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 API20-2E 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 (TOB10 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

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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 O₂ 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 alginites. Another are secreted to the outside of the cell they include exotoxin A, exoenzyme S, pyocyanin and hemolysin, in addition to the protease, lecinthinase, 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
extracted 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**

<table>
<thead>
<tr>
<th>Primer</th>
<th>sequences 5′-3′</th>
<th>amplification size</th>
<th>Nₒ</th>
<th>Cycle</th>
<th>PCR conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16SrDNA-f</td>
<td>FGGGGGATCTTTCGGACCT</td>
<td>956 Bp</td>
<td>25</td>
<td></td>
<td>95 °c for 2 min. 95 °c for 20 sec annealing 58 °c for 20 sec . extension 72 °c .40 sec .final extension 72 °c for 5 min .cooling at 4°c</td>
<td></td>
</tr>
<tr>
<td>16SrDNA-r</td>
<td>TCCTTAGAGTGCCCACCG</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

**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), imipenime (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](image1)

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, H2S 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

<table>
<thead>
<tr>
<th>Samples</th>
<th>Nₒ</th>
<th>Nₒ of isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>75</td>
<td>22</td>
<td>36.66 %</td>
</tr>
<tr>
<td>Gills</td>
<td>75</td>
<td>18</td>
<td>30.0 %</td>
</tr>
<tr>
<td>Intestine</td>
<td>75</td>
<td>15</td>
<td>25.0 %</td>
</tr>
<tr>
<td>Muscles</td>
<td>75</td>
<td>5</td>
<td>8.33 %</td>
</tr>
<tr>
<td>Total</td>
<td>300</td>
<td>60</td>
<td>100 %</td>
</tr>
<tr>
<td>Prevalence</td>
<td>300</td>
<td>60</td>
<td>20 %</td>
</tr>
</tbody>
</table>

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).

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 Figure 5).

<table>
<thead>
<tr>
<th>Virulence factors</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-Heamolysis</td>
<td>+</td>
</tr>
<tr>
<td>Protease</td>
<td>+</td>
</tr>
<tr>
<td>Lecithinase</td>
<td>+</td>
</tr>
<tr>
<td>Phospholipase</td>
<td>+</td>
</tr>
</tbody>
</table>
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*).

<table>
<thead>
<tr>
<th>Antibiotics (µg)</th>
<th>Sensitive No</th>
<th>Intermediate No</th>
<th>Resistant No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin (AX)</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Tetracycline (TE 30)</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Ceftriaxone (CE 30)</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Cefotaxime (CFM 5)</td>
<td>6%</td>
<td>5</td>
<td>42</td>
</tr>
<tr>
<td>Gentamicin (CN 10)</td>
<td>16%</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>Tobramycin (TOB10)</td>
<td>0</td>
<td>42</td>
<td>8</td>
</tr>
<tr>
<td>Impenime (IMP 10)</td>
<td>86%</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Ciprofloxacin (CIP 10)</td>
<td>80%</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Levofloxacin (LEV 5)</td>
<td>74%</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Amikacin (AK 30)</td>
<td>24%</td>
<td>38</td>
<td>0</td>
</tr>
</tbody>
</table>

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


