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Molecular identification of virulence genes of *Pseudomonas aeruginosa* isolated from fish (*Cyprinus carpio*) in Mosul city

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Abstract

Depending on the nature of the fish's environment, they are susceptible to many pathogens, including bacterial causes, so the goals of the current study are isolation and molecular identification of *Pseudomonas aeruginosa* with its prevalence rate and detected virulence genes from fresh common carp fish. The swabs were taken from the gills, skin, intestine and muscles of 75 fish samples from variable localities from Mosul city during the period September to December in the year 2021. The prevalence percentage of bacteria was 26.66% which was confirmed by traditional microbiological tests which included (phenotype culture, microscopically features and API-test) and molecular identification. The isolates formed 42.5, 37.5, 15, 5% from gills, skin, intestine and muscles, respectively. The molecular results of forty isolates determine that *Pseudomonas aeruginosa* have *rpoB* 100%, and virulence genes *oprL*, *toxA*, and *algD*, which are express the outer membrane protein, exotoxin A and alginate respectively occur as 97.5% for the *oprL* gene and 100% of both *toxA* and *algD* genes.

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Introduction

Pseudomonas aeruginosa is considered one of the primary pathogens in fresh fish thus cause of high economic losses and high mortalities among fish (1). *P. aeruginosa* is a Gram-negative bacillus, motile by unipolar flagella. Aerobic bacteria catalase and oxidase positive, produce pyocyanin and pyoverdine pigments. It is firmly adaptable to many environments, including the aquatic environment (2). An opportunistic pathogen accountable for the severe death of fish and spoilage of fresh fish. *P. aeruginosa* it can cause severe lesions in fish, including, gill necrosis, hemorrhagic septicemia, congested kidney and friable liver especially when exposure to stress factors and changing in environmental condition as decreasing in O_2 and variation in temperature (3). Moreover, *P. aeruginosa* can cause a problem for consumers associated with public health, some reports indicate that contaminated fish with enterotoxigenic *Pseudomonas* causes diarrhea and skin infection especially in the immunocompromised patient (4). The pathogenicity of *Pseudomonas aeruginosa* is related to its possession of many different virulence factors, some of them that related to the cell surface of bacteria that include; flagella, Pilli, lipopolysaccharide (LP), and alginate (*algD*), which contribute to the adhesion of bacteria in a host cell and colonization, as well as contribute in the transformation of active proteins. Therefore, cause recurrent infections (5). Others includes extracellular enzymes and toxins such as exotoxin S and exotoxin A, exotoxin A play a decisive role in inhibiting the protein biosynthesis process of host cell therefore, they aid for invasion and distribution of bacteria, thus accelerating of diseases progression (6). Toxin A is considered one of the influential virulence factors of this bacterium because it is accountable for inhibiting the protein biosynthesis in a host cell, which play a potent role in their pathogenicity. Toxin A encoded in the (*toxA*) gene is considered a prominent member of secretion system type II (T2SS) for *P. aeruginosa* (7). The invasion by this bacterium

harboring this virulence factor leads to an increase in morbidity despite antibiotics use. The virulence mechanisms in *P. aeruginosa* varies depending on the infection environment (3). Many infections are caused by *P. aeruginosa* as a result of high resistance against antibiotics (8), which is due to the synergistic relationship between the effect of the low permeability of the cellular outer membrane and the existence of multidrug efflux pump in turn directly excludes antibiotics out of the cell (9). Lipoproteins play an important role in many processes necessary for bacterium cells, as adapting the bacteria to the environment and in bacterial infections outer membrane protein of this bacterium which contributes to resisting the antibiotics and disinfectant. Outer lipoprotein encoded in *oprL* gene (2) limited on Pseudomonads. Therefore, it could be a good maker that is used for detection of this bacterium in environmental and clinical samples and utilized as pathogenicity assessment for *P. aeruginosa*; therefor, recent studies used *oprL* gene for rapid detection of *P. aeruginosa* by PCR test (9,10). Alginate (Exopolysaccharide) is another virulence factor that protects the bacteria against antibiotics, disinfectants, and from the host's immune defense. Alginates contribute to bacterial adhesion to host cell (10), *algD* gene which encodes to (GDP-mannose dehydrogenase enzyme) that is formed the first unit in biosynthesis process of alginate (11). RNA polymerase Beta subunit which encoded in (*rpoB*) gene, was applied as a signature to diagnose this bacterium. Recent studies were focused on using *rpoB* genes for detecting *P. aeruginosa* due to high molecular identification ratio properties for the confirmation of the *P. aeruginosa* isolates. The researcher evaluated this bacterium that was isolated from fish by using the *rpoB* gene that gave 99.5% (5), also another study advises using the *rpoB* gene on the identification of this bacterium (10).

Several studies revealed the virulence factors of *Pseudomonas aeruginosa* from a different source without paying attention to fish samples in Iraq. So, the goals of the current study are isolation and molecular identification of *P. aeruginosa*, and detected some genes encoding virulence factors from fresh fish (*Cyprinus carpio*) in Mosul city.

Materials and methods

Fish samples

A total of 75 samples of fresh fish (*Cyprinus carpio*) ranging between 100-350 g in weights were collected randomly from local markets of fish in Mosul city. For the period from September to December 2021, each sample was placed in a sterile plastic bag and transported directly to the microbiology laboratory under cooling conditions.

Bacteriological examination

Swabs of skin and gills also one gram of intestine and muscle were taken aseptically and placed in tryptone soya broth then incubated at 25°C for 24 h. Then one loopfull from the cultivated TSB, was streaked on each tryptone soya agar, blood agar and MacConkey agar. The plates were incubated at 25°C for 24-48 h. The Suspected singles colonies were picked up and streaked on the cetrimide agar supplemented with nalidixic acid, and glycerol then incubated the plates at 25°C for 24-48 h (12). Phenotypic identification of isolates was done microscopically by Gram stain and biochemically by using oxidase, catalase, indole, methyl red, Voges-Proskauer, citrate utilization, and urease tests (13). All isolates were confirmed by using API E-test (BioMerieux). Then forty isolates were selected for molecular identification of *Pseudomonas aeruginosa*.

Extraction of DNA

According to the manufactured company *Pseudomonas aeruginosa* isolates were subjected to genomic DNA extraction (Jena Bioscience, Germany). Fresh colonies of *P. aeruginosa* cultivated on BHIA for 24h were suspended in an Eppendorf tube for the Lysis of cells, followed by protein precipitation step. The supernatants were separated in a 1.5 ml Eppendorf microcentrifuge with 300 µl Isopropanol 99 %. Then the tubes were centrifuged and discarded the supernatant, then the draining tubes. Small pellets of DNA were washed using washing buffer by inverting it several times before being centrifuged. Then the supernatant was discarded, dried the tubes at room temperature, added 100 µl of Hydration solution for DNA hydration, and incubated it at 65 °C for one hour. The extracted DNA was stored under -20 °C for the following use (14,15).

Detection of *Pseudomonas aeruginosa and* their virulence genes by PCR reaction

All forty samples were screened for *P. aeruginosa* using *rpoB* primers, *rpoB*- f (CAGTTCATGGACCAGAACAACCCG') and *rpoB*-r(ACGCTGGTTGATGCAGGTGTTC') synthesis in Macrogen, Korea. The program of amplification is described in (Table 1). The genes (*oprL*, *toxA* and *algD*) which are encoding for bacterial virulence factors are molecular identification depending on the primers in (Table 2). The PCR reaction mixture for all protocols was carried out according to the manufacturer's instructions. The master mix reaction was prepared by adding 12.5 µl of 2X Taq Premix (Ge-Net, Bio- Korea), 1 µl of each forward and reverse primers, 8 µl of PCR grade water, finally 2.5 µl of the DNA template. PCR cycling conditions were done in (Table 2) using a thermal cycler (Bio-Rad, T100, Bio-Rad - USA). Then the products of PCR were separated by 1.2% of agarose gel (Promega, USA), which contained Prime Safe Dye by (Ge-Net, Bio- Korea). Electrophoresis conditions of include (75 V- 300 mA -1h) using Wide Mini -Sub Cell GT (BioRad, USA). After that, the gel was observed using the (Gel-doc-Ez) system to revealing the specified bands (16,17).

Table 1: PCR setting program of amplification

Primer	No Cycle	Adjusted temperature	Time	Discretion	References
	1	94°C	3min	Initial DNA denaturation	
		94°C	1min	DNA denaturation	
rpoB	30	58°C	1min	Primer annealing	5
		72°C	2min	Primer extension	
	1	72°C	2min	Final extension	

Table 2: Features of primers and PCR setting program amplification

Primer	Sequence	bp	PCR setting system	References
oprL-f *oprL*-r	ATG GAA ATG CTG AAA TTC CTT CTT CAG CTC GAC GCG	504	196 °C 5min initial DN denaturation 96 °C 1 min DNA denaturation 3055°C 1min annealing 72°C 1 min extension 1 72 °C 10 min final extension	18
toxA-f *toxA*-r	GGT AAC CAG CTC AGC CAC AT TGA TGT CCA GGT CAT GCT TC	354	1 94 °C 1 min initial DNA denaturation 30 94 °C 30 sec DNA denaturation 55 °C 1 min annealing 72 °C 1min extension 1 72 °C 10 min final extension	18
algD-f *algD*-r	ATGCGAATCAGCATCTTT CTACCAGCAGATGCCCTC	1310	1 94 °C 5min initial DNA denaturation 35 94 °C 30 sec DNA denaturation 61°C 1min annealing 72 °C 1 min extension 172 °C 7min final extension The reactions were set for cooling at four °C	5

Results

Pseudomonas aeruginosa isolates appeared as Gram-negative rods, motile, catalase, and oxidase tests positive produced yellow green, pyoverdine and blue green exopigmentation, pyocyanin on cetrimide agar, lactose non fermented on MacConkey agar, beta hemolysis on blood agar. The isolates were confirmed using the API-E test. Through bacteriological tests for fish samples, we obtained 80 isolates of *pseudomonas aeruginosa* 42.5, 37.5, 15, 5% from the gills, skin, intestine, and muscles, respectively (Table 3). The amplification PCR products showed the target identified DNA fragment which indicating that the bacteria *P. aeruginosa* possess high DNA (Figure 1). All 40 isolates were analyzed and presented positive results *rpoB* gene (Figure 2). The PCR results for *P. aeruginosa* showed that, *oprL*, *algD*, and *toxA* virulence genes were detected in all forty strains 97.5% for *oprL* and 100% for the other two genes. The *oprL* gene was amplified in all positive isolates giving the product of 505 bp (Figure 3). *algD* and *exoA* genes were amplified in all isolates 100% giving a product of 1310 bp and 350 bp, respectively (Figures 4 and 5).

Table 3: The number and percentage of *pseudomonas aeruginosa* isolated from fresh fish

Samples	No	No of isolates	Percentage
Gills	75	34	42.5%
Skin	75	30	37.5%
Intestine	75	12	15%
Muscles	75	4	5 %
Total	300	80	100 %
Prevalence	300	80	26.66

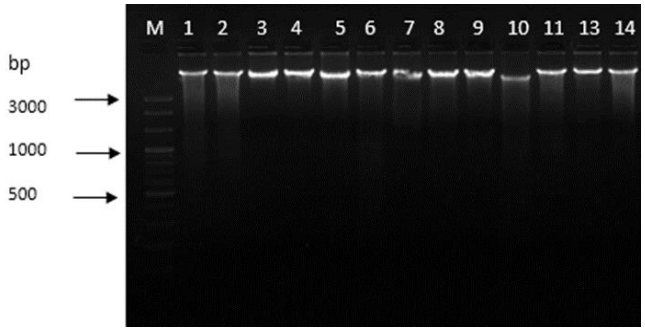

Figure 1: PCR final products of *pseudomonas aeruginosa*.

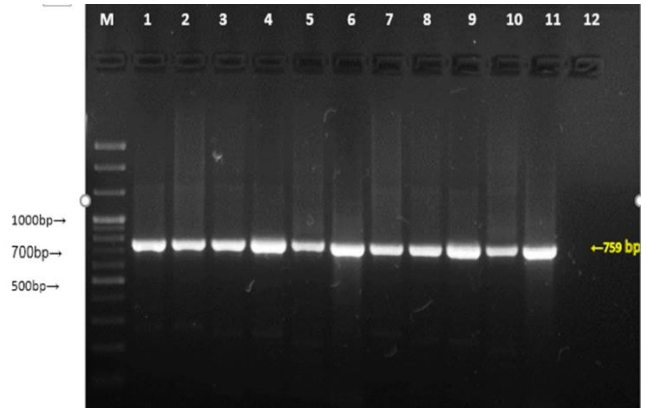

Figure 2: PCR final products for *Pseudomonas aeruginosa* using the *rpoB* gene. Well, M; DNA marker 100 bp. Well 1-11 positive fish samples giving 759 bp; well 12 negative control.

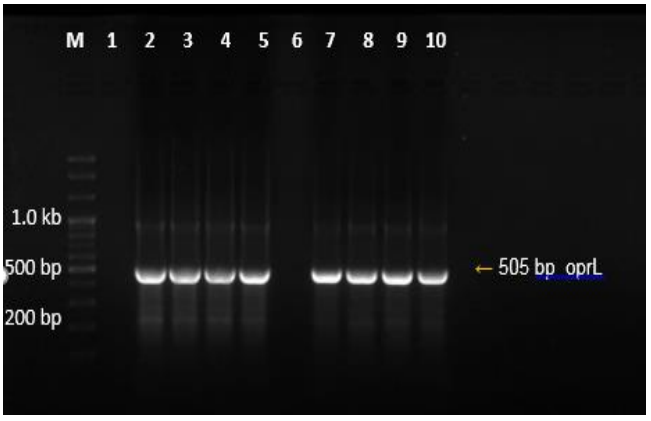

Figure 3: PCR products for the *OprL* gene. Lane M, standard DNA, well 2-5 and 7-10 positive *P. aeruginosa* fish samples giving 505 bp product size; well 1: negative control.

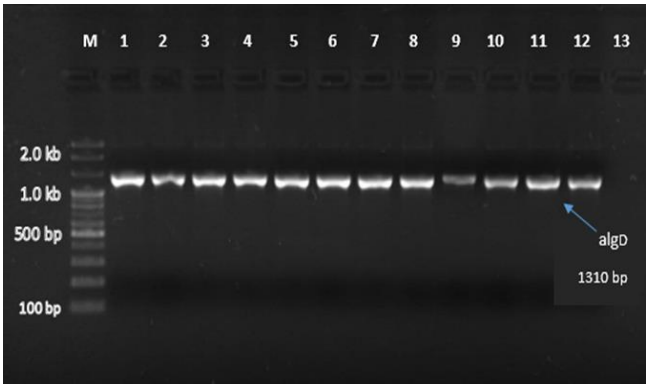

Figure 4: PCR products for the *algD* gene. Lane M, standard DNA; well 1-12 positive samples gave 1310 bp product size; well 13 negative control.

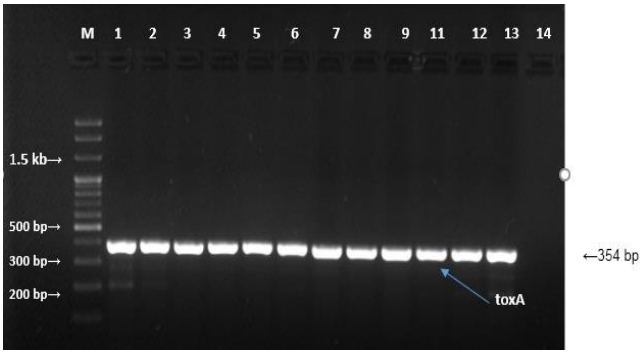

Figure 5: PCR products for *toxA* gene. Lane M, standard DNA; well 1-13 positive fresh fish samples giving 354 bp product size.; well 14: negative control.

Discussion

In the current study we obtained eighty isolates of *P. aeruginosa* from the gills, skin, intestine and muscles specimens from the fish market. The highest percentage of isolation reached 42.5% from gills followed by skin 37.5%, then intestine formed 15%, finally isolates from muscles formed 5%. The prevalence rate of *P. aeruginosa* reached 26.6%. This result was close to Hana *et al*. (19) and Yaseen *et al*. (20) finding, who obtained 30, 22% and Abd El Tawab *et al.* (1) who obtained 16.7%, 25% from the skin and gills respectively, while higher than Sanhoury *et al.* (21) who isolated it at a percentage 10.5%. The variations in the ratio may be due to the host immunity, the number of bacteria, seasonal and environmental variation (21,22). These results indicated to the possibility of serious fish diseases such as septicemia when fish are under stressed or unsuitable environmental conditions. this is considered the main cause of economic losses and effects on public health due to eating, handling and transporting (4).

The PCR results revealed that all *P. aeruginosa* harbored the *rpoB* gene. This result is consistent with the finding of Benie *et al.* (5) who indicated that 99.5% of his isolates were contained the *rpoB* gene. Therefore, it is advised to use the *rpoB* gene that gives reliable results because it has high molecular identification properties to confirm the *P. aeruginosa*, also Tayeb *et al.* (23) reported that using sequencing of *rpoB* as routine identification of Pseudomonas strains.

The *rpoB* gene performance on identification of *P. aeruginosa* strains could be illustrated by the fact that the differentiation between the species so close to this bacterium was obtained by the molecular analyses of this gene (5). Also, Baskan *et al.* (10) indicate that the *rpoB* gene give more accurate results than the 16S rRNA gene therefor; the *rpoB* gene could be used in the detection of *P. aeruginosa* in contaminated food products (10).

The study appeared that *oprL* genes were present in 97.5% of *P. aeruginosa* isolates that produce positive PCR amplicons products at 504 bp. These findings in consent with the findings each of Abd-El-Maogoud *et al.* (9), Algammal *et al.* (18), and Mehri *et al.* (24) who showed that all tested strains 100% harbored this gene. The *oprL* gene contributes significantly to the resistance of this bacterium to antibiotics and disinfectants, as it directly affects the efflux systems, which in turn affects the permeability of the cell membrane to antibiotics (3), as well as *oprL* could be a reliable factor for diagnosis of *P. aeruginosa* in environmental and clinical isolates (20).

The study showed that all tested isolates contained *algD* genes. The results disagree with the studies of Benie *et al.* (5) (5) who recorded 77.5 and 54.5% from fresh and smoked fish respectively. The *algD* gene encodes for alginate production. Alginate is one of the essential virulence factors for this bacterium. It is an exopolysaccharide that represents the mucous layer and has a high viscosity, and provides protection for the bacteria cell from defensive factors of the host such as phagocytic cells (2), also participates in the formation of the biofilm and play a role in resistance to antibiotics (11).

Also, this study showed that toxin A which encoded in the *toxA* gene were present 100% in all forty isolates, produced positive PCR amplicons products at 354 bp. This result is incompatible with Baskan *et al.* (10), and Siriken *et al.* (25) that all fish isolates harbored the *toxA* gene. The presence of this gene is associated with the pathogenicity of this bacterium, because it responsible for inhibiting the protein biosynthesis in the host cell. Thus, it facilitates the dissemination of bacteria through all tissue (2,7).

Conclusion

This study concludes that the *rpoB* gene is the identification gene for *P. aeruginosa*, which has variable virulence factors encoded by genes *oprL*, *algD* and *ToxA* responsible for antibiotic resistance, biofilm formation, and protein biosynthesis inhibition. Therefore, may be constitute serious diseases on common carp fish, thus consequently causing economic losses and affecting consumers' health.

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Conflict of interest

The authors declare that he has no conflict of interest.

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التعريف الجزيئي لجينات الضراوة للزوائف الزنجارية المعزولة من أسماك الكارب في مدينة الموصل

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الخلاصة

تتعرض الأسماك للعديد من مسببات الأمراض بما في ذلك المسببات الجرثومية وذلك تبعا لطبيعة ببيئتها. لذلك هدفت الدراسة الحالية الى العزل والتوصيف الجزيئي وتحديد نسبة انتشار الزوائف الزنجارية من عينات اسماك الكارب الشائع وتحديد بعض جينات الضراوة. أخذت المسحات من الخياشيم والجلد والأمعاء والعضلات من ٧٥ عينة من الأسماك من مناطق مختلفة من أسواق مدينة الموصل خلال الفترة من أيلول إلى كانون الأول للعام ٢٠٢١، بلغت نسبة انتشار هذه الجراثيم ٢٦,٦٦٪ وهذا ما تم تأكيده بواسطة كل من الاختبارات الميكروبيولوجي التقليدية والتي تضمنت (الصفات الزرعية والمظهرية والمجهرية) والتعرف الجزيئي. شكلت العزلات ٤٢,٥، ٣٧,٥، ١٥، ٥٪ لكل من الغلاصم والجلد والأمعاء والعضلات على التوالي. كشفت النتائج الجزيئية لأربعين عزلة من الزوائف الزنجارية بانها تمتلك الجين *rpoB* بنسبة ١٠٠٪ وكل من جينات الضراوة *oprL* و *toxA* و *algD* والتي تعبر عن بروتين الغشاء الخارجي و الذيفان الخارجي والألجينات على التوالي وبنسبة ٩٧,٥٪ لجين *oprL* و١٠٠٪ لكل من الجينين *toxA* و *algD*.

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