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Nile tilapia (*Oreochromis niloticus*) as an aquatic vector for *Pseudomonas* species: Quorum Sensing Association with Antibiotic Resistance, Biofilm Formation and Virulence

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Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) produces a suite of virulence factors that are coordinated by Quorum Sensing (QS) contributing to its disease-causing ability in aquaculture. The present study is first of its kind to obtain information regarding the presence and distribution of five QS genes, three virulence genes viz: lasI, lasR, rhlI, rhlR, rhlAB, toxA, aprA and plcH and seven of the Extended-spectrum βlactamases (blaVEB, blaPER, blaTEM,, blaSHV, blaCTX-M1, blaCTX-M2 and blaCTX-M3) of Pseudomonas species isolated from fish meat by direct PCR. Bacterial identification was based mainly on conventional biochemical techniques using the Vitek 2, automated system. Phenotypic sensitivity of antibiotics was established by the agar disc diffusion technique through 16 various antimicrobial drugs. Quantification of their in vitro production of numerous virulence genes outside the cell that are QS dependent namely, pyocyanin, elastase, alkaline protease, biofilm and cytotoxicity of Vero cell was as well executed. Fifteen genes demonstrated an enormous variety in their association. The total number of Pseudomonas species isolates were 30/100 to be identified by the API 20NE system as *P. aeruginosa* 12/30 (40%), *P. fluorescens* 8/30 (27%), *P. putida* 6/30 (20%) and *P. alkylphenolia* 4/30 (13%). The outcomes of this study have great significance for the strategic designation of QS quenching.

Keywords: Nile tilapia; pseudomonas; antibiotic resistance; biofilm formation, virulence genes

1. Introduction

Tilapia was named firstly by the Scottish zoologist Andrew Smith in 1840 [1]. Recently, it's a public name used to numerous species and genera of fish previously recognized as Tilapia in the family Cichlidae [2]. Numerous species of Tilapia were separated into three genera, *Oreochromis*,

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Sarotherodon and the rest stayed as Tilapia but subsequently separated into their own genera based on the reclassification carried out by Trewavas [3]. Although, the change in their specific taxonomy, individual species are remain mostly named as Tilapia. The data delivered by Froese and Pauly [4] on the Nile tilapia as *Oreochromis niloticus* (*O. niloticus*), differentiated it from other subspecies.

Nile tilapia is a perch like freshwater fish of a family (Cichlidae) that is widely distributed in tropical countries. Cichlids provide a valuable source of food in some areas, and many are popular in aquariums [3,4]. Approximately 50 countries except Antarctica raises the Nile tilapia for agricultural purposes [5] and recently is distributed worldwide.

In ancient Egypt, the Nile perch (a large predatory fish found in lakes and rivers in northeastern and central Africa, widely caught for food or sport) and tilapia were very dominant symbols. The name of the tilapia in Latin is thiape and in Tswana is panto (Niger-Congo languages) [6]. The ancient Egyptians named the tilapia "in.t" and had its own symbol (Gardiner k1 in the list). In China, tilapia is recognized as loufei 罗非鱼 since the Nile was named "niLOU" and Africa "FEIzhou" = LOUFEI. In several olden poetries, tilapia was not related only to sun-up and the light blue of turquoise, but also to the heart for the reason that of its attractive red shines. Tilapia powerfully symbolized the fertility and life of the sun and was also connected with defense as the female protected her young during danger by putting them in her mouth. The tilapia also cleans the lotus stem from germs and it looks like a lotus flower and stem emerges from her mouth. Otherwise, the Nile perch (*Lates niloticus*), was connected with the darkness and lazurite (a bright blue metamorphic rock). The *Lates niloticus* is a large predatory fish found in lakes and rivers in northeastern and central Africa, widely caught for food or sport and was recognized in Nilotic Luo languages as Mbuta. So it’s not surprising that that the two fish are represented by the solar boat

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because one represents the dark night and the other the bright day (https://www.gigalresearch.com/uk/article-201301.php). Tilapia was a symbol of the Renaissance in ancient Egyptian art and was also linked to Hathor (a sky goddess). It was as well believed to defend the sun god on his day-to-day journey through the heavens [7].

O. niloticus [10], *O. aureu* [11] and *O. mossambicus* [12] are considered the most three popular fished and eaten species of Nile Tilapia. Nevertheless, *O. niloticus*, the commonly recognized as Nile Tilapia, can be traced back 4 thousand years to Olden Egypt yet. In America, the popularity of Tilapia has raised in the last two decades to become the 4th most commonly consumed seafood after shrimp, salmon and tuna. Generally, Food and Agriculture Organization (FAO) in the United States stated that tilapias represent the second-most imperative cultured finfish next to the cyprinids in many parts of the world and commonly cultured by several smallholders. Nile tilapia (*O. niloticus*) occupies sixth place among cultured species, where it provides food and jobs, as well as local export incomes. According to statistics 2015, about 5.6 million tons, is the outcome of the Egyptian domestic production of fish Tilapia, consequently, Egypt ranks the third place in the world with a productivity rate of 875,000 tons. Through the 2013 FAO reports, which included import, export and production as well as re-export, the total production rate of Tilapia was 1.2 million tons at 3.7 billion US $.

Aquaculture of the Nile tilapia dates to Ancient Egypt, where Egypt produced a significant amount representing about 13.8 percent of the world's farmed fish at that time. Recently, there are several major projects in Egypt, the most important of which was the project that was established on Birkat Ghalioun in the region of Nile Delta and another giant project alongside the Canal zone was also established. Aquaculture is a new industry in the East and North Africa region, but the situation is different in Egypt as it has been farmed since the 14th century before Christ [14]. and

has been recognized in Arab Republic of Egypt from the starting point of written history; tomb friezes date back to 2500 before Christ and demonstrate the production of tilapia from pools [13]. Egypt and Iran are considered the largest two countries controlling fish production in the region, with a contribution rate reached to about 95% (74% and 21% in 2014, respectively).

Aquaculture is one of the most vulnerable sectors of the various epidemic diseases, taking into account that the best use of veterinary antibiotics has a great impact in the treatment of chronic diseases that lead to low growth rates and lack of food conversion rate as well as help in the fight against epidemic diseases that cause high mortality rates [15]. Alternatively, the poor usage of antimicrobials in fish farming has led to major problems, the most important of which is the establishment of antibiotic resistance [16]. Fisheries impact on the food safety and community health can be cooperated once food protection is not fully understood rolled during the aquaculture supply chains [17]. More than 50% of fish production is dependent on aquaculture, taking into consideration that food safety and public health problems are limited in this sector [17]. Misapplication of antibiotics worldwide is well-known as the corner stone of the development and spread of antimicrobial resistance (AMR) [18-20]. At present, AMR causes 700,000 deaths worldwide every year, and by 2050, the number of deaths could reach 10 million [21]. FAO, the World Health Organization (WHO) and the World Organization for Animal Health (OIE) are currently developing the best strategies to address the risk of antibiotic resistance worldwide [22]. In recent times, the Codex Alimentarius Commission has developed recommendations on how to get rid of farm residues as well as taking into account the recommendations made by the Risk Management Department for Veterinary Drug Residues. Two hundred and eleven species and 18 subspecies of the genus *Pseudomonas* were included in the Prokaryotic Name's list with Standing in Terminology at January 2014 (http://www.bacterio.net/pseudomonas.html). *Pseudomonas*

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aeruginosa is one of the 10 most dangerous malignant strains in the world as it causes major health problems for both humans and animals [24]. *P. aeruginosa* is a bacterium of great clinical importance with a negative effect on patients suffering from cystic fibrosis, dangerous burns and immunodeficiency as well as patients with inserted medical devices. Generally, *P. aeruginosa* has the aptitude for biofilm formation on the medical exteriors, leading to hospitalized illnesses. *P. aeruginosa* has developed tolerance to the majority of antimicrobial drugs and is today considered an important bacterium on the Centers for Disease Control and Prevention (CDC) ESKAPE bacterial list [25]. A high threat of plant-animal-human transfer of antibiotic resistant *Pseudomonas* species is seems likely to occur in all surroundings.

Several virulence factors can be produced by *P. aeruginosa* which are matched by a cell density monitoring mechanism named Quorum Sensing (QS) [26] and that provide significantly to their aptitude for disease induction. QS not only controls virulence factors produced by *P. aeruginosa*, but also in the formation of biofilms [27]. It is widely confirmed that QS systems are highly capable of regulating the virulence of microorganisms in the majority of fish farms [28]. It has recently emerged that QS molecules can be found in different foods as in fish produced by certain members of the bacterial association [29]. There are two QS systems named las and rhl in *P. aeruginosa* [30]. At high cell density, LasR and RhlR, which are associates of the big family of LuxR-type proteins, bind their cognate AIs, dimerize, bind DNA, and stimulate expression of genes coding roles needed for pathogenicity and formation of biofilms along with additional methods not included in food-related pathogenesis such as its major role in food contamination appearing as slime at their surfaces [30-37]. As QS has been stated to be compulsory for formation of biofilms and in the pathogenicity of *P. aeruginosa*, the outcomes of this effort have unlimited significance and crucial for the strategic plan for prevention and control of biofilms [38].

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The virulence genes of antibiotic resistant have been found in *P. aeruginosa* isolated from fish [39-42]. Consequently, fish represent one of major aquatic reservoirs of developed macrolide tolerance genes in the surrounding water environment. On the other hand, the existence of AMR in the other species of *Pseudomonas*, isolated from fish which could act as vectors has not been studied thoroughly [43]. Although, some information is available regarding the frequency of Multidrug-Resistant (MDR) *Pseudomonas* infections in Egypt, our motivation was to provide starting point data for additional studies into the determination of prevalence, antibiotic resistance, virulence and QS pheno- and genotypic traits in Nile tilapia, to show the potential public health concerns

2. Materials and Methods

2.1. Fish sampling

A total of 100 Nile tilapia (*Oreochromis niloticus*) were purchased as recently dead, ready to cook, from different supermarkets in Giza during the year 2017. After samples collection, the fish were sited in stretched polystyrene fish boxes, enclosed with a plastic film and then transferred and keep cold in the laboratory and finally handled for bacteriological examination through 2-3 h for detection of *Pseudomonas* spp. All samples were processed in the Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Egypt.

2.2. Isolation and phenotypic analysis of Pseudomonas spp.

The swabs collected from the fish matrix (e.g. kidney, liver, spleen, ascitic fluid and brain) were inoculated directly onto Columbia Blood Agar (CBA) with 5% defibrinated sheep blood (Oxoid Ltd., England) plates and incubated at 30°C for 18-24 h in CO_2 incubator (5%). *P.*

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169 *aeruginosa* was identified by microscopic morphology, catalase, oxidase and urease activity,
170 casein and starch hydrolysis, citrate and indole utilization, and methyl red–Voges–Proskauer and
171 gelatin liquefaction tests, using standard microbial techniques. Growth with or without the
172 production of pigment was determined on various growth media, such as Mueller–Hinton II agar,
173 *Pseudomonas* F medium (King B medium; fluorescein or pyoverdin, a green/yellow pigment) or
174 *Pseudomonas* P medium (King A medium; pyocyanin, a blue/green pigment) at 37°C. In the
175 absence of visible pigment, colonies were examined using UV illumination [119]. *P. aeruginosa*
176 was further grown on cetrimide (Difco) isolation agar and tryptic soy agar (TSA), and streaked on
177 Mueller–Hinton agar to assess purity at 37°C for 18 h. Species identifications were done by the
178 Vitek automated identification system using the API 20NE strips (BioMérieux) as stated by the
179 manufacturer's guidelines. The API database was used for detection of various Pseudomonas
180 species.
181
182 *2.3. Phenotypic potential virulence markers*
183 *2.3.1 Hemolytic activity.*
184 To assess the haemolysin produced by Pseudomonads [120], three *Pseudomonas* strains: *P.*
185 *aeruginosa* RRALC3, *P. aeruginosa* PA14 (ATCC 15442) and *P. aeruginosa* PA01(ATCC 15692)
186 were inoculated on CBA plates. The plates were incubated for three successive days and then
187 examined for the presence of obvious, brown tinged with green and no-zone which is a sign of
188 partial (α), complete (β) and no (ɤ) hemolysis, correspondingly.
189
190 *2.3.2. Elastin Congo red test*

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191 Elastin Congo red (Sigma-Aldrich, USA) was used to determine the elastolytic activity of the
192 culture supernatants based on the method designated by Caballero et al. [121].
193
194 *2.3.3. Alkaline protease assay*
195 The activity of alkaline protease was measured according to the method considered by Howe
196 and Iglewski [122]. Briefly, 0.7 ml of the buffer (20 mM Tris-HCl, 1 mM $CaCl_2$, pH 8) and 0.3 ml
197 of the enzyme-containing fraction were added to 10 mg of the Hide powder azure substrate
198 ((Sigma-Aldrich, USA). The reaction mix (1 ml) was hatched for one hour at 37 ^{o}C with continuous
199 rotation. Substrate which not dissolved was discarded by centrifugation at 4,000*g* for 5 min.
200 Afterword, the absorbance of the reaction mix was detected at $OD_{595\ nm}$.
201
202 *2.3.4. Pyocyanin quantitative analysis*
203 The quantification of pyocyanin is mainly depended upon the absorbance rate of liberated
204 pyocyanin at 520 nm in acidic solution [123]. From a stationary-phase culture (~16 h) in LB broth,
205 5 ml supernatant was mixed with 3 ml of chloroform. From the chloroform phase, the pyocyanin
206 was then extracted into 1 ml of 0.2 N HCl, which gives it dark red color, representing the existence
207 of pyocyanin and the optical density at 520 nm (OD_{520}) was then determined.
208
209 *2.3.5. Vero ingestion assay*
210 Vero cells (African green monkey kidney) were purchased from the American Type Culture
211 Collection (ATCC) (Manassas, VA) and cultured in Dulbecco's minimum essential medium
212 (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), per ATCC procedures. Based
213 to the methods described formerly [124], bacterial adhesion and invasion were identified. After

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214 washing the monolayer, 100 µl of the bacterial suspensions (1×10^6 cfu/mL) were added, to the
215 wells of a microplate. After 1 h of incubation in 5% CO_2 at 37°C, the inoculum was discarded and
216 then washing of Vero cells with PBS were carried out for 3 times to eliminate non-associated
217 microorganisms. Lysis solution (0.025% trypsin and 1% Tween 20 in PBS) was added to the Vero
218 cells and incubated for 30 min at 37°C, and the colony counting method was used to detect the
219 total number of allied bacteria (adherent and invaded). Invasion was then quantified via gentamicin
220 protection (invasion) assay as described above with the exception of after one hour of incubation
221 and before adding of the lysis solution, the infected monolayer was treated with 300 µg/ml of
222 gentamicin solution for 1 h for killing the extracellular bacteria. Calculation of the adherent
223 bacteria was carried out by the variance between the whole number of associated and invaded
224 bacteria.
225
226 *2.3.6. Biofilm formation*
227 Both qualitative and quantitative methods for detecting biofilm formation were applied to
228 discriminate between the tendency of isolate surfaces attachment and biofilm production. The
229 Congo red dye assay was used for quantification of biofilm formation, whereas; the tube method
230 was used for the qualitative investigation of attachment to glass surfaces and subsequence biofilm
231 formation throughout disturbance and fluid flow, and the quantitative analysis of attachment and
232 biofilm formation on plastic surfaces throughout static circumstances were also detected by the
233 microtiter plate method.
234
235 *2.3.7. Congo red (CR) dye uptake*

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The capability of taking up Congo red dye was detected by 50 mg/ml of Congo red dye on the agar plates [125]. Of each bacterial suspension, 5 µl were speckled onto the plates, and then incubated for 24 h. The isolates which showed biofilm formation were seen as black colonies with crystalline texture, while colonies that appeared as red color were described as unproductive or medium-productive biofilm isolates.

2.3.8. Static Biofilm studies (Microtiter plate assay (MTP))

According to the standards of Stephanovic et al. [126], PAO1 and *P. aeruginosa* with strong ability of biofilm formation were used as a reference strain. Fresh isolates were diluted in 5ml of tryptic soy broth (TSB) (Oxoid, UK) supported with 0.5% w/v NaCl and incubated at 37^{o}C to an optical density of 0.8 at 620nm. The cultures were diluted 1:40 with fresh TSB/NaCl and 150µl was added to each well of microtitre plate. All microtitre plates were closed with parafilm and incubated at 37^{o}C for 48h. Treatment was with active HDPs in TSB for 24h or dH_2O as a positive control. Biofilm staining was with crystal violet (CV). Concisely, after washing three times with dH_2O, CV was applied to the biofilms for 15min and removed, biofilms were washed three times with dH_2O, and the remaining CV solubilized with 30% v/v acetic acid. The A_{620nm} was detected via a plate reader (LabSystems Multiskan). The mean OD_{620nm} value of positive control was used as standard throughout scoring of biofilm production. Those values >0.2 were deliberated as great producers of biofilm whereas; values <0.081 were classified into none or low producers of biofilm. OD_{620nm} values higher than the standard but within 0.081 and 0.2 were considered as moderate producers of biofilm. Each analysis was done for three consecutive times to confirm the results. The amount of biofilm production was detected by using the formula: BF = AB/CW, where BF is the biofilm formation, AB is the OD_{620nm} of stained attached bacteria and CW is the OD_{620nm} of

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stained control wells containing bacteria-free medium only (unspecific or abiotic factors). Sixteen wells per each strain were examined for each analysis, and the analyses were carried out in triplicate, which resulted in 48 wells per each examined strain and control.

2.4. Antimicrobial susceptibility testing and screening for ESBLs

2.4.1. Phenotypic antimicrobial sensitivity testing of the Pseudomonas spp. isolates

The sensitivity of *Pseudomonas* spp. to various antimicrobial drugs was achieved by the Kirby–Bauer test according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [127]. The antimicrobials utilized for the sensitivity testing are deliberated by the WHO (Table 1) [128]. The upcoming antibiotic discs carefully chosen for examination are the most prescribed and frequently used in Egypt and were selected for testing consistent with their significance to human and animal health [129,130] and on the WHO's critically significant antibiotic list [131]. All bacterial isolates were examined against 16 antimicrobial drugs: Ampicillin (10µ), Amikacin (30µ), Aztreonam (30µ), Chloramphenicol (30 µ), Cefotaxime (30µ), Ceftazidime (30µ), Ceftriaxone (30µ), Cephalothin (30µ), Ciprofloxacin (5µ), Cefepime (30µ), Gentamicin (10µ), Sulphamethoxazole/Trimethoprim (25µ), Ampicillin/sulbactam (20µ), Nalidixic acid (30µ), Imipenem (10µ), Tetracycline (5µ) (Oxoid, UK). The multiple antibiotic resistance (MAR) index was estimated according to the method labelled previously by Devarajan et al. [93]. *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were utilized as reference strains.

In our study, we followed the standards for describing multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) that was formed through a joint initiative by the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) [132]. MDR was well-defined as resistant to no less than one drug in three or more antimicrobial classes, XDR was recognized as resistant to ≥1 agent in all but ≤2

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antimicrobial classes and in addition, PDR was recognized as resistant to all antimicrobial drugs in all different classes.

2.4.2. β-lactamase detection using Nitrocefine disks (Cefinase®)

The production of β-lactamase was confirmed using a nitrocefin assay. AmpC phenotypic detection AmpC production was proven by the disk approximation test which carried out with the susceptibility *Pseudomonas* isolates to cefoxitin. To carry out this method, 30 µg of cefoxitin disk was sited in a 20 mm plate center far from a ceftriaxone disk (30 µg) and ceftazidime disk (30 µg). The plate was then incubated for 18-24 h at 35 °C. Cefoxitin was used as an inducer of the AmpC enzyme and the positivity was measured when the flattening halo around the ceftriaxone and/or ceftazidime disk was noticed.

2.4.3. Confirmatory detection of Extended Spectrum-β Lactamase (ESBL) activity positive isolates using Combined Disc Diffusion Test

The test inoculums (0.5 McFarland turbidity) were dispersed onto Mueller-Hinton agar. Phenotypic confirmatory test of ESBL was done on *Pseudomonas* spp. by the double-disc synergy technique with paper disks containing ceftazidime and cefotaxime alone, or in mixture with clavulanic acid (30 µg ceftazidime, 30/10 µg ceftazidime/clavulanic acid, 30µg cefotaxime, 30/10 µg cefotaxime/clavulanic acid). The plate was incubated for 18-24 h at 37 °C. The bacteria were deliberated to be forming ESβL when A above or equal 5 mm increase in a zone diameter for either antimicrobial drug investigated in combination with Clavulanic acid versus the zone diameter of the drug when examined alone equals ESβL.

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305 *2.4.4. Preparation of genomic DNA and genetic characterization*

306 *Pseudomonas* isolates were grown in 3 ml BHI broth for 18–24 h at 37°C. Afterword, 200 µl
307 aliquots were moved to Eppendorf tubes and centrifugation was then carried out for 2 min at
308 13,000 × *g*. In 200 µl of pure water, the pellets of organism were re-suspended by mixing. Of pure
309 cultures, the DNA was extracted via the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as
310 stated by the manufacturers' protocols.

311

312 *2.4.5. Amplification of ESBL genes*

313 Examination of β-lactamase genes was done by PCR techniques. Primers were designated for
314 amplification of the $bla_{\text{TEM-1}}$, bla_{TEM2}, bla_{SHV1}, bla_{SHV2}. PCR circumstances for the SHV gene
315 included an initial denaturation step for 5 min at 95°C, shadowed by 32 cycles of 94°C for 1 min,
316 57°C for 1 min and 70°C for 1 min, with a final extension step at 72°C for 10 min. For TEM, the
317 amplification cycle consisted of 5 min at 95°C, followed by 30 cycles of 94°C for 30 s, 55°C for 1
318 min and 72°C for 1 min, with extension at 72°C for 10 min. For CTX-M, the amplification cycle
319 consisted of 5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 1
320 min, with extension at 72°C for 10 min. ATCC 25922 *E. coli* strain was utilized as a negative
321 control in all PCR analyses. *K. pneumoniae* 6064 was used as positive control strain for SHV,
322 whereas *E. coli* 971was used as positive control strain for TEM and CTX-M.

323

324 *2.4.6. PCR for Detection of QS Genes*

325 Oligonucleotide primers are listed in Table 2. The PCR was implemented in a 25-µL reaction
326 mixture holding a half volume of PCR mix (32 mM (NH4)2SO_4, 125 mM Tris-HCl (pH 8.8), 0.02%
327 Tween 20, 2 mM dNTPs, 2.5 mM $MgCl_2$, and DNA polymerase 0.05 U/µL; BioMix Red; BioLine

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Germany), 100 pmol of each primer, and 2µL of bacterial DNA template. Considerations for the magnification cycles were denaturation for 1 minute at 94°C, annealing of primers for 1 minute at 52°C, and primer extension for 1.5 minutes at 72°C, for 30 cycles.

2.4.7. Amplification of virulence genes using PCR

The frequency of virulence genes was detected by PCR. The amplification of tested virulence genes was carried out with particular primers as shown in Table 2. PCR was performed in a total volume of 25 µl containing 2 µl template DNA, 0.25 µM of each primer, 0.2 mM deoxyribonucleoside triphosphates, 1x reaction buffer, 2 mM $MgCl_2$ and 1.5 U Prime *Taq* DNA polymerase (GeNet Bio). Amplification of DNA was carried out according to the following procedures: initial denaturation (94 ºC for 5 min), followed by 25–30 cycles of denaturation (94 ºC for 35–45 s), annealing (53–62 ºC, from 45 s to 1 min) and extension (72 ºC, from 45 s to 1 min 35 s), with a single final extension of 7 min at 72 ºC.

All PCR reactions were overlaid with oil and amplifications were completed in a PCR thermal cycler (Perkin Elmer). The amplicons of PCR were then examined on a 1.5% agarose gel, stained with ethidium bromide and imagined by Gel Documentation System.

2.5. Statistical analysis

Statistical analyses, we used the R package. The function *rrcor* from package Hmisc was used for correlation analyses. All analyses were done with a $p<0.05$, $p<0.01$ and $p<0.001$ significance cut-off. The principal component analysis was carried by using the function *prcomp* and plotted by using the function *ggbiplot* from package devtools. Twenty-three phenotypic (antibiotic resistance profile) and genotypic (resistance, virulence and quorum sensing genes) data from the

strains were used for PCA. Since no resistance to Amikacin was observed, we removed this data for the statistical analyses.

3. Results

3.1. Culture Results

A total of *Pseudomonas* species recovered from fish meat were 10/100 (10%) to be recognized by the API 20NE system as *P. aeruginosa* 12/30 (40%), *P. fluorescens* 8/30 (26.7%), *P. putida* 6/30 (20%) and *P. alkylphenolia* 4/30 (13.3%).

3.2. Production of cell-to-cell signaling-dependent virulence factors.

To describe the 30 various isolates, we measured there *in vitro* productivity of numerous virulence genes that are reliant on an active cell-to-cell signaling circuitry, namely, pyocyanin, elastase, alkaline protease, biofilm and Vero cell cytotoxicity were examined as labelled formerly in the fish meat isolates of *P. aeruginosa*, *P. fluorescens*, *P. putida* and *P. alkylphenolia* (n=30). The *Pseudomonas* isolates were capable of yielding different virulence factors outside the cell which organized by the cell-to-cell signaling circuitry and demonstrated them with mutable degrees (Table 3).

3.3. Distribution of the Virulence genes toxA, aprA and plcH

As recorded in Table 3, 19 of the *Pseudomonas* isolates did not carry any of the three virulence genes (*P. aeruginosa* n=7, *P. fluorescens* n=5, *P. putida* n=4 and *P. alkylphenolia* n=3). The virulence gene *toxA* gene was evident in seven of the *Pseudomonas* isolates (*P. aeruginosa* n=3,

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373 *P. fluorescens* n=2, *P. putida* n=2 and *P. alkylphenolia* n=0), six of the and *aprA* gene (*P.*
374 *aeruginosa* n=3, *P. fluorescens* n=2, *P. putida* n=0 and *P. alkylphenolia* n=1) and nine of the
375 *Pseudomonas* isolates carried the *plcH* gene (*P. aeruginosa* n=4, *P. fluorescens* n=3 *P. putida* n=1
376 and *P. alkylphenolia* n=1).
377
378 *3.4. Screening the fish meat isolates for Extended-spectrum βlactamases (ESBLs) genes*
379 *3.4.1.* bla_{VEB}, bla_{PER}, bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M1}$ $bla_{CTX\text{-}M2}$ $bla_{CTX\text{-}M3}$
380 The 30 *Pseudomonas* isolates were screened by PCR for the seven genes listed in Table 2 and
381 the screening results are summarized as follows (Table 4): Interestingly, three of the isolates did
382 not express any of the seven ESBLs genes. The bla_{VEB} gene was expressed in four of the isolates;
383 while each gene of bla_{SHV}, bla_{TEM} and $bla_{CTX\text{-}M3}$ was found in three isolates; the gene $bla_{CTX\text{-}M1}$ was
384 expressed in one isolate only; leaving the genes bla_{PER}, and $bla_{CTX\text{-}M2}$ to be found in one isolate for
385 each gene. The seven genes showed an enormous variety in their association as shown in Table 8.
386 One prominent feature was that two of the *P. aeruginosa* and two of the *P. alkylphenolia* did not
387 express any of the seven genes under assay.
388
389 *3.4.2. Analysis of the rhlR/rhlI and lasR/lasI genes in cell-to-cell signaling-deficient fish meat*
390 *isolates*
391 We tested the reliability of the cell-to-cell signaling genes *lasI*, *las*R, *rhlR, rhlAB* and *rhlI* in
392 all 30 *Pseudomonas* fish meat isolates by PCR. While one (*P. fluorescens*) of the 30 *Pseudomonas*
393 isolates carried the five QS genes, seven of the 30 *Pseudomonas* isolates were deficient of the five
394 QS genes under investigation (*P. aeruginosa* n=3, *P. fluorescens* n=2, *P. putida* n=2 and *P.*
395 *alkylphenolia* n=0) (Table 3). The screening results of the 30 *Pseudomonas* isolates are

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summarized as follows: *lasI* gene was present in 43.3% of the isolates (*P. aeruginosa* n=4, *P. fluorescens* n=4, *P. putida* n=3 and *P. alkylphenolia* n=2); *las*R gene was present in 16.7% of the isolates (*P. aeruginosa* n=2, *P. fluorescens* n=3, *P. putida* n=0 and *P. alkylphenolia* n=0); *rhlR* gene was present in 20% of the isolates (*P. aeruginosa* n=3, *P. fluorescens* n=3, *P. putida* n=0 and *P. alkylphenolia* n=0); *rhl*AB gene was present in 36.7% of the isolates (*P. aeruginosa* n=3, *P. fluorescens* n=4, *P. putida* n=4 and *P. alkylphenolia* n=0); and *rhlI* gene was found in 43.3% of the isolates (*P. aeruginosa* n=6, *P. fluorescens* n=4, *P. putida* n=2 and *P. alkylphenolia* n=1). In Table 4 the 30 *Pseudomonas* isolates exhibited seven different QS genes combinations.

3.5. Phenotypic Antimicrobial Resistance Characterization

The findings of antibiotic sensetivity testing of the 30 *Pseudomonas* isolates are shown in Table 5. Total resistance was evident to Ampicillin (30/30 100%). A high prevalence of resistance to Sulphamethoxazole/Trimethoprim, (28/30, 93.3%), Tetracycline and Nalidixic acid (29/30, 96.7% each) while totally susceptible to Amikacin (30/30 100%). Some isolates were tolerated to β-lactamase antibiotics such as cefotaxime (8/30, 26.7%) and Ampicillin/sulbactam (9/30, 30%). The 30 *Pseudomonas* isolates were no XDR and PDR but were MDR, with the exception of one isolate while not MDR but was on the other hand XDR and were resistant to two to twelve antimicrobial drugs signifying one to six classes (Table 6).

3.6. The correlation between the formation of QS-dependent virulence factors and antibiotic sensitivity in fish meat isolates of Pseudomonas

In order to determine the independent contribution of each phenotypic virulence factor to the antimicrobial drug resistance of *P. aeruginosa*, *P. fluorescens*, *P. putida* and *P. alkylphenolia*,

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pyocyanin, elastase and alkaline protease production were assayed. An interesting observation in the current investigation was the relationship between insufficiency in the production of virulence factors and increased tolerance to various antimicrobials. The isolates that were insufficient for production of virulence factors were mostly more resistant to antibiotics and important statistical correlations were noted for certain agents. This comprised a shortage of pyocyanin, elastase and alkaline protease production and increased resistance to Tetracycline, Ampicillin, Nalidixic acid, Sulphamethoxazole/Trimethoprim and Ciprofloxacin (Table 7).

3.7. Association between Antibiotic Resistance Extended-spectrum βlactamases (bla_{VEB}, bla_{PER}, bla_{TEM}, bla_{SHV}, bla_{CTX-M1} bla_{CTX-M2} bla_{CTX-M3}), Virulence (toxA, aprA, plcH, rhlI) and QS (lasI, lasR, rhlR, rhlAB) genes

The 15 genes demonstrated an enormous variety in their association as shown in Table 8. One prominent feature was that one of the *P. aeruginosa* did not express any of the 15 genes under assay.

3.8. Correlation Analyses

The influence of resistance genes in the prevalence of antibiotic resistance was evaluated by using correlation analyses. For that, we used *prcomp* function in the Hmisc package. Despite of that, only four correlations showed significance level higher than 0.05 (data not shown). Besides, all correlations were related to extended spectrum β-lactamase (SHV, TEM and PER).

3.9. Principal Component Analyses

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In order to verify which characteristics, represent the sample, we carried out a principal component analysis (PCA) using all phenotypic (antibiotic resistance profile) and genotypic (resistance, virulence and quorum sensing genes) data from the strains. The plot was generated by using the ggbiplot function from devtool package. Based on the results, the *P. alkylphenolia* seems to be the most different of the species identified, being well clustered. However, the low number of isolated specimens could be the reason of that (Figure 1). Moreover, only six (resistance to Chloramphenicol, Aztreonam, Cefepime and Ceftriaxone, VEB and CTX-M1 resistance genes) of the 23 characteristics represent almost 70% of the variation (Figure 1).

4. Discussion

During the last and present decade, several reports have been published on the virulence factors of the opportunistic pathogens of the *Vibrio* species, including extracellular toxin [44], siderophore [45], metalloprotease [46,47], type III secretion system[48], phospholipase, caseinase, and gelatinase [49], as controlled by QS systems. A lot of evidence has demonstrated that weakening of QS in virulent strains of *Aeromonas* spp. led to insignificantly in decreased death rate concerning their relevant hosts burbot [50], larvae of salt-water shrimp and enormous freshwater prawn [51]. Yet, no available reports were published as to the antibiotic resistance, virulence and QS genes and their assortment in *Pseudomonas* species isolated from fish.

Therefore, in our current study, freshwater fish from landing centers and retail stores were investigated for the existence of ESBL-generating pseudomonads. Our findings propose the existence of different ESBL-generating pseudomonads resilient to manifold antimicrobial drugs comprising carbapenems, fluoroquinolones and cephalosporins. The isolates were examined for the relevant genes accountable for the ESBL+ phenotype.

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Everywhere in nature, *Pseudomonas sp*. belong to a group of microorganisms of most ecological significance, as they are pathogens that are relevant and lead to different diseases in both humans and animals as they are part of the natural microorganisms that live in the pharynx and mucous membranes as well as human skin [24,52]. Even though they play a significant role as plant pathogens [53] and as spoilage organisms, their role as biocontrol agents against the causes of plant diseases has recently emerged [54]. Pseudomonas species has the ability to produce volatile compounds and amino acids degradation, therefore they may lead to taints of fish products [55-59]. The spoilage or pathogenic Pseudomonads which live in various environment is considered main cause of emerging MDRGs [60] and representing public health threat.

P. aeruginosa has been considered one of the most significant unscrupulous bacteria in human, producing several local or systematic infections from benign to life threatening [61]. In contrast, *P. putida* is an infrequent reason human infection. Nevertheless, there are a number of studies indicated that *P. putida* can cause various illnesses for example eye and urinary infections, pneumonia and soft tissue infections [62-65] as well as its colonization in a haemato-oncological patient [66]. Moreover, some studies have supplied proof for the transfer of antimicrobial resistance genes (ARGs) from *P. putida* to *P. aeruginosa* and have discussed the role of *P. putida* as a source for ARGs [67-69].

P. fluorescence is not considered a pathogen in humans and although they are less harmful than *P. aeruginosa* can cause bacteremia, with most described cases being related either to contaminated blood transfusion or tainted equipment linked with intravenous infusions. In spite of the fact that, *P. fluorescence* is one of the leading causes of pulmonary disease, certain studies have been shown to be present in respiratory specimens. In addition, a robust relationship between *P. fluorescens* and human disease has been emerged, in that nearly 50% of patients suffered from

Crohn's disease developed serum antibodies against *P. fluorescens*. Overall, these studies are starting to high spot a far more common, interesting, and possibly complex relationship between humans and *P. fluorescens* throughout health and illness [70].

Pseudomonas alkylphenolica is a significant species in the breakdown of toxic alkylphenols and massive formation of bioactive polymannuronate polymers [71] and is well-matched forbiofilter applications to get rid of gaseous p-cresol, which is a most important infamous gas released from swine farms [72].

Spoilage relies on the density of the population and the connections between the bacteria constituting the environments of seafood involving QS [59,73,74]. In newly caught seafoods from temperate waters, microflora is created chiefly by aerobic rods-shapes and psychrotrophic Gram-negative microorganisms, whose growth is possible at 0^{o}C and optimal at around 25^{o}C. *Pseudomonas* spp. and a little other Gram-negative psychrotrophic microorganisms govern seafoods kept aerobically at freezing conditions [59].

Previously, cytotoxicity prompted by pyocyanin was revealed in a human embryonic lung epithelial cell line (L-132), a rainbow trout gonad cell line (RTG-2) and a *Spodoptera frugiperda pupal* ovarian cell line (Sf9) [75] while in the present work the cytotoxicity of the 30 *Pseudomonas* spp. was demonstrated in the Vero cells where *P. aeruginosa* induced a moderate cytotoxic effect while *P. fluorescens*, *P. putida* and *P. alkylphenolia* were weak in their cytotoxicity which could be attributed to the variance in the spreading of the QS and virulence genes in the four species. Milivojevic et al. [52] compared formation of biofilm, pyocyanin and hemolysin activity, and movement patterns of bacteria with the capability to destroy the Vero cells. The maximum positive link between hemolysis and the swarming aptitude was identified previously by non-parametric statistical analysis [52]. The learning method utilized on the virulence facts detected the maximum

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511 comparative prognostic significance of the immersed production of biofilm for the toxicity of the
512 cell, could not be depended on as an indicator of the infection aptitude [52].

513 The QS of *Pseudomonas* exhibit multiple signals with multiple functions [76]. QS systems
514 were widely established to be included in organization of pathogenicity of bacteria in aquaculture
515 industry [28]. The *P. aeruginosa las* QS signal 3-oxo-C12-HSL has a toxic effect on the cells [77].
516 *P. aeruginosa* QS-activated virulence factors comprise elastase, proteases, pyocyanin, lectin,
517 swarming movement, rhamnolipids, and toxins. The LasR–3OC12HSL complex stimulates
518 transcription of target genes containing those coding virulence factors. RhlR (C4HSL) stimulates
519 target genes, comprising those coding proteases, elastase, pyocyanin, and siderophores (iron
520 carrier). Consequently, most genes are believed to be carried by LasR or RhlR. For example, a
521 DlasR mutant, which is faulty for rhlI generation, expresses virulence factors initially described to
522 be LasR-dependent [78]. Seemingly, low level rhlI and rhlR expression indorses the subsequent
523 piling up of C4HSL and auto-induction of the RhlI/RhlR system. These results are considered to
524 be medically relevant because the clinical isolates of the *P. aeruginosa* isolates hold mutations in
525 lasR [79]. Biofilm production is an extra QS-controlled action in *P. aeruginosa*. Albeit regulation
526 of biofilm creation in *P. aeruginosa* mostly relies on further ecological signals, QS organization
527 of rhamnolipids, crowded movement, and likewise siderophores represent a significant role in the
528 production of biofilm in *P. aeruginosa* [80-83]. The control of biofilm differentiation and integrity
529 by RhlR and las QS in *P. aeruginosa in vivo* and *in vitro,* which makes an inextricable connection
530 between QS and biofilm formation [84]. Swarming motility has been concerned in initial phases
531 of biofilm formation created by *P. aeruginosa* [85]. QS is thought to dominate the swarming
532 motility produced *P. aeruginosa* through production of rhamnolipid, as the *rhlAB* genes are QS-
533 regulated [86] and the Rhl QS system was believed to be necessary for anaerobic biofilm existence

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[87]. Several QS signals have been established to display antimicrobial activities [88]. Gram-positive bacteria can be inhibited by The *P. aeruginosa las* QS signal 3-oxo-C12-HSL [77].

Pyocyanin produced by *P. aeruginosa* is an active molecule, which have wide-spectrum antimicrobial activity in different applications such as aquaculture industry and agriculture. The MDR *P. aeruginosa* strains were recovered from both fresh and smoked fish with a frequency rate of 33.1% and 20.0% respectively [42]. This finding was matched with the result that many androgen receptor (AR) gene pools in extraordinary levels were noticed in fish feed samples [42,89,90]. Furthermore, samples taken from skin and intestine of fish contain a huge quantity of microbial ART and AR genes which as well matching with the findings stated by Ye et al. [89]. As fish and animal by-products, frequently rich in microbial ART, are utilized in feeding of fish as an imperative source of protein, it is predictable that there is a huge pool of AR genes and possibly AR gene carrying microbes in the food of fish. ART bacteria even multiple antibiotic resistant microorganisms were demonstrated in different sources of animal feedstuffs, for instance avian foods [90], livestock food constituents [91], and extracted protein products deriving from avian, cow, and fish [92]. Without suitable treatment, the diet of fish carrying the AR gene produced during feed processing is likely to be a risk factor for the spread of AR bacteria in the fish farms and consequently in the food chain. Actually, numerous genera of ART bacteria were present in manifold types of samples. The aquatic ecosystem described by Devarajan et al. [93] is considered a hotspot for spreading and acquisition of antimicrobial resistance as a result of infection with emerging pollutants resulting from anthropogenic actions. They reported the culturing and description of 141 species of *Pseudomonas* from aquatic residues receiving moderately untreated hospital and mutual wastes from three different geographic areas: Congo, India, and Switzerland. *P. putida* (42%) and *P. aeruginosa* (39%) were the most common species

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of *Pseudomonas*. The findings of [93] demonstrated a prevalent incidence of antimicrobial resistance in aquatic environmental deposits getting untreated/treated wastewater and how these up-to-date causes of pollution, lead to the distribution of bacterial tolerance in the aquatic surroundings. These types of antibiotic resistant bacteria are considered a public hazard to people, because they can act as a vector for the keeping and prevalence of ARGs [94-99]. These microbes in the deposit of the receiving ecosystems were deliberated by Devarajan et al. [93] to be permanent and may perhaps consider essential in keeping and scattering these multiple antibiotic resistant bacteria due to human drinking of water, fisheries or agricultural products by surface water for the irrigation system.

The existence of extended spectrum b-lactamase (ESBLs) and metallo b-lactamase (MBLs) genes amongst different types of microbes represents a significant risk, as they cause resistance to a huge panel of betalactams [100,101]. Furthermore, chromosomally encoded resistance mechanisms, are copious in various types of gram-negative microorganisms especially in Pseudomonas spp., which play a vital role in increasing the level of antimicrobial resistance [102]. *bla*SHV-27 is the only other SHV variant commonly stated as chromosomally found in *E. coli* recovered from fish farms together with nonESBLs *bla*SHV-1, *bla*SHV-11, *bla*SHV-25, and *bla*SHV-26 [103]. None of the *P. aeruginosa* isolates were MDR [39].

Using of various antimicrobial drugs in fish farms was considered one of the significant causes for introducing multi-drug resistant bacteria and increasing the attentiveness of ARGs in the Ecosystem for fish farming [104,105]. The high use of antibiotics especially sulfonamides and chloramphenicol in fish farms has been stated [105-107]. The incidence of ARGs acquired from aquatic animal skin microbiota is unreliable with the deposits, water and aquatic animal gut [98,108, 109]. In this investigation, aquatic animal skin microbiota represents additional vital place

for spreading of ARGs. In addition, the movement of aquatic animal may help the spread and proliferation of ARGs in the aquaculture. The difference of agriculture design might explain this phenomenon. Comparison with marine aquatic animals, freshwater animals could simply obtain more antibiotic residues from hospitalized wastes, sewage of plants, wastewater of town and etc. Therefore, freshwater animals settled more ARGs. The variations of geographic area and anthropogenic environmental changes could illustrate the condition, which might reflect the variety in using of antimicrobial drugs. However antibiotic resistance genes (ARGs) have naturally developed, unselective usage of antimicrobial drugs in both human and animal has led to selection and distribution of multi-drug resistant bacteria. ARGs established in fish farms may be resulted from manifold sources such as effluents that can come from sewage, wastes of farms and may be dispersed through stormwater. As well as the effluent from hospitals can carry an important pool of ARGs. Rowe et al. [110] used a comparative metagenomic approach and revealed that the plenty of ARGs in liquid wastes arriving the catchment area of river is greater than that in the receiving environment.

The MAR index is considered as a decent risk assessment tool and the value of the MAR index (nominally 0.200) has been used to differentiate low- and high-risk regions where antibiotics are overused [111]. Such analysis provides a clear knowledge regarding the numbers of bacteria which show resistant to various antibiotics in the risk zone of susceptibility investigation. Based on our findings, most isolates had MAR indices of 0.25, confirming that there was high antibiotic use and high selective pressure in these environments. However, the practical significance of such an analysis in a developing country as previously recorded in Jamaica, may be lost because antibiotic use and abuse are widespread [112].

Consequently, as a result of the emergence of antibiotic resistance, it is necessary to develop new methods in order to control bacterial infections biofilms became a must. Conversely, QS becomes a possible goal for using it as a new strategy of treatment as a result of its role in microbial contamination, antibiotic resistance and formation of biofilms. One such chance can be recognized by discovering Quorum quenching (QQ) due to its expected advantages [76,113] and gaining significance as a modern method to monitor bacterial biofilms in medicinal and industrial areas, aquaculture, and water treatment plants [114,115].

5. Conclusions

Our findings highlight for the first time the diffusion of MDR *Pseudomonas* sp. isolates carrying resistance, QS and virulence genes in fish from aquaculture or the River Nile in Egypt. Using of antimicrobial agents in food-producing animals chooses for antibiotic resistance that can be transferred to individuals through food or additional ways of transmission. To keep the efficiency of therapeutically significant antimicrobial drugs, veterinary doctors, agriculturalists, regulatory agencies, and all other shareholders are advised to accept the guidelines released by the WHO in 2017 and work concerning application of these guidelines [116]. These instructions indorse diminutions in the frequently used antimicrobial drugs in aquaculture, comprising complete limit of antibiotic usage as a growth promoting agent and for prevention of disease. Furthermore, these instructions advice that antimicrobials recognized as critically significant for individuals are not applied in food-producing animals for handling or infection control except sensitivity testing reveals the medication to be the only treatment option. In addition, and because of an adjacent relationship between the QS system and antibiotic resistance, biofilm formation and virulence of aquatic microorganisms, ecological strategies are the desired option to solve the

difficulties of gaining of antimicrobial resistance and the scattering of resistant genes when antimicrobial agents or sanitizers are used to treat microbial infections [117,118].

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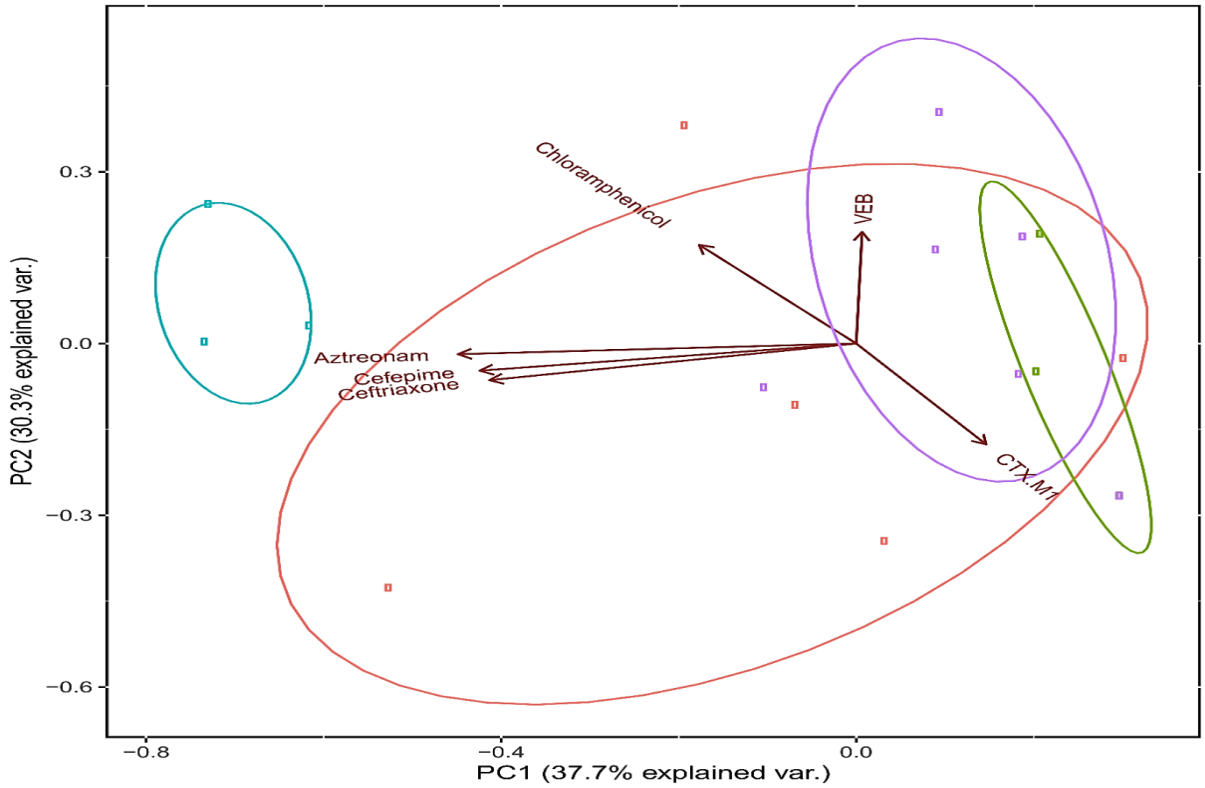

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1041 **Figure 1.** Principal component analysis of isolates. The components are showed by the arrows.
1042 The six components (resistance to Chloramphenicol, Aztreonam, Cefepime and Ceftriaxone, VEB
1043 and CTX-M1 resistance genes) represents 68% of the sample variation. The isolates from *P.*
1044 *putida*, *P. aeruginosa*, *P. alkylphenolia* and *P. flouorescens* are represent by colors red, green, blue
1045 and purple, respectively.

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Table 1. List, classification and prioritization of antimicrobials categorized as critically important in human and veterinary medicine.

Antibiotic	Disc concentration	Antimicrobial class	Medical importance	Prioritization criterion
Gentamycin	10 µg	Aminoglycosides	Critically Important Antimicrobials	C1, C2, P2, P3
Amikacin	30µ	Aminoglycosides	Critically Important Antimicrobials	C1, C2, P2, P3
Cefotaxime	30µ	Cephalosporins (3rd, 4th and 5th generation)	Critically Important Antimicrobials	C1, C2, P1, P2, P3
Ceftazidime	30µ	Cephalosporins (3rd, 4th and 5th generation)	Critically Important Antimicrobials	C1, C2, P1, P2, P3
Ceftriaxone	30µ	Cephalosporins (3rd, 4th and 5th generation)	Critically Important Antimicrobials	C1, C2, P1, P2, P3
Cefepime	30µ	Cephalosporins (3rd, 4th and 5th generation)	Critically Important Antimicrobials	C1, C2, P1, P2, P3
Imipenem	10 µg	Carbapenems and other penems	Critically Important Antimicrobials	C1, C2, P1, P2
Aztreonam	30 µg	Monobactams	Critically Important Antimicrobials	C1, C2. P1
Ciprofloxacin	5 µg	Quinolones and fluoroquinolones	Critically Important Antimicrobials	C1, C2, P1, P2, P3
Nalidixic acid	30µ	Quinolones and fluoroquinolones	Critically Important Antimicrobials	C1, C2, P1, P2, P3
Ampicillin	10 µg	Penicillins	Critically Important Antimicrobials	C1, C2, P2, P3
Ampicillin/sulbactam	20µ	Penicillins	Critically Important Antimicrobials	C1, C2, P2, P3
Sulphamethoxazole/Trimethoprim	25 µg	Sulfonamides, dihydrofolate reductase inhibitors and combinations	Highly Important Antimicrobials	C2, NA
Tetracycline	5 µg	Tetracyclines	Highly Important Antimicrobials	C1, NA
Chloramphenicol	30 µg	Amphenicols	Highly Important Antimicrobials	C2, NA
Cephalothin	30µ	Cephalosporins (1st generation)	Highly Important Antimicrobials	C2, NA

Prioritization criterion 1 (P1): High absolute number of people, or high proportion of use in patients with serious infections in health care settings affected by bacterial diseases for which the antimicrobial class is the sole or one of few alternatives for treating serious infections in humans. **Prioritization criterion 2 (P2):** High frequency of use of the antimicrobial class for any indication in human medicine, or high proportion of use in patients with serious infections in health care settings, because use may favor selection of resistance in both settings. **Prioritization criterion 3 (P3):** The antimicrobial class is used to treat infections in people for whom there is evidence of transmission of resistant bacteria (e.g., non-typhoidal *Salmonella* and *Campylobacter* spp.) or resistance genes (high for *E. coli* and *Enterococcus* spp.) from non-human sources.

NA: not available

Criterion 1 (C1): The antimicrobial class is the sole, or one of limited available therapies, to treat serious bacterial infections in people. **Criterion 2 (C2):** The antimicrobial class is used to treat infections in people caused by either: (1) bacteria that may be transmitted to humans from non-human sources, or (2) bacteria that may acquire resistance genes from non-human sources.

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1067 **Table 2. PCR-specific oligonucleotide primers, amplicon size and conditions for the *ampC*,**

1068 **class A β-lactamase, quorum sensing and virulence genes**

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Gene	Target Enzyme (s)	Sequence	Amplicon Size (bp)	Cycle Number	Annealing Temperature (°C)	Conferred Resistance (or Purpose)	References
Extended-spectrum βlactamases (ESBLs) belong mostly to class A of the Ambler classification scheme							
bla$_{VEB}$	*VEB*	5'- CGACTTCCATTTCCCGATGC -3' 5'- GGACTCTGCAACAAATACGC -3'	643	35	60˚C 45 sec	ESBL enzymes keep aptitude to deliberate penicillins resistance and discuss expanded-spectrum cephalosporins resistance. Unaffected susceptibility for cephamycins, cefoxitin, and cefotetan.[136]	133, 134
bla$_{PER}$	**PER**	5′- ATGAATGTCATTATAAAAGC -3′ 5′- AATTTGGGCTTAGGGCAGAA -3′	925				
bla$_{TEM}$	**TEM**	F: 5' GAGTATTCAACATTTCCGTGTC -3' 5'- TAATCAGTGAGGCACCTATCTC -3'	861				
bla$_{SHV}$	**SHV**	5'- AAGATCCACTATCGCCAGCAG -3' 5'- ATTCAGTTCCGTTTCCCAGCGG -3'	231				
bla$_{CTX-M1}$	**CTX-M1**	5'- GACGATGTCATCGGCTGAGC -3' 5'- AGCCGCCGACGCTAATACA -3'	499	30	55˚C 45 sec	These enzymes are not very thoroughly associated with TEM or SHV β-lactamases in that they illustrate only about 40% identity with these two frequently isolated β-lactamases [136]	
bla$_{CTX-M2}$	CTX-M2	5'- GCGACCAGGTTAACTACAATCC -3' 5'- CGGTAGTATTGCCCTTAAGCC -3'	351				
bla$_{CTX-M3}$	CTX-M3	5'- CGCTTTGCCATGTGCAGCACC -3' 5'- GCTCAGTACGATCGAGCC -3'	307				
Quorum-Sensing genes							
lasI	LasI	5' CGTGCTCAAGTGTTCAAGG 3' 5' TACAGTCGGAAAAGCCCAG 3'	295	30	60 ºC 1 min	LasI and RhlI are enzymes that synthesize the acyl-HSL signal molecules *N*-3-oxododecanoyl homoserine lactone(3-oxo-C12-HSL) and butanoyl homoserine lactone (C4-HSL), respectively. LasR and RhlR are transcriptional activator proteins that exactly connect the signal created by their cognate synthases to control transcripton of target genes.	135
*las*R		5'AAGTGGAAAATTGGAGTGGAG 3' 5'GTAGTTGCCGACGACGATGAAG 3'	130				
rhlR		5' TGCATTTTATCGATCAGGGC 3' 5' CACTTCCTTTTCCAGGACG 3'	133				
rhlAB		5' TCATGGAATTGTCACAACCGC 3' 5' ATACGGCAAAATCATGGCAAC 3'	151				
rhlI	RhlI	5' TTCATCCTCCTTTAGTCTTCCC 3' 5' TTCCAGCGATTCAGAGAGC 3'	155	35	60 ºC 40 sec		
Virulence genes							
toxA		5' GGAGCGCAACTATCCCACT 3' 5' TGGTAGCCGACGAACACATA 3'	150	35	55 ºC 30 sec		
aprA		5' GTCGACCAGGCGGCGGAGCAGATA 3' 5'GCCGAGGCCGCCGTAGAGGATGTC 3'	993				
plcH		5' GAAGCCATGGGCTACTTCAA 3' 5' AGAGTGACGAGGAGCGGTAG 3'	307				

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Table 3. Results of phenotypic tests for the investigation of virulence factor production

Pseudomonas biotype	Phenotypic Virulence factors					Virulence genes			Quorum sensing genes				
	Blood hemolysis	Elastin Congo red assay	Alkaline protease assay (PU/ml)	Pyocyanin assay (µg/ml)	Vero cell assay	*toxA*	*aprA*	*plcH*	*lasI*	*lasR*	*rhlR*	*rhlAB*	*rhlI*
aeruginosa	β	5.5	0.65	4.3	++	_	_	_	_	_	_	_	+
aeruginosa	β	6.5	6.4	4.17	++	_	_	+	+	_	+	_	_
aeruginosa	β	7.8	7.6	2.97	++	+	+	+	_	+	+	_	+
aeruginosa	β	8.7	8.7	2.04	++	_	_	_	_	_	_	_	_
aeruginosa	β	3.7	7.8	3.19	++	_	_	_	_	+	+	_	+
aeruginosa	β	7.6	6.7	2.86	++	+	_	_	+	_	_	+	+
aeruginosa	β	5.6	6.7	0.15	++	_	_	_	_	_	_	_	_
aeruginosa	β	7.8	7.8	4.3	++	_	_	_	_	_	_	_	_
aeruginosa	α	7.6	6.4	2.04	++	+	+	+	+	_	_	+	+
aeruginosa	α	4.8	6.5	2.75	++	_	_	_	_	_	_	_	_
aeruginosa	α	8.6	3.7	1.21	++	_	_	_	_	_	_	_	_
aeruginosa	α	5.6	7.4	4.17	++	_	+	+	+	_	_	+	+
fluorescens	α	7.6	5.8	4.17	+	_	_	_	_	_	_	_	_
fluorescens	α	4.4	7.6	4.57	+	_	_	_	+	_	_	+	_
fluorescens	α	3.4	5.6	2.97	+	_	_	_	_	_	_	_	+
fluorescens	α	4.6	7.6	0.86	+	_	_	_	+	+	+	_	+
fluorescens	α	3.3	5.0	2.04	+	+	_	+	_	+	+	_	+
fluorescens	α	6.7	5.4	5.6	+	_	+	+	_	_	_	+	_
fluorescens	γ	4.6	5.6	1.03	+	_	_	_	+	+	+	+	+
fluorescens	γ	6.5	7.6	4.04	+	+	+	+	+	_	_	+	_

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putida	γ	7.7	5.8	8.0	+	+	_	_	_	_	_	+	+
putida	γ	2.3	8.7	7.58	+	_	_		_	_	_	+	_
putida	γ	2.5	5.6	5.92	+	+	_	+	+	_	_	_	_
putida	γ	3.3	6.5	4.57	+	_	_	_	+	_	_	+	+
putida	γ	5.4	3.3	4.3	+	_	_	_	_	_	_	_	_
putida	γ	2.3	3.2	2.04	+	_	_	_	+	_	_	+	_
alkylphenolia	γ	4.3	3.4	4.3	+	_	+	_	_	_	_	_	+
alkylphenolia	γ	2.6	4.5	2.04	+	_	_	_	+	_	_	_	_
alkylphenolia	γ	4.5	5.4	5.92	+	_	_	+	_	_	_	_	_
alkylphenolia	γ	4.5	5.4	5.92	+	_	_	_	+	_	_	_	_

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Table 4. QS genes combinations

QS genes combinations	Number of gene combinations	Number of isolates	Percentage
rhlAB	**None**	**3**	**10**
rhlI	**None**	**3**	**10**
lasI	**None**	**3**	**10**
lasI, rhlR	**2**	**1**	**3.3**
rhlAB, rhlI	**2**	**1**	**3.3**
lasI, rhlAB	**2**	**3**	**10**
lasR, rhlR, rhlI	**3**	**3**	**10**
lasI, rhlAB, rhlI	**3**	**4**	**13.3**
lasI, lasR, rhlR, rhlI	**4**	**1**	**3.3**
lasI, lasR, rhlR, rhlAB, rhlI	**5**	**1**	**3.3**

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Table 5. Investigation of phenotypic antibacterial resistance and antibiotic resistance genes in fish meat isolates of *Pseudomonas*

Pseudomonas biotype	Antibiotics																				Antibiotic resistance genes						
	Sulphamethoxazole/ Trimethoprim	Amikacin	Imipenem	Tetracycline	Ampicillin	Nalidixic acid	Chloramphenicol	Gentamicin	Ciprofloxacin	Aztreonam	Ampicillin/sulbactam	Cefepime	Ceftriaxone	Cephalothin	Cefotaxime	Ceftazidime	MAR_{index}	Biofilm (MTP)	Degree of Biofilm production	$Biofilm_{index}$	*SHV*	*TEM*	*PER*	*VEB*	*CTX-M3*	*CTX-M2*	*CTX-M1*
aeruginosa	R	S	S	R	R	R	R	S	R	S	S	S	S	R	S	S	0.3	0.2	MBP	0.2	+	_	_	+	_	_	_
aeruginosa	R	S	S	R	R	R	S	S	R	S	S	S	S	S	S	S	0.3	0.1	NBP	0.1	_	_	_	_	_	_	+
aeruginosa	R	S	S	R	R	R	S	S	R	S	S	S	S	S	S	S	0.3	0.2	MBP	0.2	_	+	_	_	_	+	+
aeruginosa	R	S	S	R	R	R	S	S	R	S	R	S	S	R	S	S	0.4	0.2	MBP	0.1	+	+	_	_	_	+	_
aeruginosa	R	S	R	R	R	R	S	S	R	S	R	S	S	R	S	S	0.4	0.2	MBP	0.2	_	_	_	+	_	+	_
aeruginosa	R	S	S	R	R	R	S	S	R	S	R	S	S	R	S	S	0.4	0.1	NBP	0.1	+	_	_	_	_	_	+
aeruginosa	R	S	S	R	R	R	S	S	R	S	S	S	S	S	S	R	0.4	0.2	MBP	0.1	_	+	_	+	_	_	_
aeruginosa	R	S	S	R	R	R	S	S	R	S	S	S	S	S	R	S	0.3	0.2	MBP	0.2	_	_	_	_	+	_	_
aeruginosa	R	S	S	R	R	R	S	S	R	S	S	S	S	R	S	S	0.3	0.1	NBP	0.1	_	_	+	+	_	_	+
aeruginosa	R	S	S	R	R	R	S	S	R	S	S	S	S	R	S	S	0.3	0.2	MBP	0.2	+	_	+	_	_	+	+
aeruginosa	R	S	S	R	R	R	S	S	S	S	S	S	S	S	S	S	0.3	0.2	MBP	0.1	_	_	_	_	_	_	_
aeruginosa	R	S	S	R	R	R	S	S	R	S	S	S	S	S	S	S	0.3	0.2	MBP	0.1	_	_	_	_	_	_	_
fluorescens	R	S	S	R	R	R	R	S	R	S	S	S	S	R	S	S	0.3	0.2	MBP	0.2	_	_	_	+	_	_	_
fluorescens	R	S	S	R	R	R	R	S	R	S	R	S	S	R	S	S	0.4	0.2	MBP	0.2	_	_	_	_	_	_	+
fluorescens	S	S	S	R	R	R	R	S	R	R	R	S	S	S	S	S	0.3	0.1	NBP	0.1	+	+	+	_	_	+	+
fluorescens	R	S	S	R	R	R	R	S	R	S	S	S	S	S	S	S	0.4	0.2	MBP	0.2	_	+	_	+	_	+	_
fluorescens	R	S	S	R	R	R	R	S	R	S	S	S	S	S	S	S	0.4	0.2	MBP	0.1	_	_	_	_	+	_	_
fluorescens	S	S	S	S	R	S	S	S	R	S	S	S	S	S	S	S	0	0.2	MBP	0.1	+	_	+	_	_	_	+
fluorescens	R	S	S	R	R	R	R	S	R	S	S	S	S	S	S	S	0.4	0.1	NBP	0.1	_	+	_	+	_	_	+
fluorescens	R	S	S	R	R	R	R	S	R	S	S	S	S	S	S	S	0.4	0.1	NBP	0.1	+	_	_	+	_	_	_
Putida	R	S	S	R	R	R	R	R	R	R	S	S	S	R	S	R	0.6	0.2	MBP	0.2	_	_	_	+	_	_	_

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Putida	**R**	S	S	**R**	**R**	**R**	S	S	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	0.6	0.2	MBP	0.1	+	_	+	_	_	_	+
Putida	**R**	S	S	**R**	**R**	**R**	S	S	**R**	S	S	S	S	S	S	S	0.3	0.2	MBP	0.2	_	+	_	+	_	+	+
Putida	**R**	S	S	**R**	**R**	**R**	S	S	**R**	S	**R**	S	**R**	**R**	S	S	0.4	0.1	NBP	0.1	_	_	_	_	_	+	+
Putida	**R**	S	S	**R**	**R**	**R**	S	S	**R**	S	**R**	**R**	S	**R**	S	S	0.4	0.2	MBP	0.2	_	+	+	_	+	_	_
Putida	**R**	S	S	**R**	**R**	**R**	S	S	**R**	**R**	S	**R**	**R**	**R**	S	S	0.6	0.2	MBP	0.1	+	_	+	_	+	_	+
alkylphenolia	**R**	S	S	**R**	**R**	**R**	S	S	**R**	**R**	**R**	**R**	**R**	**R**	S	S	0.6	0.2	MBP	0.2	+	_	_	+	_	_	_
alkylphenolia	**R**	S	S	**R**	**R**	**R**	**R**	S	**R**	**R**	S	**R**	**R**	**R**	S	S	0.6	0.1	NBP	0.1	_	_	_	_	_	_	_
alkylphenolia	**R**	S	S	**R**	**R**	**R**	**R**	S	**R**	**R**	S	**R**	**R**	**R**	S	S	0.6	0.2	MBP	0.2	_	_	+	+	_	_	_
alkylphenolia	**R**	S	S	**R**	**R**	**R**	**R**	S	**R**	**R**	S	**R**	**R**	**R**	S	S	0.6	0.2	MBP	0.1	_	_	_	_	_	_	_

Ampicillin (10 µg), Amikacin (30 µg), Aztreonam (30 µg), Chloramphenicol (30 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), Ceftriaxone (30 µg), Cephalothin (30 µg), Ciprofloxacin (5 µg), Cefepime (30 µg), Gentamicin (10 µg), Sulphamethoxazole/Trimethoprim (25 µg), Ampicillin/sulbactam (20 µg), Nalidixic acid (30 µg), Imipenem (10 µg), Tetracycline (5 µg)
NBP: non biofilm producer
MBP: moderate biofilm producer
MTP: Microtiter plate assay

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Table 6. Antimicrobial resistance profiles of *Pseudomonas* spp. isolated from fish meat to various antibiotics

Antibiotics	n= of antibiotics	n= of antibiotic classes	n= of *Pseudomonas* isolates	n= of XDR *Pseudomonas* isolates	n= of MDR *Pseudomonas* isolates	n= of PDR *Pseudomonas* isolates
AMP, ATM	2	1	1	1	**0**	**0**
SXT, TE, AMP, NA	4	4	1	**0**	1	**0**
SXT, TE, AMP, NA, CIP	5	4	4	**0**	4	**0**
SXT, TE, AMP, NA, CIP, CAZ	6	4	1	**0**	1	**0**
SXT, TE, AMP, NA, CIP, CTX	6	4	1	**0**	1	**0**
SXT, TE, AMP, NA, CIP, KF	6	4	2	**0**	2	**0**
AMP, NA, C, CIP, ATM, SAM	6	4	1	**0**	1	**0**
SXT, TE, AMP, NA, C, CIP	6	5	4	**0**	4	**0**
SXT, TE, AMP, NA, C, CIP, KF	7	5	2	**0**	2	**0**
SXT, TE, AMP, NA, CIP, SAM, KF	7	4	2	**0**	2	**0**
SXT, IPM, TE, AMP, NA, CIP, SAM	7	5	1	**0**	1	**0**
SXT, TE, AMP, NA, C, CIP, SAM, KF	8	5	1	**0**	1	**0**
SXT, TE, AMP, NA, CIP, SAM, CRO, KF	8	4	1	**0**	1	**0**
SXT, TE, AMP, NA, CIP, SAM, KF, FEP	8	4	1	**0**	1	**0**
SXT, TE, AMP, NA, CIP, ATM, FEP, CRO, KF	9	4	1	**0**	1	**0**
SXT, TE, AMP, NA, C, CN, CIP, ATM, KF, CAZ	10	6	1	**0**	1	**0**
SXT, TE, AMP, NA, CIP, ATM, SAM, FEP, CRO, KF	10	4	1	**0**	1	**0**
SXT, TE, AMP, NA, C, CIP, ATM, FEP, CRO, KF	10	5	2	**0**	2	**0**
SXT, TE, AMP, NA, CIP, ATM, SAM, FEP, CRO, KF, CTX	11	5	1	**0**	1	**0**
SXT, IPM, TE, AMP, NA, C, CIP, ATM, CAZ, FEP, CRO, KF	12	6	1	**0**	1	**0**

AMP: Ampicillin, AK: Amikacin, ATM: Aztreonam, C: Chloramphenicol, CTX: Cefotaxime, CAZ: Ceftazidime, CRO: Ceftriaxone, KF: Cephalothin, CIP: Ciprofloxacin, FEP: Cefepime, CN: Gentamicin, SXT: Sulphamethoxazole/Trimethoprim, SAM: Ampicillin/sulbactam, NA: Nalidixic acid, IPM: Imipenem, TE: Tetracycline

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Table 7. The correlation between the production of QS-dependent virulence factors and antimicrobial susceptibility in fish meat isolates of *Pseudomonas* (n = 30)

	Rates of resistance to antimicrobials (%)															
	Sulphamethoxazole/ Trimethoprim	Amikacin	Imipenem	Tetracycline	Ampicillin	Nalidixic acid	Chloramphenicol	Gentamicin	Ciprofloxacin	Aztreonam	Ampicillin/sulbactam	Cefepime	Ceftriaxone	Cephalothin	Cefotaxime	Ceftazidime
Elastin Congo red	93.3	0	3.3	96.7	100	96.7	40	3.3	96.7	26.7	30	23.3	23.3	56.7	26.7	26.7
Alkaline protease	93.3	0	3.3	96.7	100	96.7	40	3.3	96.7	26.7	30	23.3	23.3	56.7	26.7	26.7
Pyocyanin	93.3	0	3.3	96.7	100	96.7	40	3.3	96.7	26.7	30	23.3	23.3	56.7	26.7	26.7

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Table 8. Association between Antibiotic Resistance Extended-spectrum βlactamases, Virulence and QS genes.

Pseudomonas biotype	Antibiotic resistance genes							Virulence genes			Quorum sensing genes				
	***bla*$_{SHV}$**	***bla*$_{TEM}$**	***bla*$_{PER}$**	***bla*$_{VEB}$**	***bla*$_{CTX-M3}$**	***bla*$_{CTX-M2}$**	***bla*$_{CTX-M1}$**	***toxA***	***aprA***	***plcH***	***lasI***	***lasR***	***rhlR***	***rhlAB***	***rhlI***
aeruginosa	+	_	_	+	_	_	_	_	_	_	_	_	_	_	+
aeruginosa	_	_	_	_	_	_	+	_	_	+	+	_	+	_	_
aeruginosa	_	+	_	_	_	+	+	+	+	+	_	+	+	_	+
aeruginosa	+	+	_	_	_	+	_	_	_	_	_	_	_	_	_
aeruginosa	_	_	_	+	_	+	_	_	_	_	_	+	+	_	+
aeruginosa	+	_	_	_	_	_	+	+	_	_	+	_	_	+	+
aeruginosa	_	+	_	+	_	_	_	_	_	_	_	_	_	_	_
aeruginosa	_	_	_	_	+	_	_	_	_	_	_	_	_	_	_
aeruginosa	_	_	+	+	_	_	+	+	+	+	+	_	_	+	+
aeruginosa	+	_	+	_	_	+	+	_	_	_	_	_	_	_	_
aeruginosa	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
aeruginosa	_	_	_	_	_	_	_	_	+	+	+	_	_	+	+
fluorescens	_	_	_	+	_	_	_	_	_	_	_	_	_	_	_
fluorescens	_	_	_	_	_	_	+	_	_	_	+	_	_	+	_
fluorescens	+	+	+	_	_	+	+	_	_	_	_	_	_	_	+
fluorescens	_	+	_	+	_	+	_	_	_	_	+	+	+	_	+
fluorescens	_	_	_	_	+	_	_	+	_	+	_	+	+	_	+
fluorescens	+	_	+	_	_	_	+	_	+	+	_	_	_	+	_
fluorescens	_	+	_	+	_	_	+	_	_	_	+	+	+	+	+
fluorescens	+	_	_	+	_	_	_	+	+	+	+	_	_	+	_
Putida	_	_	_	+	_	_	_	+	_	_	_	_	_	+	+
Putida	+	_	+	_	_	_	+	_	_		_	_	_	+	_
Putida	_	+	_	+	_	+	+	+	_	+	+	_	_	_	_
Putida	_	_	_	_	_	+	+	_	_	_	+	_	_	+	+

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Putida	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–
Putida	+	–	+	–	+	–	+	–	–	–	+	–	–	+	–	
alkylphenolia	+	–	–	+	–	–	–	–	+	–	–	–	–	–	+	
alkylphenolia	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	
alkylphenolia	–	–	+	+	–	–	–	–	–	+	–	–	–	–	–	
alkylphenolia	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	