DETECTION OF pelA AND ASSOCIATED VIRULENCE GENES IN EMERGING MULTI-DRUG AND EXTENSIVELY DRUG-RESISTANT (MDR AND XDR) PSEUDOMONAS AERUGINOSA ISOLATED FROM OREOCHROMIS NILOTICUS

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Summary


Pseudomonas aeruginosa is one of the common pathogens causing serious infections in fish, consequently high economic losses in freshwater aquaculture. This study was carried out to investigate the prevalence, antibiotic sensitivity, and distribution of virulence genes (oprL, toxA, exoS, fliC and pelA) and antibiotic resistance genes (blaTEM and blaCTX-M) related to natural infection of P. aeruginosa among some cultured freshwater fish. A total of 100 Oreochromis niloticus fish were randomly collected from EL-Manzla lake in Port-Said Governorate, Egypt. Clinical and bacteriological examinations were performed on P. aeruginosa, recovered from O. niloticus (32/100; 32%). The PCR results revealed that all isolates harboured 16srRNA conserved gene, oprL virulence gene, whereas only 87.1%, 84.3% were positive for the toxA and pelA gene, respectively. In addition only 37.5% and 15.7% were positive for the fliC and exoS gene, respectively. Depending on the antibiotic sensitivity and molecular analysis of resistance genes, 12.5% of the tested isolates exhibited multi-drug resistance (MDR) to four antimicrobial classes (penicillins, cephalosporins, tetracyclines and sulfonamides) and harboured the blaTEM and blaCTX-M resistance genes as well as oprL, toxA and pelA virulence genes. MDR to three antimicrobial classes (penicillins, cephalosporins and fluoroquinolones) was demonstrated by 9.4% of the tested isolates which harboured the blaTEM and blaCTX-M resistance genes as well as oprL, toxA and pelA virulence genes. Of tested isolates, 12.5% exhibited extensive drug resistance (XDR) to five antimicrobial classes (penicillins, cephalosporins, tetracyclines, aminoglycosides and sulfonamides) harbouring the blaTEM and blaCTX-M resistance genes as well as oprL, toxA, exoS and pelA virulence genes. The emergence of MDR and XDR strains is regarded as a public health issue and indicates improper treatment and a bad prognosis of infections caused by P. aeruginosa.

Key words: antibiotic sensitivity, MDR, Oreochromis niloticus, PelA, P. aeruginosa, virulence genes
INTRODUCTION

Aquaculture is an emerging and the fastest food-producing economy that requires regular developments (Yanong & Francis-Floyd, 2002). Fish is an essential food due to the high palatability and great nutritional value, richness in calcium and phosphorus and a reasonable supply of vitamins (Austin & Austin, 2012). Fish diseases caused by bacterial infections are the major causes of high mortality and economic losses among fish and seafood farms (Austin & Austin, 2012). The majority of fish infectious diseases are opportunistic; this implies that the simple presence of the pathogen in the fish environment is insufficient to cause an outbreak of disease (Yanong & Francis-Floyd, 2002). Bacterial diseases in Egyptian aquaculture are primarily caused by agents from families Pseudomonadaceae, Vibrionaceae and Aeromonadaceae (Abdel-moneam et al., 2020). Pseudomonas spp. is a Gram-negative rod-shaped bacterium belonging to the Pseudomonadaceae family, which is considered as one of the most dangerous agents in fishes, causing significant losses to fish farms (Kholil et al., 2015). Pseudomonas aeruginosa is an opportunistic, common pathogen prevalent in marine and freshwater aquaculture (Thomas et al., 2014). P. aeruginosa is part of the natural gut flora of healthy fish. However, any change in normal environmental conditions may lead to outbreaks (Roberts, 2001) and has high intrinsic antibiotic resistance, including novel antibiotics and susceptibility changes resulting in loss of their effectiveness (Hancock & Speert, 2000).

Antibiotics used in the aquaculture environment and improper usage pose a threat of resistance that can be transmitted from aquaculture bacteria to humans and animals, representing a pivotal element of risk for animal health and public health (Algammal et al., 2020a). Septicaemia caused by significant variations of Pseudomonas strains is hard to treat due to resistance of Pseudomonas toward various antibiotics (Abdullahi et al., 2013). Also, susceptible bacteria that do not have an established resistance genetic basis can have a deeply reduced susceptibility when a biofilm is developed. The formation of biofilms is an essential survival mechanism for P. aeruginosa as 80% of all microbial infections, and biofilms are associated with resistance to antibiotics and antiseptics (Costa et al., 2018; Kozirog et al., 2018).

Molecular technologies have enabled the faster identification of microbial species by amplifying sequences and information for the composition and complexity of bacterial populations (Kim et al., 2007; Wu et al., 2010). Most Pseudomonas species display different resistance patterns to several antimicrobial agents (Quinn et al., 2011). Principally, P. aeruginosa may develop resistance to several antimicrobial agents due to efficient antibiotic efflux pumps, modifying enzymes, target mutants and the permeability of its cell membrane (Mesquita et al., 2013; Walsh, 2000; Algammal et al., 2020a). Consequently, molecular identification of most antibiotic resistance genes must be done to prevent the development of antibiotic-resistant isolates with public health effect (Dalmasso et al., 2009). P. aeruginosa’s resistance to β-lactam antibiotics including penicillins and cephalosporins (such as cefotaxime) is primarily due to the extended-spectrum beta-lactamases (ESBLs). Genes conferring this type of resistance are blaCTXM and blatEM (Algammal et al., 2020c; Algammal et al., 2021).
P. aeruginosa's ability to cause infections in hosts is linked to its ability to regulate virulence genes in response to environmental conditions (Mulcahy et al., 2008; Bialvaei et al., 2015). P. aeruginosa has one or more toxins and proteins, which are thought to stimulate the organism spread at the infection site, disruption of the host immune system and suppression of DNA synthesis, leading to damage to the host genome and disease (Bradbury et al., 2010; Jabalameli et al., 2012) like oprL; outer membrane lipoprotein-L, toxA; exotoxin A, exoS; exotoxin S gene, flIC; flagellar-mediated chemotactic motility and Pel: polysaccharide gene helping intercellular adhesion for the formation of biofilms. Therefore, the present study concentrates on identification of P. aeruginosa isolates and their antibiotic sensitivity patterns. In addition, the presence of the virulence genes as well as the ESBLs resistance genes blaTEM and blaCTX-M was performed to examine their potential for bacterial pathogenicity and biofilm formation with their phenotypic antibiotic resistance and the effect of PelA gene on biofilm formation in examined P. aeruginosa isolates.

MATERIALS AND METHODS

Animal ethics

Following appropriate guidelines and recommendations, well-trained scientists performed the handling of fish and all experimental protocols in line with the guidelines of the Suez Canal University Animal Ethics Review Committee, Suez Canal University, Egypt.

Fish sampling

A total of 100 Nile Tilapia fish (Oreochromis niloticus) – 50 apparently healthy and 50 moribunds were randomly collected from EL-Manzla lake in Port-Said Governorate, Egypt, during the period from August 2019 to February 2020. Fresh fish samples were transferred individually in strong sterile plastic bags and kept in an icebox to the laboratory of microbiology for further bacteriological examinations. Samples from the intestine, liver, kidney and spleen were collected aseptically as described by Yanong (2003).

Clinical and postmortem examination

Clinical examination of fish for the presence of external and internal lesions was carried out as described previously (Austin et al., 2007).

Identification of P. aeruginosa

All samples were processed within 4 hours of collection. One gram of each sample was transferred aseptically into tryptic soya broth (Oxoid, UK) then incubated at room temperature for 24 hours to enrich the samples. A loopful from tryptic soya broth was directly streaked onto Pseudomonas base medium supplemented with gentamicin and MacConkey’s agar (Oxoid, UK), and incubated at 30 °C for 18–24 h under aerobic condition (APHA, 1992). All purified colonies were stained with Gram’s stain and examined microscopically for morphological characteristics. They were identified using multiple biochemical tests: catalase, oxidase, indole, methyl red, Voges Proskauer, citrate utilisation, arginine hydrolases, lysine decarboxylase, ornithine decarboxylase, development of H2S, fermentation of mannitol, glucose, maltose and lactose as well as their motility test detected by stabbing into semi-solid agar medium (MacFaddin,1985; Koneman et al., 1988). The ATCC27853 of the P. aeruginosa
strain was used as standard control. In addition, the identified isolates were detected using a species-specific set of primers targeting 16S rRNA gene of P. aeruginosa as shown in Table 1.

*Antimicrobial susceptibility of P. aeruginosa isolates*

The recovered isolates were tested to different antimicrobial agents (Oxoid), using a disc diffusion method (CLSI, 2016). The test was carried out using Muller Hinton agar plates (Oxoid) incubated at 30 °C for 24 h exhibited in Table 2. The inhibition zones were measured with the reference data.

*Biofilm assay of P. aeruginosa isolates*

Phenotypic screening of the biofilms formed by the isolates was tested by tryptic soy broth (Oxoid, UK) tube (Kadam et al., 2013).

*PCR detection of the virulence and antibiotic-resistant genes*

The detection of pelA, toxA, βlC, oprL and exoS virulence genes and blaTEM and blaCTX-M antimicrobial-resistance genes was carried out by PCR. Bacterial DNA was extracted according to the QIAamp DNA Mini Kit information (QIAGEN Sciences Inc., Germantown, MD, USA/ Cat. No. ID 51326). The reaction volume was therefore adjusted to 25 μL as follows: 5 μL of 10× PCR reaction buffer, 1 μL 200 μM of dNTP mix (10 mM), 2 μL of bacterial-DNA, 0.4 μL of Taq DNA Polymerase (5 U/μL), 1 μL of each used primer (0.1–0.6 μM); sterile water was added up to 25 μL. The ATCC27853 genomic DNA (template) of the standard *P. aeruginosa* strain served as positive control. PCR reactions included positive and negative control (generously obtained by the National Animal Research, Dokki, Gizza, Egypt). All recovered isolates of *P. aeruginosa* were screened for virulence genes using five sets of primers targeting oprL, toxA, pelA, βlC and exoS genes reported in previous studies (Matar et al., 2002; Xu et al., 2004; Winstanley et al., 2005; Ghadaksaz et al., 2015). Additionally, the resistance patterns to antibiotics of all recovered isolates were evaluated, including β-lactams (blaCTXM, blaTEM) were investigated using two sets of specific primers (Colom et al., 2003; Archambault et al., 2006). The agar gel electrophoresis was carried out to separate the obtained PCR-products using 1.5% agarose stained with ethidium bromide 0.5 μg/mL and then the gel was photographed. Oligonucleotide sequences (Thermo Fisher Scientific, Waltham, MA, USA) and the thermal-cycling protocols are described in Table 1.

*Statistical analysis*

The Chi-square was carried out to analyse the antibiotic sensitivity tests, virulence prevalence, and antibiotic resistance genes. Besides, correlation analysis was performed among the pelA gene and biofilm formation. All data were analysed using statistical software (R-software, version 4.0.2; https://www.r-project.org/). The significance level was P<0.05).

**RESULTS**

*Clinical and postmortem observations*

In the current study, a total of 100 *O. niloticus* were collected randomly and examined. The moribund *O. niloticus* revealed signs of haemorrhagic septicemia. Most naturally infected fish had the same clinical signs, including haemorrhages on external body surfaces, mainly on the ventral side of the abdomen and abdominal desti-
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Cycling conditions (35 cycles)*</th>
<th>Amplified product (bp)</th>
<th>Reference</th>
</tr>
</thead>
</table>

*All genes involved initial denaturation at 94°C for 5 min followed by final extension at 72°C for 10 min.
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Fig. 1. Haemorrhages on external body surfaces, mainly on the ventral side of the abdomen and abdominal distention (A); haemorrhagic septicaemia with typical sign of haemorrhagic liver, necrotic gills and congestive spleen (B).

Fig. 2. PCR amplification of specific gene of P. aeruginosa (16SrRNA) at 956 bp. Lane L: ladder (100 bp), lane P: positive control, lane N: negative control, lanes 1–11: positive samples.

Bacteriological assay

The bacteriological examination revealed that 32 isolates were microscopically identified as P. aeruginosa being motile, Gram-negative straight or slightly curved rods, non-capsulated, non-sporulated, arranged in short chains. On Pseudomonas base agar, P. aeruginosa colonies were large, circular, smooth, moist, convex surface, glistening. On nutrient agar, P. aeruginosa colonies were smooth, large, translucent, produced a sweetish aromatic odour with a yellowish-green fluorescent pigment. On MacConkey’s agar, P. aeruginosa exhibited flat, smooth, non-lactose fermenting colonies with a regular edge. All recovered Pseudomonas aeruginosa colonies reacted positively to oxidase, catalase, gelatin liquefaction, arginine hydrolysis, methyl red and Simon's citrate and negatively to indole, Vogues Proskauer, lysine decarboxylase and ornithine decarboxylase tests. All recovered isolates were detected for the amplification of species-specific 16S rRNA gene (Fig. 2). Regarding the prevalence of P. aeruginosa in different organs, the intestine (34.3%; 11/32) was the most com-
monly infected organ, followed by the liver (28.1%; 9/32), kidney (25%; 8/32) and spleen (12.5%; 4/32).

**Antimicrobial susceptibility of P. aeruginosa isolates**

The antimicrobial susceptibility testing was conducted on 32 representative isolates of *P. aeruginosa*. Statistically, the tested strains showed different resistance patterns to various antimicrobial agents (P<0.0001). The tested strains were sensitive to meropenem (84.3%) followed by gentamycin (78.1%) and both norfloxacin and ciprofloxacin (71.8%), while showing remarkable resistance to amoxicillin and penicillin G (100%), cefotaxime (84.3%), oxytetracycline (31.2%), and trimethoprim-sulfamethoxazole (25.1%). In addition, many strains showed multi-drug resistance (MDR) to seven antimicrobial agents: amoxicillin, penicillin G, cefotaxime, oxytetracycline, ciprofloxacin, gentamicin and trimethoprim-sulfamethoxazole. Four strains (4/32; 12.5%) showed extensive drug resistance (XDR) to six antimicrobial agents: amoxicillin, penicillin G, cefotaxime, oxytetracycline, streptomycin and trimethoprim-sulfamethoxazole. The examined isolates showed resistance patterns and average AMR of 0.468 (Table 2).

**Biofilm formation of P. aeruginosa isolates**

Out of the 32 *P. aeruginosa* isolates, 87.5% (28/32) produced biofilm, but 12.5% (4/32) were non-biofilm producers, as described in Table 3.

**Molecular typing of the virulence and antimicrobial resistance genes of isolates**

Several virulence and antimicrobial resistance genes associated with *P. aeruginosa* were selected based on previous publications and were examined in the 32 *P. aeruginosa* strains. The results showed that the 32 tested strains harboured oprL gene (100%) with specific amplicon size

<table>
<thead>
<tr>
<th>Table 2. The distribution of antibiotic sensitivity patterns among recovered isolates (n=32)</th>
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<tbody>
<tr>
<td><strong>Antibiotic classes</strong></td>
</tr>
<tr>
<td><strong>Penicillins</strong></td>
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<tr>
<td></td>
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<tr>
<td><strong>Cephalosporins</strong></td>
</tr>
<tr>
<td><strong>Fluoroquinolones</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td><strong>Aminoglycosides</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Carbenemems</strong></td>
</tr>
<tr>
<td><strong>Tetracyclines</strong></td>
</tr>
<tr>
<td><strong>Sulphonamides</strong></td>
</tr>
<tr>
<td><strong>Chi square</strong></td>
</tr>
<tr>
<td><strong>P value</strong></td>
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</tbody>
</table>
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Table 3. Biofilm production of P. aeruginosa isolates

<table>
<thead>
<tr>
<th>Biofilm type</th>
<th>Percentage</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong biofilm producer</td>
<td>75.0%</td>
<td>(21/28)</td>
</tr>
<tr>
<td>Moderate biofilm producer</td>
<td>14.3%</td>
<td>(4/28)</td>
</tr>
<tr>
<td>Weak biofilm producer</td>
<td>10.7%</td>
<td>(3/28)</td>
</tr>
</tbody>
</table>

of 504 bp (Fig. 3A, Table 4) while 27 strains (87.1%) were positive to toxA gene with amplicons size of 396 bp (Fig. 3B, Table 4). Also, 25 strains (84.3%) were positive to the pelA gene with fragment size of 786 bp (Fig. 3C, Table 4), and another 12 isolates were positive for fliC gene (37.5%) with fragment size of 180 bp (Fig. 3D, Table 4). Furthermore, six (18.75%) tested P. aeruginosa strains were positive to exoS gene with specific amplicon size of 118 bp (Fig. 4C, Table 4). The statistical analysis revealed a significant difference in the prevalence of various virulence genes among the tested strains (P<0.0001).

Concerning the distribution of the antimicrobial resistance genes, all strains (100%) were positive for the blaTEM gene with a specific amplicon size of 516 bp, while 27 strains (87.1%) harboured the blaCTX-M gene with a specific amplicon size of 593 bp (Table 4, Fig. 4A,B). There was a strong positive correlation (r=1, P<0.05) between resistance to antibiotics (amoxicillin, and penicillin G) and blaTEM gene and between resistance to cefotaxime and blaCTX-M gene (Fig. 5).

Depending on the antibiotic sensitivity and molecular analysis of resistance genes, most of the tested isolates exhibited multi-drug resistance (MDR) to four antimicrobial classes (penicillins, cepha-
Table 4. The distribution of virulence and antimicrobial resistance genes in the recovered *P. aeruginosa* isolates (n=32)

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Number</th>
<th>%</th>
<th>Chi square (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virulence determinant genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>oprL</em></td>
<td>32</td>
<td>100.00</td>
<td>23.392</td>
</tr>
<tr>
<td><em>toxA</em></td>
<td>27</td>
<td>87.10</td>
<td>0 (0.0001057?)</td>
</tr>
<tr>
<td><em>pelA</em></td>
<td>25</td>
<td>84.30</td>
<td></td>
</tr>
<tr>
<td><em>fliC</em></td>
<td>12</td>
<td>37.50</td>
<td></td>
</tr>
<tr>
<td><em>exoS</em></td>
<td>6</td>
<td>18.75</td>
<td></td>
</tr>
<tr>
<td>Antimicrobial-resistance genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>blaTEM</em></td>
<td>32</td>
<td>100.00</td>
<td>0.42373 (non-significant)</td>
</tr>
<tr>
<td><em>blaCTX-M</em></td>
<td>27</td>
<td>87.10</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. A. PCR amplification of *blaTEM* gene of *P. aeruginosa* (516 bp); B: PCR amplification of *blaCTX-M* gene of *P. aeruginosa* (593 bp); C: PCR amplification of *exoA* gene of *P. aeruginosa* (118 bp). Lane L: ladder (100 bp), lane P: positive control, lane N: negative control.

losporins, tetracyclines, and sulfonamides) and harboured the *blaTEM* and *blaCTX-M* resistance genes as well as *oprL*, *toxA* and *pelA* virulence genes, whereas 12.5% of tested isolates exhibited extensive drug resistance (XDR) to five antimicrobial classes (penicillins, cephalosporins, tetracyclines, aminoglycosides and sulfonamides) and harboured *blaTEM* and *blaCTX-M* resistance genes as well as *oprL*, *toxA*, *exoS* and *pelA* virulence genes (Table 5).

AMR index in all isolates was >0.2, indicating high contamination and high resistance (Table 5).

Correlation between biofilm and *pelA* gene

*PelA* gene was expressed heavily (25/28; 89.2%) among the biofilm-producing isolates. Our results showed that the phenotypically positive isolates for biofilm production *in vitro* were genotypically posi-
Detection of pelA and associated virulence genes in emerging multidrug- and extensively drug ...

tive for the pelA gene and were also found to be resistant to beta-lactam antibiotics (cefotaxime, amoxicillin and penicillin G). The correlation between biofilm-producing isolates and the presence of pelA among recovered isolates was strong and positive (r=0.99, P<0.05). A total of 89.2% of biofilm-producing isolates harboured the PelA gene.

**DISCUSSION**

*Pseudomonas* infection has been recognised as one of the most prevalent bacterial infections among fish. It appears to become a stress-related disease of freshwater fish, particularly under conditions as overcrowding and is regarded as one of the main causes of bacterial haemorrhagic septicaemia in fish (Abd El-Rhaman *et al.*, 2002; Eissa *et al.*, 2010). It can only produce an outbreak if usual environmental circumstances have changed (El-Nagar, 2010). The *P. aeruginosa* prevalence of 32% in this study was fairly in agreement with earlier reports e.g. Eissa *et al.* (2010), who concluded that *Pseudomonas* was found in 30.83% of the examined *O. niloticus* in Egypt and Eid *et al.* (2016) who recorded a *P. aeruginosa* incidence of 27.06 % in apparently healthy *Magil cephalus* and *Oreochromis niloticus* fish. El-Nagar (2010) found out *P. aeruginosa* in 22% of examined *T. zillii*. On the other hand, Zorrilla *et al.* (2003) observed *Pseudomonas* species less frequently among

**Fig. 5.** Correlation between antimicrobial resistance genes and the tested antimicrobial agents. The size and density of circle colours represents the value of the correlation coefficient (r), black and white colour refers to the negative and positive correlations, respectively.
**Table 5.** The frequency of the phenotypic multidrug-resistance and the antibiotic-resistance (AR) genes among the retrieved strains (n=32)

<table>
<thead>
<tr>
<th>Number of strains</th>
<th>%</th>
<th>Phenotypic multi-drug resistance</th>
<th>Virulence genes</th>
<th>AR genes</th>
<th>AMR index</th>
<th>Type of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>37.5</td>
<td>amoxicillin, penicillin G, cefotaxime</td>
<td><em>OprL, toxA, flIC, pelA</em></td>
<td>bla&lt;sub&gt;TEM&lt;/sub&gt;, bla&lt;sub&gt;C-TXM&lt;/sub&gt;</td>
<td>0.36</td>
<td>DR</td>
</tr>
<tr>
<td>5</td>
<td>15.6</td>
<td>amoxicillin penicillin G</td>
<td><em>OprL</em></td>
<td>bla&lt;sub&gt;TEM&lt;/sub&gt;</td>
<td>0.27</td>
<td>DR</td>
</tr>
<tr>
<td>4</td>
<td>12.5</td>
<td>amoxicillin, penicillin G, cefotaxime, oxytetracycline, trimethoprim/sulphamethoxazole</td>
<td><em>OprL, toxA, pelA</em></td>
<td>bla&lt;sub&gt;TEM&lt;/sub&gt;, bla&lt;sub&gt;C-TXM&lt;/sub&gt;</td>
<td>0.54</td>
<td>MDR</td>
</tr>
<tr>
<td>4</td>
<td>12.5</td>
<td>amoxicillin, penicillin G, cefotaxime, oxytetracycline, trimethoprim/sulphamethoxazole, streptomycin</td>
<td><em>OprL, esoS, toxA, pelA</em></td>
<td>bla&lt;sub&gt;TEM&lt;/sub&gt;, bla&lt;sub&gt;C-TXM&lt;/sub&gt;</td>
<td>0.63</td>
<td>XDR</td>
</tr>
<tr>
<td>3</td>
<td>9.4</td>
<td>amoxicillin, penicillin G, cefotaxime, ciprofloxacin, norfloxacin</td>
<td><em>OprL, toxA, pelA</em></td>
<td>bla&lt;sub&gt;TEM&lt;/sub&gt;, bla&lt;sub&gt;C-TXM&lt;/sub&gt;</td>
<td>0.54</td>
<td>MDR</td>
</tr>
<tr>
<td>2</td>
<td>6.3</td>
<td>amoxicillin, penicillin G, cefotaxime, gentamicin</td>
<td><em>OprL, esoS, toxA, pelA</em></td>
<td>bla&lt;sub&gt;TEM&lt;/sub&gt;, bla&lt;sub&gt;C-TXM&lt;/sub&gt;</td>
<td>0.45</td>
<td>MDR</td>
</tr>
<tr>
<td>2</td>
<td>6.3</td>
<td>amoxicillin, penicillin G, cefotaxime, oxytetracycline</td>
<td><em>OprL, toxA</em></td>
<td>bla&lt;sub&gt;TEM&lt;/sub&gt;, bla&lt;sub&gt;C-TXM&lt;/sub&gt;</td>
<td>0.45</td>
<td>MDR</td>
</tr>
</tbody>
</table>

Average AMR = 0.468

AMR index = a/b; a = the number of antibiotics to which the isolates are resistant; b = total number of tested antibiotics (n=11); XDR: non-susceptible to ≥ one agent in all but ≤ two antimicrobial classes; MDR: non-susceptible to ≥ one agent in ≥ three antimicrobial classes; DR: non-susceptible to < one agent in < three antimicrobial classes.
the examined marine fish (15.27%), while Algammal et al. (2020a) demonstrated high prevalence of *P. aeruginosa* in all moribund *O. niloticus* and *C. gariepinus* with a total prevalence of 56.8% and 55.4%, respectively. Differences in prevalence can be due to geographical distribution, environmental factors, host susceptibility, and season of sample selection (Algammal et al., 2020b). Regarding the morphological characteristics of *P. aeruginosa*, all the recovered strains showed the typical phenotypic features, culture and biochemical characteristics of *P. aeruginosa*. These observations are almost the same as those of Aprameya et al., (2013).

Regarding the frequency distribution of *Pseudomonas aeruginosa* in different organs, the intestine was the organ containing the highest prevalence of *P. aeruginosa* in *Oreochromis niloticus* (34.3%). This finding confirmed data of Hatai et al. (1975), who indicated that *P. aeruginosa* was typically located in the intestine of the fish and by Noga (2010), who considered *P. aeruginosa* as part of the normal flora of fish.

Concerning antibiotic sensitivity, the recovered strains were sensitive to meropenem, gentamicin, ciprofloxacin and norfloxacine, while showed high resistance to amoxicillin, penicillin G and cefotaxime, in agreement with data that ciprofloxacin was a more effective antibiotic against *P. aeruginosa* than other antibiotics (Mesaros et al., 2007), and the finding that gentamicin was effective drug against *P. aeruginosa* (Khalil et al., 2010). Akinbowale et al. (2007) recorded that *P. aeruginosa* was sensitive only to ciprofloxacin and gentamicin but resistant to amoxicillin. In addition, a resistance to cefotaxime of 77.7%, nearly similar to our results was reported (Algammal et al., 2020a). These findings are similar to those recorded by Eid et al. (2016), who observed resistance to penicillin and amoxicillin. *P. aeruginosa* expresses frequently antibiotic resistance and can acquire this resistance through plasmids, transposons or naturally (Sheetal & Srivastava, 2016). In general, this resistance promotes *P. aeruginosa*'s participation in nosocomial infections, food poisoning and production of biofilms (Tremblay et al., 2014). Globally, to control bacterial pathogens in fish farms, producers use multiple antibiotics without discrimination, destroying fish and water and increasing the risk to consumer safety (Meireles, 2008). The use of antibiotics and the development of antibiotic resistance genes could influence the emergence of multidrug-resistant strains (Farhan et al., 2019; Abd El-Baky et al., 2020; Algammal et al., 2020b).

In the present study, the PCR results showed that the 32 tested strains harbouring oprL gene (100%) while 27 strains (87.1%) were positive for toxA gene, 25 strains (84.3%) were positive to the pelA gene and 12 strains (37.5%) were positive to the fiC gene. Furthermore, 6 of tested *P. aeruginosa* strains were positive for exoS gene (18.75%). These results are in line with previously recorded data (Nikbin et al., 2012; Uğur et al., 2012; Abdullahi et al., 2013; Khattab et al., 2015). The high virulence of *P. aeruginosa* is primarily due to its ability to produce many virulence factors that can contribute in different ways to their pathogenicity (Markey et al., 2013; Jayaseelan et al., 2014). The outer membrane lipoprotein L (oprL) is a potent pro-necrosis factor for cells (Picot et al., 2001), a protein found only in this organism; it could be a reliable factor for rapid identification of *P. aeruginosa* in clinical samples (De Vos et

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In addition, it plays essential roles in the interaction of the bacterium with the environment as well as the inherent resistance of *P. aeruginosa* to antibiotics where the presence of these specific outer membrane proteins is implicated in efflux transport systems that affect cell permeability (Markey et al., 2013). Exotoxin A is an extracellular product of virulent *P. aeruginosa* that is embedded in the chromosome by the *toxA* gene. In the host cell, it functions by inhibiting protein biosynthesis in animals, fish and humans, similarly to the action of diphtheria toxin (Pollack, 2000; Michalska & Wolf, 2015; Aljebory, 2018). The exotoxin S gene (*exoS*) is a toxic virulence factor causing death in the infected cell. It causes a decrease in DNA synthesis and viability of host cells (Kaufman et al., 2000; Mitov et al., 2010). *Pseudomonas aeruginosa* movement is driven by encoded proteins involved in flagellar-mediated chemotactic motility, including *flIC*, which encodes flagellin that is considered a subunit protein that polymerises to form the filaments of bacterial flagella (Wolfgang, 2004; Song, 2014). The *pelA* gene is involved in the formation of biofilm among the isolates due to variability strain-to-strain in the contribution of PelA to biofilm formation (Colvin et al., 2011). Biofilms provide *P. aeruginosa* with a high colonising, food alteration and resistance to antibiotics, disinfectants and antibiotic power (Meseret et al., 2014).

Regarding the distribution of the antimicrobial resistance genes, the examined strains harboured *blaTEM* and *blaCTX-M* genes with a total prevalence of 100% and 84.3%, respectively in agreement with reported data (Ishida et al., 2010; Ndi & Barton, 2012). Regarding the antibiotic sensitivity and molecular analysis of resistance genes, 34.3% of the tested isolates exhibited multidrug resistance (MDR) to four antimicrobial classes and 12.5% of tested isolates exhibited extensive drug resistance (XDR) to five antimicrobial classes (Table 5). The marked increase in antimicrobial resistance of enteric pathogens in developing countries has become a serious issue, so the evaluation of antibiotic sensitivity is required to select the most appropriate antibiotic drug that will be used. However, studies are concentrated on the human-terrestrial animal transmission of antimicrobial resistance (AMR) (Barton, 2000), focusing less on water ecosystems, especially fish. The intense interaction between humans, livestock and the aquatic ecology indicates that AMR can be transmitted to the aquatic environment. The prevalence of naturally resistant bacteria in the aquatic and soil environment may also transfer antibiotic resistance genes into fish bacteria (Cantás et al., 2013).

In conclusion, *P. aeruginosa* is one of the most critical emerging fish pathogens isolated from *O. niloticus*. The presence of multidrug resistant (MDR) and extensive drug resistant (XDR) *P. aeruginosa* strains poses a high danger necessitating to pay attention to the proper use of antibiotics. The most frequent antibiotic resistance genes with *P. aeruginosa* isolated from fish were *blaTEM* and *blaCTX-M* genes which encouraged resistance patterns to penicillin G, amoxicillin and cefotaxime, respectively. The routine use of the antibiotic sensitivity test is required to prevent potential public health concerns from antibiotic-resistant strains. The most common virulence genes associated to *P. aeruginosa* were oprL, *toxA*, *flIC*, and *exoS* genes. Biofilm associated with the presence of *pelA* cannot be neglected.
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