTICLE

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

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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 35ºC, 16 (64%) and 8 (32%) showed strong and moderate biofilm production ability, respectively. At 25ºC, 21 (84%) were biofilm producers, of which, 8 (32%), 7 (28%) and 6 (24%) were strong, moderate and weak, respectively. At 4ºC, 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

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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 contravenes 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 94ºC for 10 min, secondary denaturation at 94ºC for 45 sec, then annealing was done at 37ºC for 1 min, extension was at 72ºC for 1.2 min and final extension was performed at 72ºC 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 match a McFarland standard tube No. 0.5 (1.5×10^8 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 4ºC, 25ºC and 35ºC 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 fixed 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 40ºC 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 and 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).

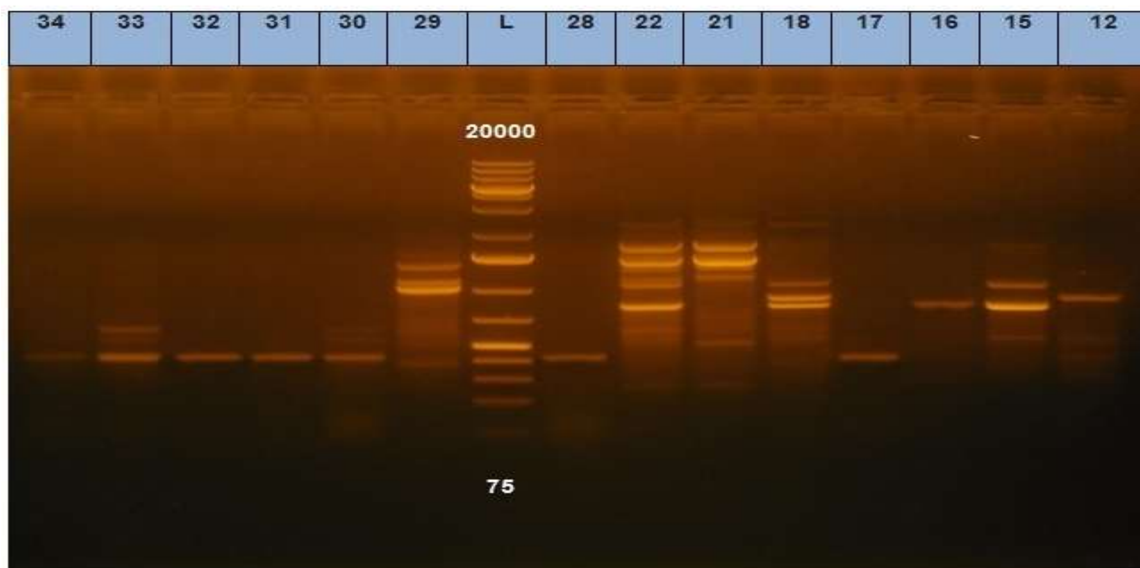

Figure 1: PCR results of RAPD-PCR fingerprinting in 1.5% agarose of *A. hydrophila* isolates from fish and humans at Damietta Governorate (L: 100bp ladder). Lanes (12, 15, 16, 17, 18, 21 and 22) from diarrheic stool, Lanes (28, 29) from non-diarrheic stool. Lanes (30, 32) from apparently healthy mugil viscera. Lanes (31, 34) from diseased mugil viscera. Lane 33 from diseased tilapia viscera.

Table 1: RAPD-PCR fingerprinting profiles and associated clusters for *A. hydrophila* recovered from fish tissues and human stool from Damietta Governorate

Profile	No. of isolates	Source	Cluster
P1	8	FND, FD, BVD, BVH, TVD	Ia
P2	3	TVD, TMD, BVH	Ib
P3	3	TVH, TMH, BVD	II
P4	5	BVH, BVD, TMH, TMD	III
P5	2	FD	IV
P6	3	FD	Va
P7	1	FND	Vb

FND: non-diarrheic stool, FD: diarrheic stool, BVD: diseased mugil viscera, BVH: apparently healthy mugil viscera, TVD: diseased tilapia viscera, TMD: diseased tilapia muscle, TVH: apparently healthy tilapia viscera, TMH: apparently healthy tilapia muscle, BMD: diseased mugil muscle.

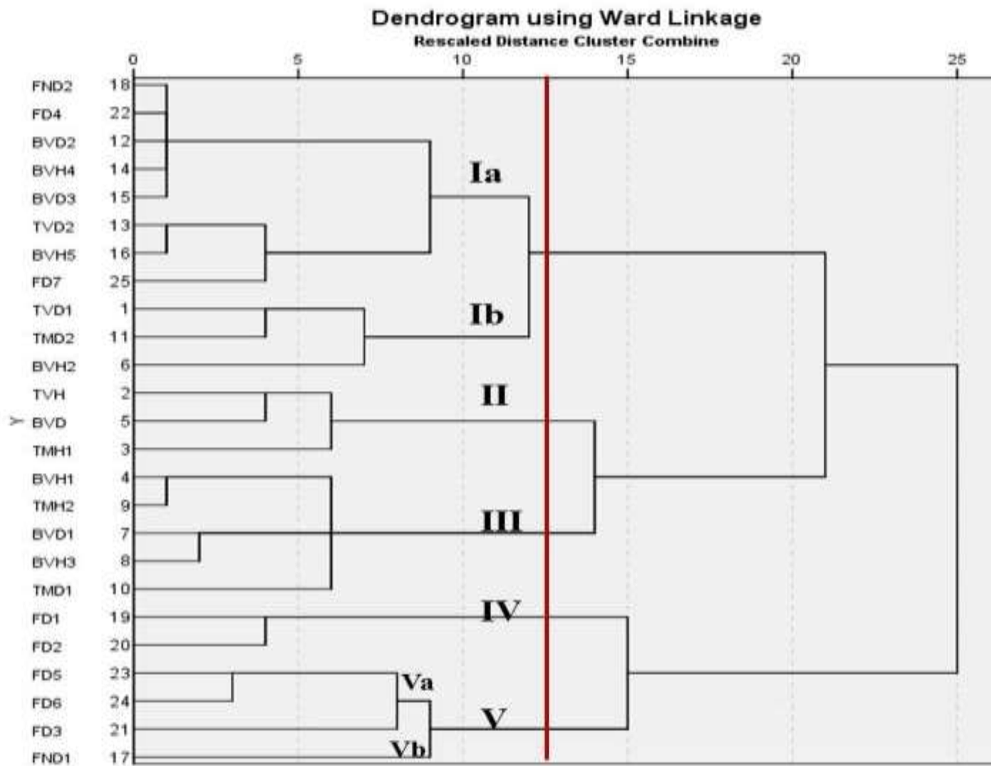

Figure 2: Dendrogram showing the relatedness of *A. hydrophila* spp. isolated from fish and human from Damietta Governorate as determined by RAPD-PCR fingerprinting using the SPSS computer software program (based on the presence or absence of each band at 12.5 linkage distance. TVD: diseased tilapia 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 SSO (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

Temperature	Non-producer	Degree of biofilm production (%, average OD± SD)			Overall biofilm producers
		Weak	Moderate	Strong	
4˚C	12 (48%, 0.029± 0.008)	5 (20%, 0.130± 0.011)	8 (32%, 0.279± 0.014)	0	13 (52%, 0.222± 0.013)
25˚C	4 (16%, 0.051± 0.009)	6 (24%, 0.171± 0.010)	7 (28%, 0.334± 0.020)	8 (32%, 0.785± 0.009)	21 (84%, 0.459± 0.013)
35˚C	1 (4%, 0.072± 0.002)	0	8 (32%, 0.297 ± 0.020)	16 (64%, 0.714± 0.018)	24 (96%, 0.575± 0.019)

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 35ºC. Interestingly, 8 (32%) and 16 (64%) were moderate and strong biofilm producers, respectively, while one isolate was non-biofilm producer. In addition, at 25ºC 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 4ºC, 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 30ºC, 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 ($p \leq 0.05$). Between temperature groups, the ability of *A. hydrophila* to form biofilm at 25ºC was significantly higher than at 4ºC. Moreover, the *A. hydrophila* ability to produce biofilm at 35ºC was significantly higher than at 4ºC (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 4ºC than at 25 and 35ºC. Moreover, strong biofilm producers were only observed at 35 and 25ºC. Rachid *et al.* [57] recorded that the increase of temperature resulted in increased bacterial ability to produce biofilm.

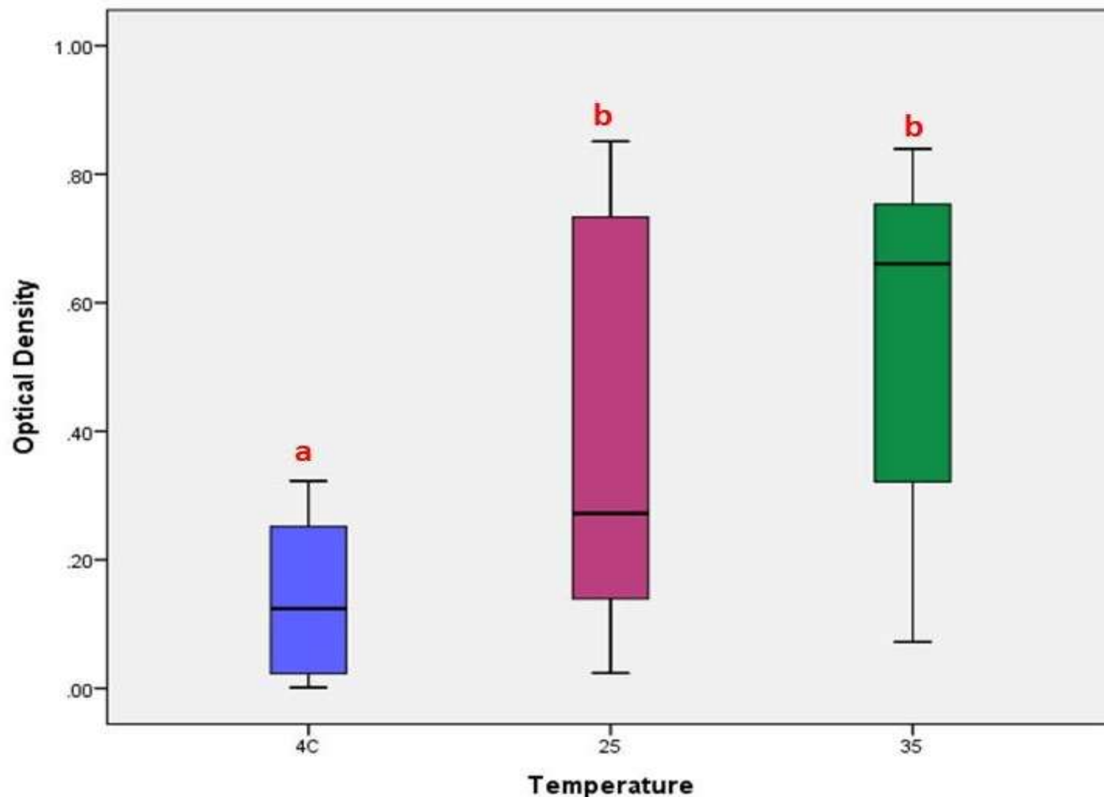

Figure 3: Boxplot showing the median optical density of biofilm formation by *A. hydrophila* spp. isolated from fish and human samples from Damietta Governorate at different temperatures (4°C, 25°C and 35°C). Different letters indicate significant difference at p ≤ 0.05.

In contrary, Nagar *et al.* [58] reported that most of *Aeromonas* spp. produced biofilm at 10ºC, while cultivation at 30 and 37ºC revealed lower biofilm production. Previous studies reported that the biofilm production ability of *Aeromonas* spp. at temperature range 25-23ºC 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 20-25ºC was the most favorable condition for biofilm formation ability of *Aeromonas* isolates, while decrease in biofilm was observed by cultivation at temperatures below 20ºC (4, 10, 15ºC) and over 25ºC (30, 35, 37ºC). 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 37ºC) [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 الأيروموناس هيدروفيلا من الأسماك والإنسان: القدرة على إنتاج البيوفيلم والعلاقة الجينية

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تم اجراء هذه الدراسة لمعرفة النمط الجيني والقدرة على إنتاج البيوفيلم لعدد ٢٥ من عترات الأيروموناس هيدروفيلا المعزول من أسماك البلطي (عدد ١٥٠) والبوري (عدد ١٤٤) وبراز الإنسان (عدد ١٠٢) في محافظة دمياط، وقد أوضحت نتائج اختبار التضاعف العشوائي المتعدد الأشكال للحمض النووي المستخدم لتقييم القرابة الجينية تصنيف عزلات الأيروموناس هيدروفيلا إلى عدد ٧ أنماط جينية مع معامل تفرقة قدره ٠.٨٤ كمؤشر للتباين، تم التأكد من وجود خمسة أفرع رئيسية باستخدام الرسم الشجري. ودل تواجد عترات من الانسان والاسماك في ذات المجموعة على احتمالية انتقالية العترات بينهما. بينما أظهرت نتائج اختبار الشريط الحيوي اختلاف قدرة العزلات مع اختلاف درجة الحرارة حيث أنه في درجة حرارة ٣٥ سليزيوس وجد أن ٢٤عزلة (٩٦%) كانت لها القدرة على إنتاج البيوفيلم، ٨ عزلة (٣٢%) قدرتهم متوسطة بينما ١٦ عزلة (٦٤%) لها قدرة قوية على إنتاج البيوفيلم، بينما في درجة حرارة ٢٥ سليزيوس أوضحت النتائج أن ٢١عزلة (٨٤%) كانت لها القدرة على إنتاج البيوفيلم، بحيث كان ٦(٢٤%) من بين تلك العزلات لهم قدرة ضعيفة على إنتاج البيوفيلم،٧عزلة (٢٨%) قدرتهم متوسطة و٨ عزلة (٣٢%) قدرتهم قوية علي إنتاج البيوفيلم، وفي درجة حرارة ٤ سليزيوس وجد أن ١٣عزلة (٥٢%) كان لها القدرة على إنتاج البيوفيلم، بحيث كان ٨ (٣٢%) و٥ (٢٠%) من العزلات لهم قدرة متوسطة وضعيفة في إنتاج البيوفيلم، على التوالي. قد وجدت علاقة معنوية بين قدرة عزلات الأيروموناس هيدروفيلا على إنتاج البيوفيلم في درجات الحرارة المختلفة، وقد أوضحت هذه الدراسة أهمية أسماك البوري و البلطي في نقل مرض الأيروموناسيس في الإنسان مع قدرة المعزولات علي إنتاج البيوفيلم مما يشكل خطر علي صحة الإنسان.