PHENOTYPIC IDENTIFICATION OF *PSEUDOMONAS AERUGINOSA* ISOLATED FROM FISH FARMS (*CYPRINUS CARPIO*) IN MOSUL CITY

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ABSTRACT

This study was aimed to isolation and phenotypic characterization of *P. aeruginosa*. 75 carp fish samples were collected from fish farms at Mosul city during (September2021 to January 2022). Each sample was placed separately in sterile plastic bags and transferred directly to the microbiology laboratory under cooling conditions. The API20-20E assay was used to confirm *P. aeruginosa* isolates. Also the isolates were confirmed molecularly by polymerase chain reaction assay using the primer 16srDNA. Through bacterial isolation were obtained (60) isolates, which formed (36.6, 30, 8.3, 25)% of each of the skin, gills, intestines and muscles, respectively. The results showed the sensitivity of *P. aeruginosa* isolates to impenime (IMP10 µg) and cephalosporin (CIP10 µg) reached (86% and 80 %) respectively, followed by levofloxacin (LEV 5 µg) (74%). But resistant (100%) to both amoxicillin (AM10 µg) and tetracycline (TE10 µg). Isolates varied in their sensitivity and resistance to other antibiotics which included, tobramycin (T0B10 µg), gentamycin (CN10 µg) and amikacin (AK30 µg), the study showed that isolates gave positive results for each hemolytic activity, protease, lecithinase and phospholipase virulence factors tests.

Key wards: *P. aeruginosa*, molecular detection, virulence factors

التوصيف المظهري لجراثيم الزوائف الزنجارية المعزولة من المزارع السمكية لأسماك الكارب في مدينة الموصل

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المستخلص

هدفت هذه الدراسة الى العزل والتوصيف المظهري للزوائف الزنجارية. جمعت 75 عينة سمك كارب من المزارع السمكية في مدينة الموصل خلال (شهر أيلول 2021وكانون الأول 2022). وضعت كل عينة بصورة منفصلة بأكياس بلاستيكية معقمة وتم نقلها مباشرة الى مختبر الاحياء المجهرية تحت ظروف التبريد. استخدم فحص API20-E لتأكيد العزل الجرثومي. كما أكد العزل جزيئيا بواسطة فحص تفاعل البلمرة المتسلسل باستخدام البادئ 16srDNA. ومن خلال العزل الجرثومي تم الحصول على 60 عزلة توزعت بنسبة (36.6، 30 ، 8.3،25)% لكل من الجلد و الغلاصم والامعاء والعضلات على التوالي .اظهرت النتائج حساسية الزوائف الزنجارية لكل من الايمبينيم(IMP10 µg) السيفالوسبورين (CIP10 µg) وبنسبة (86%، 80%)على التوالي يليها الليفوكساسلين (LEV 5 µg) بنسبة(74%)، فيما كانت مقاومة لكل من الاموكسيسيلين (AM10 µg) والتتراسايكلين(TE10 µg) بنسبة 100%.وتباينت في حساسيتها ومقاومتها لكل من المضادات الحيوية الاخرى والتي شملت كل من التوبرامايسين (T0B10 µg) والجنتامايسين (CN10 µg) والاميكاسين(AK30 µg) اظهرت النتائج إيجابية العزلات لكل من اختبارات عوامل الضراوة والتي شملت النشاط التحللي للدم، البروتييز واللسثنيز والفوسفولايبيز.

كلمات مفتاحية: الزوائف الزنجارية، الكشف الجزيئي، عوامل الضراوة

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INTRODUCTION

Diseases of fish are considered the essential troubles in fish farms, that is caused economic losses due to fish mortality and treatment cost (26). Bacterial infections are major threats in fishes, *Pseudomonas* infection constitute the most common bacterial infection among fresh fish under stress condition (12, 27). In addition to *Pseudomonas* plays a role in fish spoilage, therefor it contributes in human infection particularly infection with *Pseudomonas aeruginosa*, thus caused problems for consumers that associated with public health especially in immunocompromised patient (11). In aquatic environment Some *Pseudomonas* species as *P. aeruginosa, P. fluorescens, P. angulliseptica* , *P. putida* and , *P.diminuta* are commonly found (39), while *P. aeruginosa* and *P. fluorescens* are considered the most frequent opportunistic pathogens that effect on fish farms (30). *P. aeruginosa* are one the most bacterial that cause diseases in fish farms, that lead to economic losses (26). *P. aeruginosa* an aerobic, motile, Gram negative rod bacteria that belonging to the family pseudomonadaceae (34). This bacterium is distinguished by their secretion of many pigments, including pyocyanin, pyoverdine, pyorubin, and pyomelanin (24). *P. aeruginosa* has highly genetic variations therefor, its adaptable to many environments (13), it is widely spread in nature, water, soil, plants and considered as a part of normal flora in the intestines of fish (37). *P. aeruginosa* can colonize gills, skin and fish intestines, then developed to pathogenic bacteria when predisposing factors are available as stress, overcrowding and environmental factors changing as decreasing in O_2 concentration and pollution (9) that lead to causing Pseudomonas septicemia, ulcer diseases including ulcerative syndrome in fish farms (30). *P. aeruginosa* has the ability to cause multiple infections due to its high ability to produce a wide range of virulence factors, some of them which are related to the bacterial cell surface, as lipopolysaccharide, pili, flagella, as well as alginates. Another are secreted to the outside of the cell they include exotoxin A, exoenzyme S, pyocyanin and hemolysin, in addition to the protease, lecithinase, phospholipase C, urease, DNase, gelatinase, proteases and siderophores . These factors aids in adhesion, invasion and colonization the epithelial cell, and in bacterial toxicity, also contribute in increasing their resistance to the phagocytosis, antibiotics and antiseptics (31). Currently, antibiotics are used in fish farms to prevent any bacterial infections. However, the emergence of antibiotics resistance is constituting a high concern for investigators as transform of bacterial antibiotics resistance to consumers (41). This study was aimed to isolate and phenotypic characterization of *Pseudomonas aeruginosa* from fish farms with detected some of virulence factors, and study their sensitivity to antibiotics.

MATERIALS AND METHODS

Collection of samples: A total of seventy-five (75) samples of (*Cyprinus carpio*) were collected from farms of fish in Mosul city, during the period from September 2021 to January 2022. Each sample was placed in sterile plastic bag and transported directly under cooling condition to the laboratory of microbiology / College of Veterinary Medicine /University of Mosul.

Bacterial isolation and identification

A swabs of skin, gills and on gram of muscles and intestine were taken under sterile condition and placed in TSB and incubated 24h at 25°C, then one loopful from cultured broth was streaked on each blood agar, Tryptone soya agar and MacConkey agar (18) the plates were incubated 24-48 hours at 25°C, then suspected singles colonies were picked up and streaked on the Cetrimide agar supplemented with nalidixic acid (CN) the plates were incubated at 25°C for 24-48 hours.

Morphological and biochemical examination test: Microscopic examinations were carried out on the suspected isolates for studying the arrangement shape, and reaction staining by Gram stain and motility test. Biochemical tests were performed on the isolates according to (17,26) and conformed by API20- E analysis -(BioMérieux, France) (17).

Extraction of DNA

3-4 colonies of fresh *P. aeruginosa* isolates on BHIA were suspended in 1.5 ml Eppendorf tubs then subjected to genomic DNA

extraction According the manufactured company (Jena Bioscience, Germany), the extracted DNA were stored under -20 °C for following using (5).

Molecular identification

P. aeruginosa isolates were screened by using 16srDNA primer, The PCR reaction mixture were carried out according the manufacturer 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 and thermocycling conditions were done using Thermal cycler (Bio.Rad - USA) as in (Table 1). Then the PCR products were separated by using 1.2% of agarose gel (Promega, USA) containing Prime Safe Dye by (Ge-Net, Bio-Korea). The conditions of electrophoresis include (75 V- 300 mA -1h) using Wide Mini - Sub Cell GT (Bio-Rad-USA). The gel was observed by using (Gel –doc- Ez) system to detecting the specified bands (4).

Table1. Sequences of primers used for PCR

Primer	sequences 5′-3’	amplicon size	N_o Cycle	PCR conditions	Reference
16SrDNA-f 16SrDNA-r (PA-SS)	FGGGGGATCTTCGGACCT CA TCCTTAGAGTGCCCACCCG	956 Bp	25	95 °c for 2 min. 95 °c for 20 sec .annealing 58 °c for 20 sec . extension at 72 °c ,40 sec .final extension 72 °c for 5 min .cooling at 4°c	3

Antibiotic sensitivity test

Antibiotic susceptibility test was done by modified discs diffusion method on Mueller Hinton agar (MHA) (40). Ten antibiotics supplied by (Bioanalyse) were used, which included amoxicillin (AX 10µg), ciprofloxacin (CIP 5µg), ceftadime(CFT 5µg), cefitraxone (CE 30µg), tetracyclin(TE 10µg) , levofloxacin (LEV 5µg), gentamycin (CN 10µg), impenime (IMP 10 µg), tobramycin (TOB 10 µg) and amikacin (Ak 30µg) . The results have been interpreted according to Clinical and Laboratory Standards Institute, USA (15).

Virulence factors test

Some virulence factors tests were performed for *P. aeruginosa* isolates, which included hemolytic activity on blood agar, producing of protease enzyme on nutrient agar supplemented with 5% skimmed milk, and producing of lecithinase and phospholipase enzyme by using nutrient agar supplemented with egg yolks 5 % respectively (16, 23).

RESULTS AND DISCUSSION

Morphological and phenotypical features showed that all *P. aeruginosa* isolates appeared as Gram negative bacilli, straight or curved. Typical colonies of *P. aeruginosa* appeared large ,irregular, pigmented with a fruity odor. The bacteria grew on MacConkey agar and showed smooth, flat, pale, lactose non fermented colonies. also isolates gave Beta hemolysis on blood agar. on citrimide agar, the colonies appeared fluorescent as yellowish green that’s due to produce pyoverdine pigment, others colonies were bluish green color as resulted from the produce of blue pigment pyocyanin, these characteristics are in consistent with each of the (1,22) (Figure1).

Figure 1. A.pyocyanin pigment of *P. aeruginosa* on citrimide agar B. pyoverdine pigment of *P. aeruginosa* on citrimide agar

The producing of pyocyanin is considered the most common characteristic of *P. aeruginosa* that distinguish it from other *pseudomonas* spp (7,19, 25), in addition to pyocyanin pigment is contribute in gene expression and biofilm formation, thus pyocyanin is considered as a virulence factor of this bacterium (22,36). Also biochemical identification showed that all isolates had positive reacted to oxidase, catalase, urease, citrate utilization and gelatin hydrolysis test, while negatively reacted to indole, methyl red, Voges- Proskauer, H_2S production test, these results was in agreement with others (6,17). The isolates of *P. aeruginosa* were confirmed by API-E20 test that specific for Gram negative bacteria (Figure2).

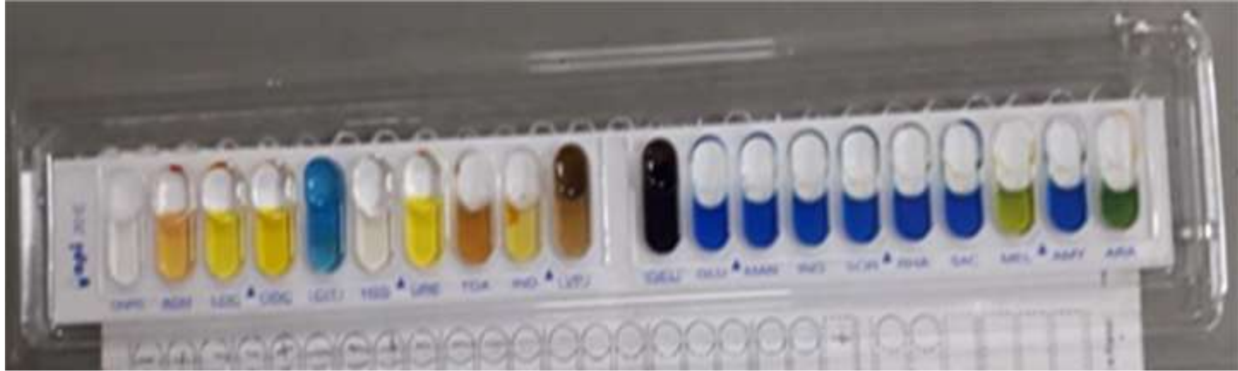

Figure 2. API20-E test for *pseudomonas aeruginosa* isolated from fish farms (*Cyprinus carpio*)

According to phenotypic and cultural characteristic of studied isolates, current study revealed 60 isolates for *p. aeruginosa* at the percentage (36.6%, 30.0 %, 25% and 8.33%) from the skin, gills, intestine and muscles from total isolates respectively, while the prevalence rate reached 20 % from total fish specimens (Table 2).

Table 2. Number and percentage of *p. aeruginosa* isolated from fish farms in Mosul city

Samples	N_o	N_o of isolates	Percentage
Skin	**75**	**22**	**36.66 %**
Gills	**75**	**18**	**30.0 %**
Intestine	**75**	**15**	**25.0 %**
Muscles	**75**	**5**	**8.33 %**
Total	**300**	**60**	**100%**
Prevalence rat	**300**	**60**	**20 %**

These results were closely to the results of (6,22) they recorded (31.57%, 27.06%) from cultured fish, while lower than (20) they recorded (45.5%). The variations in the incidence of *P. aeruginosa* could be due to host susceptibility, method of catching and seasonal variation (43). Skin fish isolates formed the high percentage of isolation (36.6%), skin is considered the primary barrier against the environmental pollutants (17). Some reports indicated that the skin of fish is the most common target for infectious agents, and the presence of close relationship between skin bacterial colonization and skin damage (28,38). Followed by gills fish isolates that formed (30.0%) this finding in disagreement with the others (18,42) they recorded (8.3%,22%) from gills respectively, gills play a role in food filtration from the water column thus it considered as a barrier of microorganism (40). *P. aeruginosa* is considered as normal flora in the gills, skin, and intestines, but it could be caused infection under stress conditions in fish farms as skin ulceration, gill necrosis (12). Many researchers indicated that fish muscle must have been sterile, therefore, isolation of *P. aeruginosa* from the muscles at percentage (8.33 %)is considered as reliable indicator that this bacterium as a cause of fish disease as septicemia due to of its transfer from its natural sites to the muscles through the blood therefore, the consumption of infected fish will be a source of concern for consumers especially in immunocompromised patients (11).For rapid and accurate identification of *p. aeruginosa* isolates, PCR assay was used to confirm the isolates by specific gene16s rDNA (3). The amplification PCR products showed the target identified DNA fragment which indicates that *P. aeruginosa* isolates possess highly DNA (Figure3). The current molecular results showed that all isolates were detected by this gene and 16srDNA produced positive amplicon at 956 bp (Figure4) this finding in agreement with others (35,37). This gene is

considered specific for species that act as a preliminary gene for revelation of *P. aeruginosa* because it provides unambiguous information even for rare isolates (3).

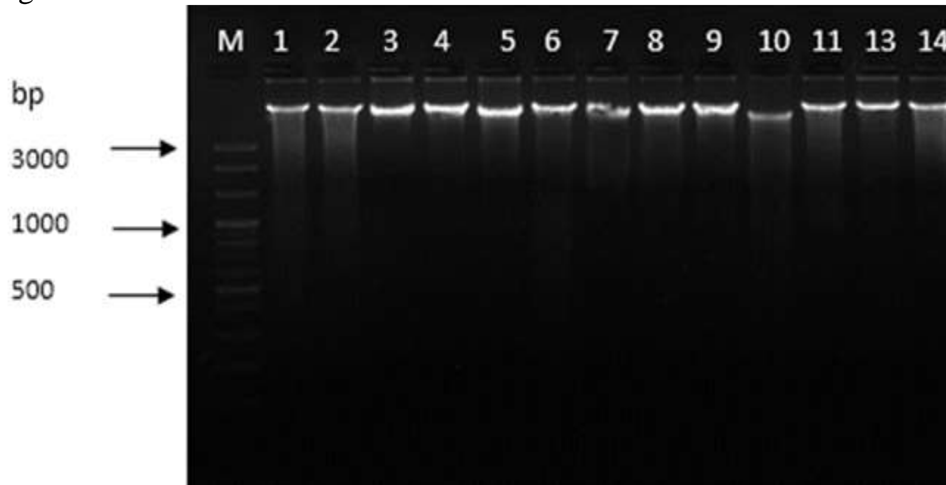

Figure 3. PCR final products of *pseudomonas aeruginosa*

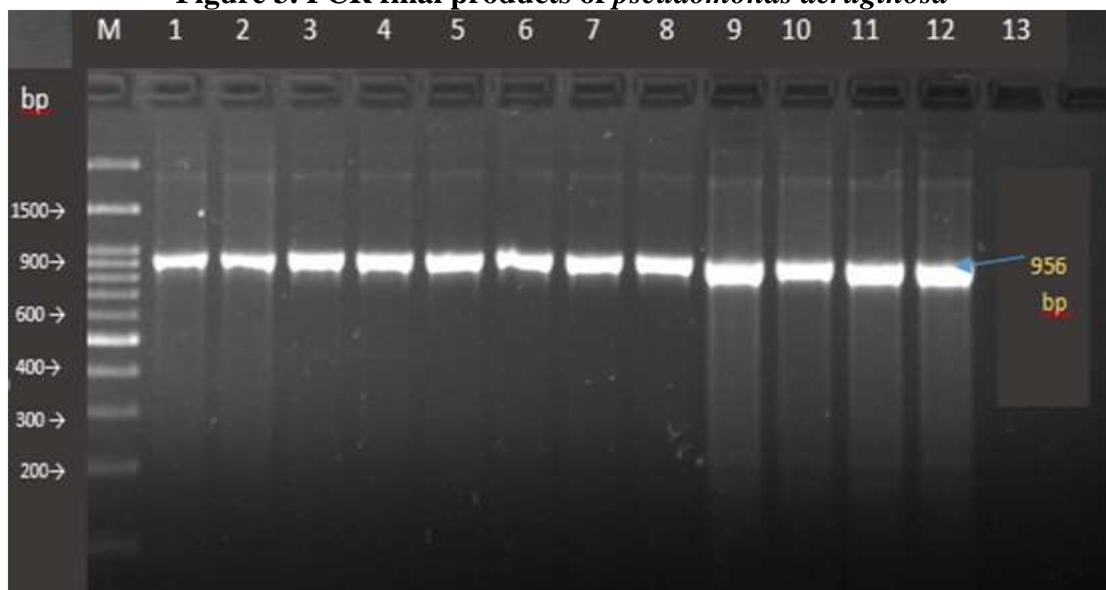

Figure 4. PCR final products for *Pseudomonas aeruginosa* using 16s rDNA gene. Well M: DNA marker (100 bp). Well (1-12) positive fish samples that giving (956 bp). Well (13) -ve control

The extent of infection of host tissues with this bacterium is depending on their capacity to produce extracellularly virulence factors. This study showed the ability of *p. aeruginosa* isolates to produce Beta haemolysis on Blood agar and gave positive results for each protease on milk agar and lecithinase , phospholipase enzymes on nutrient agar supplemented with 5% of egg yolk , the results was showed by the formation of a clear zone around the colonies (Table 4 and Figure5).

Table 4 . Virulence factors tests of for *pseudomonas aeruginosa* isolated from fish farms (*Cyprinus carpio*)

Virulence factors	Results
B-Heamolysis	+
Protease	+
Lecithinase	+
Phospholipase	+

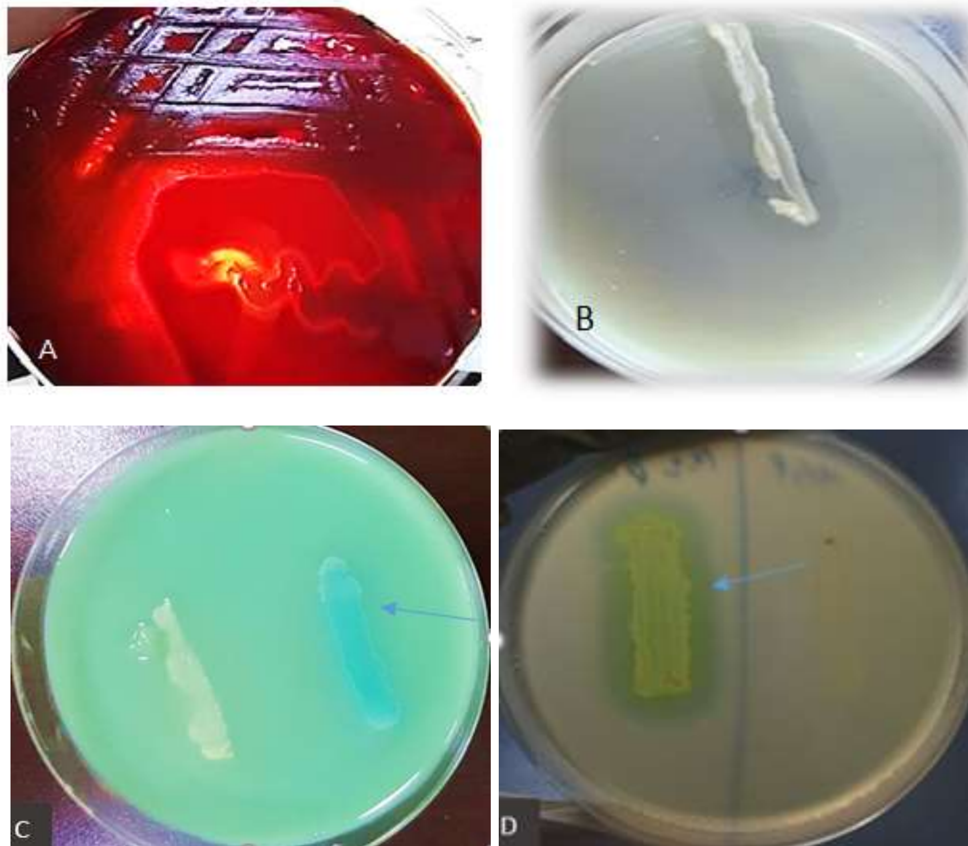

Figure 5. A. β haemolysis on Blood agar
B. producing of protease enzyme on nutrient agar with 5% skimmed milk by *p. aeruginosa* . appeared the clear zone around the colonies
C. producing of phospholipase enzyme nutrient agar with 5%yolk egg by *p. aeruginosa.* appeared blue colors colony when treated with hydrated copper sulphate
D. producing of Lecithinase enzyme on nutrient agar with 5%yolk egg by *p. aeruginosa* . appeared the clear zone around the colonies

Haemolysin is considered one of the most effective virulence factors of *p. aeruginosa* as its destroying the tissue thus releases the elements that necessary for this bacterium such as iron thus, contributed to enhancing the multiplication of bacteria in host tissues (24). Most of *p. aeruginosa* isolates had positive results for each enzymes protease, lecithinase and phospholipase at high percentage, these virulence factors play a potent role in the mechanism of infection protease which contribute in tissue invasion by lysis of some protein as collagen and elastase. especially in muscles tissue, and separating the close fusion between epithelial cells, it also analyzes the Fibronectin and inhibit anti-proteinase and stimulate the secretion of mucus (31,47). phospholipase is act to disrupted the phospholipid in cell membrane and in the activity of blood hemolysis (14). The antibiotic sensitivity test results showed that isolates were sensitive (86%) to the antibiotics impenime and (80 %) to ciprofloxacin and (74 %) to levofloxacin, while all of them were resistant (100%) to amoxicillin and tetracycline, but the isolates were resist at percentage (90%, 84 %) for each ceftriaxone and cefotaxime respectively, while isolates showed intermediate sensitivity to gentamicin amikacin and tobramycin in different percentage as in (Table 5).

Table 5. Antibiotic sensitivity test of fifty *pseudomonas aeruginosa* isolates from fish farms (*Cyprinus carpio*).

Antibiotics (µg)	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Amoxicillin (AX)	0	0	0	0	50	100%
Tetracycline (TE 30)	0	0	0	0	50	100%
Ceftriaxone (CE 30)	0	0	5	10%	45	90%
Cefotaxime (CFM 5)	3	6%	5	10 %	42	84%
Gentamicin (CN 10)	8	16%	17	34%	25	50%
Tobramycin(TOB10)	0	0	42	84%	8	16%
Impenime (IMP 10)	43	86%	5	10%	2	4%
Ciprofloxacin(CIP10)	40	80 %	10	20 %	0	0
Levofloxacin (LEV 5)	37	74 %	9	18%	4	8%
Amikacin (AK 30)	12	24%	38	76%	0	0

These results were in agreement with Algammal *et al*.,2020 (6) who mentioned that *P. aeruginosa* isolated from fish farm were resist to amoxicillin, cefotaxime , tetracycline at percent (83.3% , 77.7% ,75.6%). while disagreement with the finding of Abdullahi *et al.*,2013 (2) that gentamicin and nalidixic acid could be more effective agents of *P. aeruginosa* in fish farms and the results of (10) the sensitivity test were showed that amikacin and ceftriaxone was more effective on this bacterium. The antibiotics resistance of *P. aeruginosa* is occur as a result of horizontal transfer of resistance genes from animal to human, that occurs by transposon genes or conjugative plasmid (21, 42).

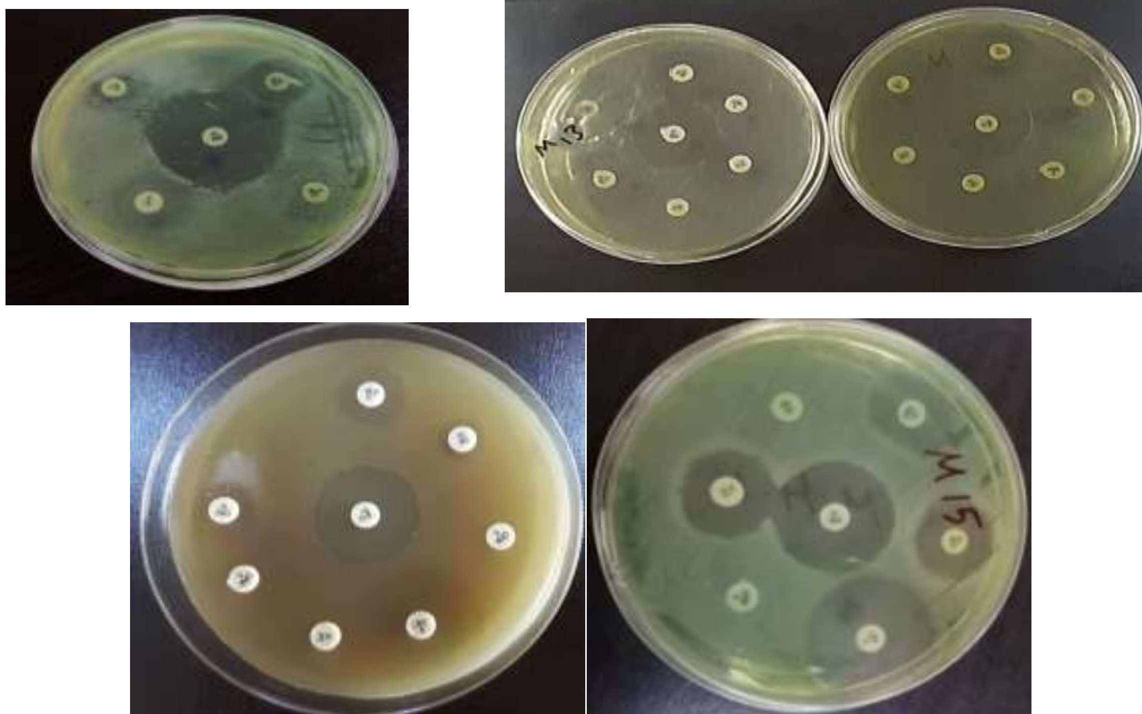

Figure 6. Disc diffusion antibiotic test of *p. aeruginosa* isolated from fish farms

From this study could be concluded that the 16srDNA gene is the identification gene for *P. aeruginosa*. Impenime and cephalosporin were more effective against this bacterium. Also *P. aeruginosa* have variable virulence factors that are responsible for its pathogenicity.

REFERENCES

1. Abd El Tawab, A.A.; A.A. Maarouf and M.G. Ahmed,2016. Detection of virulence factors of *Pseudomonas* species isolated from fresh water fish by PCR.BENHA Vet Med Journal. 30(1) :199-207.
DOI: 10.21608/bvmj.2016.31364

2. Abdullahi ,R.; S. Lihan; B.S. Carlos; M.L. Bilung ; M.K .Mikal and F. Collick, 2013. Detection of oprL gene and antibiotic resistance of *Pseudomonas aeruginosa* from aquaculture environment. European Journal of Experimental Biol, 3(6): 148- 152. http://www.pelagiaresearchlibrary.com

3. AbuElala, N. M.; R. M. AbdElsalam; S. Marouf; M. Abdelaziz and M. Moustafa, 2016. Eutrophication, ammonia intoxication, and infectious diseases: interdisciplinary factors of mass mortalities in cultured Nile tilapia Journal of Aquatic Animal Health, 145: 187-198. DOI: 10.1080/08997659.2016.1185050

4. Ahmed, I.M; S.Y.A. Al-Dabbagh and Dh. M. Jwher, 2021. Molecular characterization of extended spectrum cephalosporin resistant *Escherichia coli* isolated from dogs. Iraqi Journal of Veterinary Sciences 35 (3):473-478. 10.33899/ijvs.2020.127032.1441

5. Al- Dabbagh, S.Y.2022. Molecular characterization of extended spectrum beta-lactamase producing *Klebsiella pneumoniae* isolated from cows in Mosul city, Iraq. Iraqi Journal of Veterinary Sciences, 36(2): (375-380). DOI: 10.33899/ijvs.2021.130341.1803

6. Algammal, A. M.; M. Mabrok; E.Sivaramasamy; F. M. Youssef; M. H.Atwa; A. W. El-kholy; H. F. Hetta; and W. N. Hozzein, 2020. Emerging MDR-*Pseudomonas aeruginosa* in fish commonly harbor oprL and toxA virulence genes and blateM, blactX-M, and tetA antibiotic-resistance genes. 2020. Nature. 10: 1-12 .doi.org/10.1038/s41598-020-72264-4

7. Alonso, B.; B.L. Fernánde; E.G. Di Domenico; M.Marín ;E.Cercenado; E. MerinoI; M.de Pablos and M.Muñoz P,2020. Characterization of the virulence of *Pseudomonas aeruginosa* strains causing ventilator-associated pneumonia.2020; BMC Infectious Diseases. 20:909 https://doi.org/10.1186/s12879-020-05534-1

8. Al-Shammari, N.A.H.;A.M.R. Al-Taee and N.R. Khamees.2019 Bacterial disease agents of *Cyprinus carpio* from some farms in Basra, Iraq. Eco. Env. and Cons. 25 (4) : 1554-1558https://www.researchgate.net/publication/338659778

9. Al-mamari, N.M.H. and A.Y.S. Al-Numa'an, 2019.Isolation and identification of *Pseudomonas aeruginosa* from some clinical and environmental sample and study its activity for the production of pyocyanin and protease. Journal of Education and Science. 28(4): 93-107.http://edusj.mousijournal.com

10. AL-Zamily, K. Y.; A. A. AL- Darwesh Shoob and N. Asal, 2016. Isolation and identification of *Pseudomonas aeruginosa* from goldfish (*carassius auratus*) and studding the antibiotic sensitivity in Al-Kufa city Euphrates Journal of Agriculture Science. 8 (1): 17-22. https://www.iasj.net/iasj/download/c35046348f46bbf1

11. Ardura, A.; A.R. Linde and V. E. Garcia, 2013.Genetic detection of *Pseudomonas* spp. in ommercial Amazonian fish. Int. J. Environ. Res. Public Health. 10:3954–3966. doi: 10.3390/ijerph10093954

12. Austin, B. and D.A. Austin, 2007. Bacterial Fish Pathogens: Diseases of farmed and wild Fishes. Edn 3, Springer-Praxis, Chichester, UK. pp:552. DOI 10.1007/978-1-4020-6069-4

13. Baskan, C.;B. Siriken; C.Kilinc and F.Siriken ,2019 Genotypic and Phenotypic Identification of Clinical Origin *Pseudomonas aeruginosa* isolates.2 Uluslararası Tarım, Çevre ve Sağlık Turky Congress pp:1466-1477. Doi 10.13140/RG.2.2.15178.72645

14. Bennki MHG. *Pseudomonas aeruginosa,* 1999. 3: 1867–1871

15. CLSI. Performance Standards for Antimicrobial Susceptibility Testing,2018. 28th ed. CLSI Supplement M100.Wayne, PA: Clinical and Laboratory Standards Institute pp:38-41.

16. Collee, J.G.; A.G. Franser; B.P.Marmion; A.Simmons; A. Mackie and McCartney,1996. Practical Medical Microbiology. 14th ed. London: Churchill, Livingstone pp :413-423.

17. Eissa, A.A,2016. Clinical an laboratory manual fish diseases. LAP Lambert Academic Publishing pp :70-75

18. Eissa, N.; E.N. Abou ElGheit ;A. Shaheen and A. Abbass,2015. Characterization of *Pseudomonas* species isolated from tilapia "*Oreochromis niloticus*" in Qaroun and Wadi-El-Rayan lakes, Egypt. Global Veterinaria. 5 (2): 116-121, DOI: 10.13140/2.1.5002.4961

19. El-Fouly, M.Z.; A.M. Sharaf; A.A.M .Shahin.;H.A. El-Bialy and A.M.A. Omara,

2014. Biosynthesis of pyocyanin pigment by *Pseudomonas aeruginosa*, Journal of Radiation Research and Applied Sciences. http://dx.doi.org/10.1016/j.jrras.2014.10.007

20. El-Hady, M.A and A.A. Samy , 2011.Molecular typing of *Pseudomonas* species isolated from some cultured fishes in Egypt. Global Veterinaria. 7(6): 576-580. https://www.idosi.org/gv/GV7(6)11/8

21. Hamid, O. Z. and S.S. Mahmood. 2021. thesynergisticeffectofgoldnanoparticle loaded with ceftazidium antibiotic against multidrug ersistance *Pseudomonas aeruginosa*. Iraqi Journal of Agricultural Sciences. 52(4):828-835. https://doi.org/10.36103/ijas.v52i4.1391

22. Hanna, M.I.; M.E. El-Hady ; M.A. Elmeadawy and A.M.A. Kenwy,2014. Contribution on *Pseudomonas aeruginosa* infection in African Catfish (*Clarias gariepinus*). RJPBCS.5(5) P:575. https://www.rjpbcs.com/pdf

23. Ibrahim, A. H. 2022. Link between some virulnce factors genes and antibacterial resistance of *Pseudomonas aeruginosa*. Iraqi Journal of Agricultural Sciences .53(5):985-993. https://doi.org/10.36103/ijas.v53i5.1612

24. Jawitz, J.L. and Adebberg, E.A,2004 . Medical Microbiology . 23^ed^ Lang – Medical Publications . California.pp:240-247.

25. Kamil, Sh. H.; R. F. ALjasani and H. I. ALShammari. 2023. Isolation, identefecation and effecincy of *Pseudomonas fluorescens* bacteria to termite *microcerotermis*. Iraqi Journal of Agricultural Sciences.54(6):1583-1593. https://doi.org/10.36103/ijas.v54i6.1859

26. Kitao, T.; T. Aoki; M.Fukudome; K. Kawano;Y. Wada and Y. Mizuno, 1993. Serotyping of *Vibrio anguillarum* isolated from fresh water fish in Japan. Journal of Fish Diseases . 6:175- 181. DOI:10.1111/J.1365-2761.1983.TB00064.

27. Kousara R.; N. Shafia; S. Andleeb ; A.N. Mazhar; T. Akhtara and S. Khalid,2020. Assessment and incidence of fish associated bacterial pathogens at hatcheries of Azad Kashmir, Pakistan. Braz. J. Biol. 80 (3) .https://doi.org/10.1590/1519-6984.217435

28. Lowrey, L.;D.C. Woodhams; L.Tacchi and I. Salinas, 2015. Topographical mapping of the rainbow trout (*Oncorhynchus mykiss*) microbiome reveals a Diverse bacterial community with antifungal properties in the skin. Applied and Environmental Microbiology, 81, 6915-6925. DOI: https://doi.org/10.1128/AEM.01826-15

29. Macfaddin, J.F, 2000. Biochemical Tests for Identification Bacteria. Lippincott Williams and Wilkns. Philadelphia, USA.pp:249-260

30. Markey, B.K.; F.C. Leonard; M. Archambault ; A. Cullinane and D. Maguire, 2013. Clinical Veterinary Microbiology. 2nd ed. Mosby. Elsevier Ltd. Edinburgh London New York Oxford Philadelphia St Louis Sydney Toronto., Clinical Veterinary Microbiology. https://www.amazon.com/Markey-DipStat/dp/0723432376

31. Martín, I. J.; M. S. Mejías and S. McClean,2021 . *Pseudomonas aeruginosa*: An audacious pathogen with an adaptable arsenal of virulence factors. Int. J. Mol. Sci. 22:3128. doi.org/10.3390 ijms22063128

32. Ndi, O. L.and M. D. Barton,2012. Resistance determinants of *Pseudomonas* species from aquaculture in Australia. J Aquac Res Development 2012, 3:1 DOI: 10.4172/2155-9546.1000119

33. Neves, P.R.;J.A. McCulloch ; E.M.Mamizuka and N. Lincopan . 2014.*Pseudomonas aeruginosa*. Elsevier Ltd. by Marjon H.J. Bennik, volume 3, pp: 1867–1871. https://books.google.iq/books?id=1b1CAgAAQBAJ&pg

34. Quinn, P.J.; M.E. Carter; B. Markey and GR. Carter ,2004. Clinical Veterinary Microbiology . 15th ed. Mosby , Elsevier Limited , London. pp: 237 – 242

35. Ravea, A. F. G.;A.V. Kussb; G.H. Peila; S.R. Ladeirac; J.P.V. Villarreald and P.S. Nascente, 2019. Biochemical identification techniques and antibiotic susceptibility profile of lipolytic ambiental bacteria from effluents. Braz. J. Biol. 79 (4):555-565. doi: 10.1590/1519-6984.05616

36. Rigan, E.; R. Dutoit ; S. Matthijs ; N.Brandt ;S. Flahaut and K.S. Belghith, 2020. Characterization of putative virulence factors *of Pseudomonas aeruginosa* strain RBC isolated from a saltern, Tunisia: Effect of metal ion cofactors on the structure and the activity of LasB). Hindawi, Bio Med Rese Inter. doi: 10.1155/2020/6047528

37. Safinska, A.P,2018. Contemporary threats of bacterial infections in freshwater fish. J Vet Res.62: 261-267. DOI:10.2478/jvetres-0037

38. Saikia ,D.J.;P. Chattopadhyay; G.Banerjee and D. Sarma,2017. Time and dose dependent effect of *Pseudomonas aeruginosa* infection on the scales of *Channa punctata* (Bloch) through light and electron. Journal of the World Aquaculture Society. doi: 10.1111/jwas.12476

39. Sırıken, B.; V. Öz and C. Başkan , 2019. *Pseudomonas aeruginosa* detection methods from fish samples, SETSCI Conference Proceedings. 9:141-145. doi.org/10.36287/setsci.4.9.083

40. SMITH, S.A, 2019. Fish Diseases and Medicine. Taylor & Francis Group, LLC.CRC press. pp:7-9. http://www.copyright.com

41.Sorum, H., 2006. in Antimicrobial resistance in bacteria of animal origin. American Society of Microbiology.PP: 213–238. doi: 10.3201/eid1207.060503

42. Ture, M. and H. Alp, 2016. Identification of Bacterial Pathogens and Determination of Their Antibacterial Resistance Profiles in some Cultured fish in Turkey. J Vet Res 60: 141-146, DOI:10.1515/jvetres-2016-0020

43. Vandepitte, J.; K. Enghak; P. Piol and C. C. Heuch, 1991. Basic laboratory Procedures in Clinical Bacteriology. World Health Organization Genera pp:37–42. https://www.scirp.org/reference/referencespapers1623584.