Research Article

AL- ANBAR JOURNAL OF VETERINARY SCIENCES

Vol. 17 Issue:1, (2024) ISSN: P-1999:6527 E-2707:0603

Detection and Characterization of Virulent *Pseudomonas aeruginosa* genes in Fresh Fish at Al-Diwaniya City Market, Iraq

Ban Ali Hassan and Asseel Abdulridha Saeed*

Department of Public Health / College of Veterinary Medicine, University of Al-Qadisiyah

*Corresponding Author: aseel.saeed@qu.edu.iq ORCID:0000-0002-8842-7548

Doi: https://doi.org/10.37940/AJVS.2024.17.1.10

Received: 3/2/2024 Accepted:13 /5/2024

Abstract

Fish meat is more susceptible to pathogen contamination than other protein-rich foodstuffs due to its high deterioration. *Pseudomonas aeruginosa* is considered one of the most common microbial strains responsible for the deterioration of fish and seafood products. the study's goals are to detect virulence variables of *Pseudomonas aeruginosa* and estimate the level of contamination in a fish sample, In this research, 120 fresh fish samples, were gathered from the local Diwaniyah city market local fish meat sample. including (60 marine fish and 60 common carp (Cyprinus carpio). The isolates were subjected to numerous biochemical, microbiological, and molecular analyses, and the results showed that about 26 samples were contaminated with *Pseudomonas* spp. performed on 21.6% of the total sample. Polymerase chain reaction (PCR) was used to amplify 539 bp, 511 bp, and 495 bp, for 16S DNA, Tox A, and Tox S respectively. A total of 21 Tox A genes were identified in 26 P. *aeruginosa* isolates, whereas the ToxS gene was detected in 17 of the same isolates. According to the results, *Pseudomonas aeruginosa* isolated from fish samples contains multiple virulence factor genes, indicating high pathogenicity, which may affect consumer health because these variants exhibit the capability to increase the pathogenesis of the microorganisms with the severity of the infection and cause economic losses

Keywards: Fish meat , Tox A Gene, Tox S Gene, PCR , *Pseudomonas Aeruginosa*

عزل وتشخيص جينات الضراوة *Pseudomonas Aeruginosa* من عينات الأسماك الطازجة في اسواق مدينة الديوانية / العراق

الخلاصة

تعتبر لحوم الأسماك أكثر عرضة للتلوث بالعوامل الممرضة من غيرها من المواد الغذائية الغنية بالبروتين بسبب تلفها العالي تعتبر بكتيريا الزائفة الزنجارية. *Pseudomonas spp* من أكثر السلالات الميكروبية شيوعاً والمسؤولة عن فساد منتجات الأسماك والمأكولات البحرية. الهدف من هذه الدراسة هوعزل بكتريا Pseudomonas aeruginosa و تحديد القدرة المرضية للعزلات التي تم الحصول عليها من عينات لحوم الأسماك المحلية، وكذلك تحديد عوامل الضراوة وتقييم مدى التلوث في عينة الأسماك. تم في هذا البحث اختيار 120 عينة من الأسماك الطازجة تشمل (60) سمكة بحرية و 60 سمكة كارب عادي ((Cyprinus carpio) من سوق مدينة الديوانية المحلي. خضعت العزلات للعديد من التحاليل البيوكيميائية والميكروبيولوجية والجزيئية، وأظهرت النتائج تلوث حوالي 26 عينة ببكتيريا Pseudomonas spp بنسبة 21.6% من إجمالي عدد العينات استخدم تفاعل البلمرة المتسلسل (PCR) لتضخيم 539 pb، و511 pb، و495 pb، لـجينات *16 S DNA*، و *Tox A*، و Tox S على التوالي. تم تشخيص 21 جين Tox A في 26 عزلة من P. aeruginosa، في حين تم اكتشاف جين ToxS في 17 عزلة من نفس العزلات. ووفقا للنتائج، فإن *Pseudomonas aeruginosa* المعزولة من عينات الأسماك تحتوي على جينات متعددة لعامل الضراوة، مما يشير إلى إمراضية عالية، مما قد يؤثر على صحة المستهلك. وذللك بسبب ان هذه المتغيرات لها القدرة على زيادة الإصابة في الكائنات الحية الدقيقة وزيادة شدة العدوى وخسائر اقتصادية .

Introduction

Fish meat has long been regarded as an essential food for optimum health; from ancient times, it has been known as a 'brain food', referring to its role in the formation of a healthy brain. Over the last several decades, research has shown the relevance of fish nutritional components in brain development and reproduction, as well as their participation in a range of other bodily activities. Fish provides excellent macro- and micronutrients compared to other protein sources (1). It is considered to be a "rich food for poor people" because it supplies significant nutrients, including high-biovalue fatty acids and protein. (2) .Fish flesh protects against a variety of common ailments, including cardiovascular disease, stroke, asthma , diabetic heart disease, Alzheimer's, cancer, and may also aid in the prevention of other disorders.(3) Both pathogenic and non-pathogenic bacteria for human , are often found in fish and fish product .

Most seafood products deteriorate, due to microorganisms, their activities and the microbial flora of freshly caught fish. . Fish that has undergone microbial deterioration may have unexpected tastes, odors, slime formation, and discolorations, which make it unfit for consumption. (4) However, only a small number of organisms known as specific spoilage organisms (SSOs) can be responsible for the unpleasant off flavors associated with seafood spoilage. (5). European Food Safety has recognized *Campylobacter, Salmonella, E. coli, Yersinia, and Listeria monocytogenes* as the bacteria responsible for the most significant infectious outbreaks around the world. Also refer to (6). Gram-negative psychotropic rods, which include *Shewanella, Pseudomonas, Vibrio, and Aeromonas* species, were mostly identified from fresh fish samples. (7 Each of these bacterial species was identified in samples of both fresh and degraded fish. Pseudomonas aeruginosa is a very adaptable pathogen that can grow in a variety of environments.

Typically, the species retreats from soil and water or settles in various anatomical Humans, animals, insects, and plants may all be present in certain places. *Pseudomonas aeruginosa* strains have been introduced into a sample of marine and local carb fish this bacterium has a number of virulence factors. Tox A and Tox S genes are extracellular toxins (exoenzyme S and exotoxin A). They necrotize colonized tissue and limit the activity of phagocytic cells, promoting dispersion (8). These factors have been scientifically demonstrated to enhance the pathogenicity of bacteria and the severity of illness. .(9) Despite the importance of knowing these traits, strains derived from local food sources are not properly documented. (10) The current research is intended to assess the prevalence of *Pseudomonas.aeruginosa* its isolation from a local fish sample in Diwaniyah City Market, and the identification of virulence genes. because these variants exhibit the capability to increase the pathogenesis of the microorganisms and the severity of the infection, which may affect consumer health.

Material and methods

1- Fish Samples collection:

One hundred twenty randomly selected samples of fresh fish (60 Cyprinus carpio and 60 marine fish) weighing between 500 and 1000 g were collected from stores in Al-Diwaniya city. The sample was put in a clean plastic bag and allowed to cool before being sent immediately to the microbiological lab. Various microbiological and biochemical tests were performed. After getting swabs from the skin, intestine, and meat, about 25 grams of muscle were cultivated in nutrient broth at 25°C for 48 hours, and then different bacterial and biochemical tests were done.

2- isolation of bacteria and Bacteriological examination :

This study involved aseptically removing 25 grams of muscle and intestine tissue. After that, it was mixed with 225 ml of peptone water. we cultivated the broth at 25°C for 24 hours. then one loopful of the cultivated broth was distributed onto MacConkey agar, blood agar, 24–48 hours at 25°C. The suspected colonies streaked for 48 hours at 25°C on cetrimide agar and chromogenic pseudomonas agar (Oxoid, England). (11) Gram stain was utilized to identify the isolates by their phenotypes. As specified by the American Society of Clinical Pathology (12), Additionally, biochemical analyses were conducted, including assays for oxidase, catalase, indole, TSI and Simon citrate. (13).

3- **Molecular methods :**

3.1 Extraction DNA

The DNA extraction kit (Add Bio/Korea) was used to extract genomic DNA from the isolates in accordance with the instructions provided by the manufacturer. Place about (1×10^9 bacterial cells) from bacterial culture in a 1.5 ml microcentrifuge tube. Spin for 1 minute at 14–16,000 x g and Include 20 µl of Proteinase K, for lysis. Combine 200µl of GB Buffer with the sample with a vortex , Every 3 minutes, invert the tube during incubation for 10 minutes . for DNA-binding Stir 200 µl of absolute into the sample lysate immediately by shaking firmly. Add the GD column to a 2 ml collection tube. After transferring the mixture to the GD column, centrifuge it at 14–16,000 x g for two minutes.. Wash Put 400 µl of W1 buffer in the GD column. Add 600 µl of wash buffer to the GD column. Dry the column matrix by centrifuging again for 3 minutes at 14-16,000 x g. The standard elution volume is 100 µl.. Put 100µl of pre-heated elution buffer, TE buffer, or water in the center of the column matrix.

3.2 Quality Estimation Of DNA

To determine the extracted DNA concentration and assess sample quality for future applications the Quantus Fluorometer(DNA IQ™ chemistries on the Quantus™ Fluorometer) was used. 199 µl of diluted quanti flour dye was combined with 1 µl of DNA (14). DNA concentrations were measured throughout a 5-minute incubation period at room temperature. Additionally, the integrity of the DNA was determined using a 2% agarose gel stained with 0.5 µg/ml of ethidium bromide. from AddBio/Korea. (15) The DNA isolated in this study was amplified using PCR. . The 16S rRNA (toxA and toxS) genes, which encode for virulence factors, are based on the primers demonstrated in Table 1. The primers that were used for this investigation were designed using NCBI Gene-Bank and applied to a 25µl PCR reaction The description of the amplification program listed in (Table 2).

Table(1) . list of primers that used in the study

Primer type	Primer sequence	Amplication size(bp)	Gene bank reference code
16 ribosomal RNA gene	F:TACCTGGCCTTGACATGCTG R:GTTCCCCTACGGCTACCTTG	539 bp	EU344794.1
ToxA gene	F:GTGCTGCACTACTCCATGGT R:GCTGGGCGAGGTAGTTGTAG	511 bP	JX026663.1
ToxS gene	F:TTTTAGGTTTTGCCGCTGCC R:CCCTTCAAGGTCATGGGCAA	459bp	L27629.1

Table (2): The PCR program used to amplify the *Pseudomonas aeruginosa* genes

Steps	℃	(time)Min:sec.	Cycle
Initial Denaturation	94	05:00	1
Denaturation	94	00:30	35
Annealing	55	00:30	
Extension	72	01:00	
Final extention	72	07:00	
Hold	15	10:00	1

Results and Discussion:

***1 Pseudomonas aeruginosa* prevalence and Molecular detection of virulence factors**

Fish is more susceptible to pathogen contamination because of its higher deterioration in comparison to other high-protein foods due to a lowered pH and higher moisture content. P. aeruginosa and other microorganisms have been identified as the primary cause of deterioration in the majority of marine products (16) The traditional microbiological tests, including phenotype culture , microscopically features and biochemical tests were used to determine Pseudomonas aeruginosa isolates which showed negative for Gram stain, and positive for oxidase, catalase, TSI, and Simmon citrate, also indicating positive growth on cetrimide and chromogenic agar due to their green blue color, uneven ends, and metallic colonies accordance to . (17). These biochemical tests results showed in (Table 3), The result of isolation *Pseudomonas . aeruginosa* among the fish samples examined from total (120) was 26 (21%), which was contaminated with the pathogen. The high percentages for common carp and marine samples were 17 (28%), and 9 (15 %) , respectively, as illustrated in (Tables 4 and 5).

Table (3) Result Of Biochemical Analysis *Pseudomonus Spp.*

Bacteria	Gram stain	Indol	Simmon citrate	Oxidase	Catalase	T.S.I
Pseudomonus spp	-	-	+	+	+	+

Table (4) shows The prevalence percentage of isolated pseudomonas aeruginosa in fresh fish sample

Type of sample	Number of sample	N.Positive
Local carb	60	17(28%)
Marine	60	9(15%)
Total	120	26(21%)

(Table 5) The distribution of 16 s ribosomal RNA genes among 26 samples of fish and virulence factors *Tox A gene* and *Tox S gene* in *pseudomonas aeruginosa* isolates.

Type of sample	Carp	Marine	Total
16 S ribosomal RNA gene	17	9	26
Tox A gene	15	6	21
Tox S gene	11	6	17

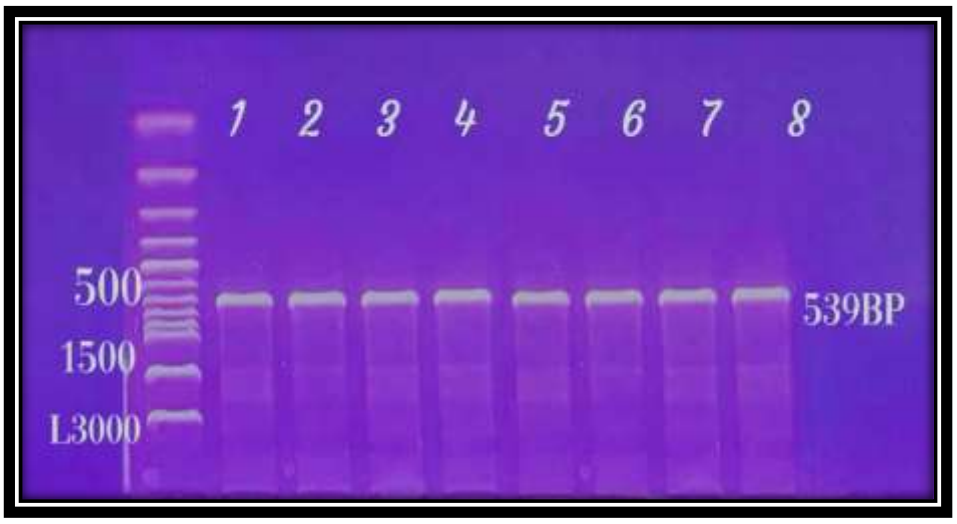
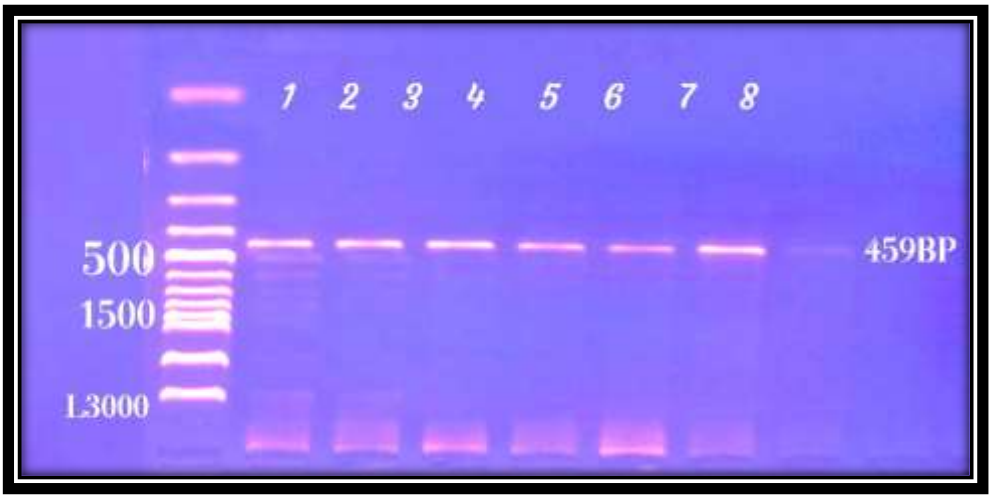


Figure (1)showing the gel electrophoresis was used to analyze the amplification of the 16S rRNA gene. Lane 1-8 positive samples for the *16S rRNA gene* of *pseudomonas aeruginosa* (which is 539 bp) M ladder (3000 bp)

Figure . (2) show the gel electrophoresis was used to analyze the amplification of the targeting ToxA gene *pseudomonas aeruginosa* at product size(511 bp) ,Lane L(3000)bP ladder , 1and 2,4,5,7, to 8, is positive result.



(Figure.3) show the gel electrophoresis was used to analyze the amplification targeting Tox S gene ***pseudomonas aeruginosa*** Product size (459 bp) lane L(3000) bp ladder from 1 to8 is positive result.

These isolates were confirmed using the PCR technique. The result of Pseudomonas. using 16S rRNA, shown in Fig. 1; Table 4; and the confirmed *Tox A gene and Tox S gene* (Fig. 2, 3; and Table 4), presents the distribution of 16S ribosomal RNA genes among 26 samples, aeruginosa strains detected by

in addition to the virulence factors. The higher contamination rate was in local carbs (17%), while it was 9 (15%) in marine samples. These results were confirmed by a specified 16S RNA

gene amplicon size of 539 bp, as shown in (Fig. 1 and Table 4) Our results of isolation from local carp seemed higher than those of research conducted in Iran (18), which found that the isolates had 5% of 470 fish samples, and 10.5%. monitored by (19) but also similar to those of studies conducted in Iraq (20) and(21). The chromosomal markers of the toxA gene and the tox S gene were used in order to identify the toxicogenic All 26 isolates were analyzed for the detection of virulence genes for (Tox A gene and Tox S gene) respectively. The PCR results for Pseudomonas aeruginosa, as in Table 5) showed a higher prevalence in carb fish samples with (15) Tox A genes and(11) Tox S genes. While in marine fish, Tox A genes and Tox S genes were detected in 6 isolates with fragment sizes of 511 bp and 549 bp Fig. (2 and 3)

These findings concur with the findings(22) and Kenneth (23)_ that most *P. aeruginosa* isolates harbored the *ToxA* gene. The availability of this gene is linked with the pathogenicity of the organism because it inhibits the host cell from synthesizing proteins (24) As a result, it promotes bacteria to proliferate quickly across every type of tissue. (25). Variations in the present period of isolation may, nevertheless, be attributed to a variety of factors, including host immunity, seasonality, environmental conditions, and bacterial diversity. As a result, it presents a significant prospective risk to the health of consumers. *pseudomonas aeruginosa*, detected in fish samples, has several virulence factor genes, indicating high pathogenicity, which might harm consumer health by increasing infection severity. Additionally, fish that are exposed to stressful or unsuitable environmental conditions may develop other severe diseases causing economic losses .

Conclusion

Pseudomonas aeruginosa isolated from fish samples contains some virulence factor genes, indicating high pathogenicity, which may affect consumer health. According to the results, handling and retail display of fish and fish products requires hygienic conditions due to the rise prevalence of these virulence genes. The increasing use of fish and seafood requires regulation of their microbiological quality which causes most of the public health and economic costs related to consumers, management and transportation.

Acknowledgments

This study was conducted as part of a master's thesis, we would like to extend our gratitude to the research assistant and laboratory involved with the Department of Public Health at Al-Qadissihiay University/Veterinary College for their assistance.

Conflict of Interest

The authors confirmed that they had no conflicts of interest.

Reference :

1- Lilly TT, Immaculate JK, Jamila P. Macro and micronutrients of selected marine fishes in Tuticorin, South East coast of India. International Food Research Journal. 2017 Mar 1;24(1).
2- Sujatha K, Joice AA, Kumaar PS. Total protein and lipid content in edible tissues of fishes from Kasimodu fish landing centre, Chennai, Tamilnadu. European Journal of Experimental Biology. 2013;3(5):252-7.
3- Bud I, Stefan R. Nutritive value of fish meat comparative to some animals meat. Bulletin of the University of Agricultural Sciences & Veterinary Medicine Cluj-Napoca. Animal Science & Biotechnologies. 2008 Jan 1;65.

4- Morshdy AE, Darwish WS, Daoud JR, Sebak MA. Estimation of metal residues in Oreochromis niloticus and Mugil cephalus intended for human consumption in Egypt: a health risk assessment study with some reduction trials. Journal of consumer protection and food safety. 2019 Mar 4;14:81-91.

5- Gram L, Dalgaard P. Fish spoilage bacteria–problems and solutions. Current opinion in biotechnology. 2002 Jun 1;13(3):262-6.

6- Lehane L, Olley J. Histamine fish poisoning revisited. International journal of food microbiology. 2000 Jun 30;58(1-2):1-37.

7- Murray PR, editor. Microbiología médica básica: hhh. Elsevier Health Sciences; 2018 Feb 23.

8- Bogiel T, Depka D, Kruszewski S, Rutkowska A, Kanarek P, Rzepka M, Leitão JH, Deptuła A, Gospodarek-Komkowska E. Comparison of virulence-factor-encoding genes and genotype distribution amongst clinical Pseudomonas aeruginosa Strains. International Journal of Molecular Sciences. 2023 Jan 9;24(2):1269.

9- Benie CK, Dadié A, Guessennd N, N'gbesso-Kouadio NA, Kouame NZ, N'golo DC, Aka S, Dako E, Dje KM, Dosso M. Characterization of virulence potential of Pseudomonas aeruginosa isolated from bovine meat, fresh fish, and smoked fish. European Journal of Microbiology and Immunology. 2017 Mar;7(1):55-64.

10- Church D, Melnyk E, Unger B. Quantitative Gram stain interpretation criteria used by microbiology laboratories in Alberta, Canada. Journal of clinical microbiology. 2000 Nov 1;38(11):4266-8.

11- Saleem H, Mazhar S, Syed Q, Javed MQ, Adnan A. Bio-characterization of food grade pyocyanin bio-pigment extracted from chromogenic Pseudomonas species found in Pakistani native flora. Arabian Journal of Chemistry. 2021 Mar 1;14(3):103005.

12- Carter GR. Essentials of veterinary bacteriology and mycology. Lea & Febiger; 1986.

13- Nakayama Y, Yamaguchi H, Einaga N, Esumi M. Pitfalls of DNA quantification using DNA-binding fluorescent dyes and suggested solutions. PloS one. 2016 Mar 3;11(3):e0150528.

14- Dehkordi FS, Tavakoli-Far B, Jafariaskari S, Momtaz H, Esmaeilzadeh S, Ranjbar R, Rabiei M. Uropathogenic Escherichia coli in the high vaginal swab samples of fertile and infertile women: virulence factors, O-serogroups, and phenotyping and genotyping characterization of antibiotic resistance. New Microbes and New Infections. 2020 Nov 1;38:100824.

15- Abdelaziz AA, Kamer AM, Al-Monofy KB, Al-Madboly LA. Pseudomonas aeruginosa's greenish-blue pigment pyocyanin: its production and biological activities. Microbial Cell Factories. 2023 Jun 8;22(1):110.

16- Ali A, Wei S, Ali A, Khan I, Sun Q, Xia Q, Wang Z, Han Z, Liu Y, Liu S. Research progress on nutritional value, preservation and processing of fish—A review. Foods. 2022 Nov 16;11(22):3669..

17- Shahrokhi GR, Rahimi E, Shakerian A. The prevalence rate, pattern of antibiotic resistance, and frequency of virulence factors of Pseudomonas aeruginosa strains isolated from fish in Iran. Journal of Food Quality. 2022 Apr 25;2022:1-8.

18- Sanhoury FA, Khalil SA, Ebied SK. Studies on some bacteria isolated from marine shrimp retained in Alexandria markets. Alexandria Journal of Veterinary Sciences. 2016 Nov 1;51(2).

19- Altaee AJ, Aldabbagh SY. Molecular identification of virulence genes of Pseudomonas aeruginosa isolated from fish

(Cyprinus carpio) in Mosul city. Iraqi Journal of Veterinary Sciences. 2022 Oct 1;36(4):953-8.

20- Magdy IH, El-Hady MA, Ahmed HA, Elmeadawy SA, Kenwy AM. A contribution on Pseudomonas aeruginosa infection in African catfish (Clarias gariepinus).

21- Khattab MA, Nour MS, ElSheshtawy NM. Genetic identification of Pseudomonas aeruginosa virulence genes among different isolates. J Microb Biochem Technol. 2015 Jan;7(5):274-7.

22- Kenneth T. Todar's online textbook of bacteriology. Bacterial Protein Toxins. 2011.

23- Rocha AJ, Barsottini MR, Rocha RR, Laurindo MV, Moraes FL, Rocha SL. Pseudomonas aeruginosa: virulence factors and antibiotic resistance genes. Brazilian Archives of Biology and Technology. 2019 Jun 9;62:e19180503.

24- Jawher IM, Hassan MG. Detection of some virulence genes of Pseudomonas aeruginosa isolated from meat at Mosul city.2022- 105-101 :

25- Abd El Tawab AA, Maarouf AA, Ahmed NM. Detection of Virulence factors of Pseudomonas species isolated from fresh water fish by PCR. Benha Veterinary Medical Journal. 2016 Mar 1;30(1):199-207.