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Prevalence of *Pseudomonas* spp. in Marine Water Fish Intended for Human Consumption

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Abstract

This study aimed at investigation of the prevalence and antimicrobial susceptibility of *Pseudomonas* spp., in two marine water fish, namely Pagrus, and *Saurus* intended for human consumption. In the current study, *Pseudomonas* spp., was isolated and identified from two marine water fishes. Fifty Pagrus fish and 50 *Saurus* (Saurida undosquamis) fish were sampled from fish markets at Sharkia governorate, Egypt. The prevalence rates of *Pseudomonas* spp. were 84%, and 40% in Pagrus fish, and *Saurus* respectively. The identified *Pseudomonas* spp., from *Saurus* was *Pseudomonas aeruginosa* (P. aeruginosa), *P. fluorescens*, *P. fragi*, *P. Cepacia*, at 20% for each. *P. alcaligenes* and *P. lundensis* were recovered at 10%. In Pagrus, the prevalence rates were 31.3%, 56.3%, and 6.2% for *P. aeruginosa*, *P. fluorescens*, *P. fragi* and *P. stutzeri*. Seven serotypes of *P. aeruginosa* were identified with the serotypes O11 at the top prevalence (42.8%), and O6, O5, O1, O8 at 19.3% for each. The virulence-associated genes were *lasB* (Elastase B gene), *exoS* (Exoenzyme S) and *pilB* (pili gene) were detected in the recovered *P. aeruginosa* at 100%, 71.4%, and 28.6%, respectively. The recovered *Pseudomonas* species had high antimicrobial resistance to erythromycin, amoxicillin, ampicillin and gentamycin at 100%, 88.5%, 65.4%, and 50%, respectively. In conclusion, Pagrus and *Saurus* fish species are considered as potential sources of multidrug resistant *Pseudomonas* spp.

KEYWORDS
Saurus, Pagrus, *Pseudomonas* spp., Drug resistance.

INTRODUCTION

Fish is a unique source of nutrients with metabolic and hormonal importance, such as omega-3 fatty acids, iodine, selenium, vitamin D, taurine, and carnitine. Fish is also a good source of high-quality protein and has a low caloric density. Regular fish consumption has a positive impact on conditions such as metabolic syndrome, obesity, diabetes, hypothyroidism, polycystic ovary syndrome, and menopausal transition (Cardoso *et al*., 2016; Mendivil, 2021; Morshdy *et al*., 2019; 2022). Vitamin D is a steroid hormone that is required to ensure proper absorption of dietary calcium and phosphate, and thus it plays an important role in musculoskeletal homeostasis. Aside from its well-known role in bone preservation, vitamin D may also play a role in metabolic and immune physiology (Lazano, 2003; Rosen, 2011; Atia *et al*., 2018). Nutritionists and other health professionals recommend it to customers' dieticians. It is demonstrated that eating fish, particularly fatty fish, lowers the risk of aging muscle degeneration. Strong scientific evidence supports the positive effects of fish consumption on human health, including cognitive development, mental health, immune system, anemia prevention, cardiovascular disease prevention, and Alzheimer's disease prevention (Augood *et al*., 2008; Béné *et al*., 2015; Golden *et al*., 2016).

Fish transports microorganisms from its natural aquatic environment, sewage, soil, contaminated harvesting areas, and contaminated utensils during handling, processing, distribution, and storage (Novoslavskij *et al*., 2016). Contamination sources of fish products could include contact with food handlers and asymptomatic carriers with infected skin lesions, and poor hands. Workers may touch cooked ready-to-eat fish products that are typically eaten without further cooking or reheating. Contact with inadequate unclean utensils or serving plates, contaminated preparation surfaces, poor sanitation in kitchens, insects, rodents, and other animals, and temperature abuse of food can all cause food-borne diseases (Morshdy *et al*., 2013; Alsayeqh *et al*., 2021; Darwish *et al*., 2022).

Microbial metabolism of both carbon and nitrogen source compounds plays an important role in the spoilage of fish and crustaceans during storage. Proteins, particularly structural proteins, can be hydrolyzed into peptides and amino acids by microbial protease, causing changes in the physicochemical properties of fish flesh (texture, moisture distribution, color, and water-holding capability) (Masniyom, 2011; Saber *et al*., 2018; Hafez *et al*., 2022).

Various bacterial pathogens affect a wide variety of aquatic species and cause significant economic losses worldwide. It is estimated that up to 50% of farmed fish are lost prior to marketing due to bacterial infection. Poor growth, high mortalities, and poor flesh quality are the primary causes of economic losses (Morshdy *et al*., 2022).

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Pseudomonas species are related to the Pseudomonadaceae family, *Pseudomonas* species-genus is a big and complicated heterogeneous group. The genus is formed from more than 255 species (Kornegay, 2000). These species differ metabolically and heterogeneous in nutrition. *P. aeruginosa* belongs to the Psudomonusasa genus and involve 13 distinct subgroups. They are *P. aeruginosa*, *P. alcaligens, P. anguilliseptica, P. caeni, P. citronellolis, P. nitroreductase, P. oleovorans, P. flavescens, P. jinjuensis, P. mendocina, P. stramina, P. resinovorans, P. pseudoalcaligenes, P. mendocina. P. aeruginosa* is widely distributed in aquatic environments and it is a normal microflora of fish, but when occur stress factors such as malnutrition, overcrowding and high temperature, and the bacteria become pathogenic and induce necrosis of gills, hemorrhagic septicemia, and distention of abdomen so causing an economic loss in fish farms. Besides, *Pseudomonas* spp., is the causative agent of food-borne illnesses for humans by eating spoiled food and raw seafood (Ardura *et al*., 2013).

P. aeruginosa is an aerobic Gram-negative motile rod with no spore forming with catalase and oxidase activity; Furthermore, this bacterium has many virulence-related determinants, including cell-mediated, and secreted virulence factors; Pilli, flagella, and lipopolysaccharide (LPS) are cell-mediated types that are commonly involved in bacterial colonization and motility, delivery of active proteins into host cells, and establishment of persistent infections (Zeng, 2004). Similarly, secreted virulence factors strengthen inflammatory processes, cause severe tissue damage, facilitate bacterial invasion and dissemination, and accelerate disease progression (Mesquita *et al*., 2013). Serious efforts to detect *P. aeruginosa* are now being made, not only for economic reasons but also for public health reasons. *Pseudomonas* infection is most caused by *P. aeruginosa*. *Pseudomonas* species are currently classified as a food-borne illness that infects humans through the consumption of spoiled foods and ready-to-eat products, as well as the manipulation of contaminated seafood (Gram and Huss, 2000). Although few studies have claimed possible disease transmission routes, people with weakened immune systems or those who work with infected fish are usually at risk (Ardura *et al*., 2013).

This study aimed TO investigate the prevalence and antimicrobial susceptibility testing of *Pseudomonas* spp., in two marine water fish species commonly consumed in Egypt, namely Pagrus spp., and *Saurus* spp. Besides, the detection of virulence associated genes in the recovered *Pseudomonas aeruginosa* isolates was done using PCR.

MATERIALS AND METHODS

Sample collection

A total of 100 marine water fish samples; 50 each of Pagrus and *Saurus* fish species were purchased randomly from various fish shops at Sharkia Governorate, Egypt. Sampling took place during October to December 2022. The samples were transported in ice box to the Food Safety Laboratory, Faculty of Veterinary Medicine, Zagazig University, Egypt and immediately tested for isolation and identification of *Pseudomonas* species.

Pseudomonas isolation and identification

Under aseptic conditions and using sterile equipment and utensils, 5 grams from the back muscles from each fish sample were mixed in 45 ml of 0.1% of sterile buffered peptone water. The mixture was homogenized by sterile homogenizer to obtain a concentration of 10-1. This mixture stood for 5 minutes at room temperature and then serial dilution was prepared according to APHA (2001). For the isolation of *Pseudomonas* spp., 0.1 ml from each dilution was spread on *Pseudomonas* agar base medium and incubated for 24- 48 hours at both 25°C, and 37°C.

Pseudomonas species identification

Suspected colonies were purified and subcultured onto nutrient agar slopes for 24 hours at 37°C. According to Cheesbrough (2000), the purified colonies were subjected to further identification either morphologically or biochemically. Proteolytic activity of the recovered isolates was examined (Scatamburlo *et al*., 2015). In addition, lipolytic activity of the recovered isolates were also tested (Meghwanshi *et al*., 2006). Serological identification of *P. aeruginosa* was done according to the previous methods (Legakis *et al*., 1982; Glupczynski *et al*., 2010).

Antimicrobial sensitivity testing

Antimicrobial susceptibility testing of the recovered *Pseudomonas* isolates towards the most commonly used antimicrobials in Egypt was done using the disk diffusion method according to Wayne (2013).

Detection of virulence associated genes

At first genomic DNA was extracted according to Sambrook *et al*. (1989). Application of PCR for identification of virulence factors was done according to Sambrook *et al*. (1989) including elastase B (*LasB*), exoenzyme S (*ExoS*), and pili (*PilB*) genes of *Pseudomonas aeruginosa*. The PCR primers and conditions were done following the procedures of Benie *et al*. (2017).

RESULTS AND DISCUSSION

In the current study *Pseudomonas* species was isolated from pagrus, and *Saurus* at with 84%, and 40%, respectively (Fig. 1). Such variation between the two species could be attributed to the differences in the hygienic measures adopted during handling of the two species (Salem *et al*., 2018). Similarly, it was reported that all fish samples collected from Assuit, Egypt were contaminated with *Pseudomonas* spp. (El-Aziz, 2015).

Table 1. Primer sequences for virulence factors of *Pseudomonas aeruginosa*.

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
LasB (F) *LasB* (R)	5′ GGAATGAACGAGGCGTTCTC ′3 5′ GGTCCAGTAGTAGCGGTTGG ′3	300	Inegrave *et al*. (2013)
ExoS (F) *ExoS* (R)	5′ CTTGAAGGGACTCGACAAGG ′3 5′ TTCAGGTCCGCGTAGTGAAT ′3	504	Strateva (2008)
pilB (F) *pilB* (R)	5′ ATGAACGACAGCATCCAACT ′3 5′ GGGTGTTGACGCGAAAGTCGAT ′3	826	

Application of PCR for identification of virulence factors including elastase B (*LasB*), exoenzyme S (*ExoS*) and pili (*PilB*) genes of *Pseudomonas aeruginosa* were adopted using the following primers.

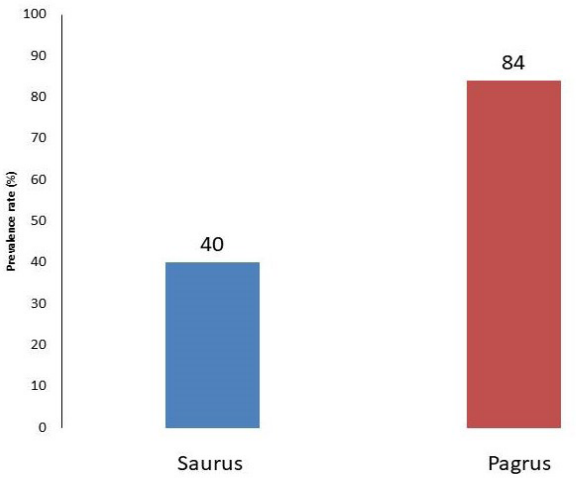

Fig. 1. Prevalence rates of *Pseudomonas* spp., in Pagrus and *Saurus* fish species.

In the present study, the recovered *Pseudomonas* spp., from *Saurus* fish were *P. aeruginosa* (20%), *P. fluorescens* (20%), *P. fragi* (20%), *P. Cepacia* (20%), *P. alcaligenes* (10%) and *P. lundensis* (10%). In pagrus fish, the recovered *Pseudomonas* spp. were *P. aeruginosa* (31.3%), *P. fluerescens* (56.3%), *P. fragi* (6.2%), and *P. stutzeri* (6.2%). In agreement with the obtained results of the present study, *P. aeruginosa* was isolated at a 31.57% from 90 fish samples in Egypt (Algammal *et al*., 2020). In addition, it was reported that the prevalence rates of *P. aeruginosa* in the fresh and smoked fish were 33.1%, and 20% (Benie *et al*., 2017). *P. aeruginosa* and *P. stutzeri* had been also isolated from the northeast of the Mediterranean region (Matyar *et al*., 2010; Ture *et al*., 2018).

The proteolytic and lipolytic activities of the identified *Pseudomonas* types were screened (Table 2). The obtained results showed that *P. Cepacia*, P. aeroginosa, *P. stutzeri*, P. lundeensis were negative for proteolytic and lipolytic activities. While *P. fluorescens* was positive for such activities. *P. fragi* was positive for the proteolytic activity and negative for lipolytic activity. It was noted that the genus *Pseudomonas* includes psychrotrophic species able to produce lipolytic and proteolytic enzymes. In particular, *P. fluerescens*, *P. putida*, *P. fragi*, stood out, and less frequently *P. aeruginosa* (Sørhaug and Stepaniak, 1997; Munsch-Alatossava and Alatossava, 2006).

Table 2. Identification of *Pseudomonas* species.

Key No	Identified bacterium	Proteolytic Activity	Lipolytic Activity
1	*Pseudomonas cepacia*	-------	-------
2	*Pseudomonas cepacia*	-------	-------
3	*Pseudomonas aeruginosa*	-------	-------
4	*Pseudomonas fluorescens*	+ve	+ve
5	*Pseudomonas fragi*	+ve	-------
6	*Pseudomonas fluorescens*	+ve	+ve
7	*Pseudomonas fluorescens*	+ve	+ve
8	*Pseudomonas fluorescens*	+ve	+ve
9	*Pseudomonas stutzeri*	-------	-------
10	*Pseudomonas fluorescens*	+ve	+ve
11	*Pseudomonas aeruginosa*	-------	-------
12	*Pseudomonas aeruginosa*	-------	-------
13	*Pseudomonas fragi*	+ve	-------
14	*Pseudomonas aeruginosa*	-------	-------
15	*Pseudomonas fluorescens*	+ve	+ve
16	*Pseudomonas aeruginosa*	-------	-------
17	*Pseudomonas fluorescens*	+ve	+ve
18	*Pseudomonas fluorescens*	+ve	+ve
19	*Pseudomonas aeruginosa*	-------	-------
20	*Pseudomonas alcaligenes*	+ve	+ve
21	*Pseudomonas fluorescens*	+ve	+ve
22	*Pseudomonas fragi*	+ve	-------
23	*Pseudomonas fluorescens*	+ve	+ve
24	*Pseudomonas aeruginosa*	-------	-------
25	*Pseudomonas fluorescens*	+ve	+ve

Table 3. Serological Identification of *Pseudomonas aeruginosa*.

Key No.	Identified bacterium	Serotype	Group
3	*Pseudomonas aeruginosa*	O5	B
11	*Pseudomonas aeruginosa*	O11	E
12	*Pseudomonas aeruginosa*	O11	E
14	*Pseudomonas aeruginosa*	O8	C
16	*Pseudomonas aeruginosa*	O1	I
19	*Pseudomonas aeruginosa*	O6	G
24	*Pseudomonas aeruginosa*	O11	E

Serotyping of the recovered *Pseudomonas* spp., was presented in Table 3. The obtained results revealed identification of *P. aeruginosa* serotype O11 which belongs to group E at 42%, serotype O1which belongs to group I at 14.3%, serotype O5 in group B is at 14.3%, Serotype O8 in group C at 14.3%, serotype O6 in group G at 14.3%. Similarly, *P. aeruginosa* was isolated from fresh fish and classified serotypes with prevalence O11 with (25.5%), O5 with (21.1%), O8 with (3.9%), O1with (2.9%) (Benie *et al*., 2017).

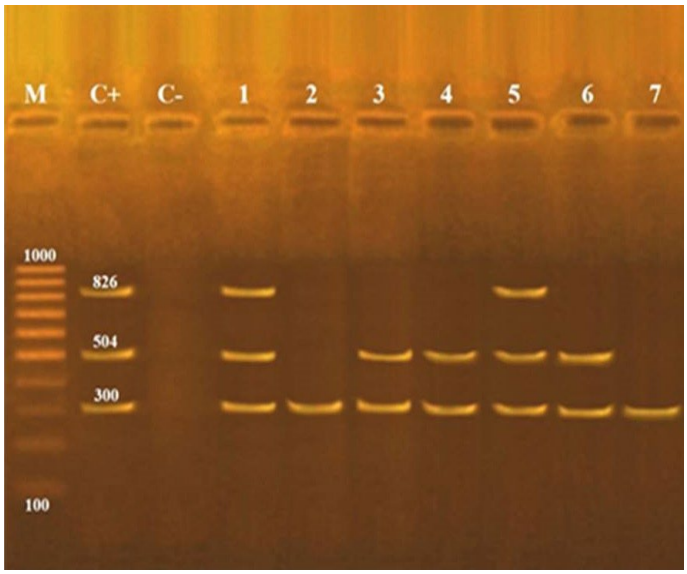

Fig. 2. Agarose gel electrophoresis of multiplex PCR of *LasB* (300 bp), *ExoS* (504 bp) and *pilB* (826 bp); virulence genes of *Pseudomonas aeruginosa*
Lane M: 100 bp ladder as molecular size DNA marker. Lane C+: Control positive strain for *LasB*, *ExoS* and *pilB* genes. Lane C-: Control negative. Lanes 1 and 5: Positive strains for *LasB*, *ExoS* and *pilB* genes. Lanes 3, 4 and 6: Positive strains for *LasB* and *ExoS* genes. Lanes 2 and 7: Positive strain for *LasB* gene.

Antimicrobials are commonly used in the intensive fish farming, beside several dead birds are discarded to major water streams in Egypt (Alsayeqh *et al*., 2021). This study was extended to investigate antimicrobial susceptibility of the recovered *Pseudomonas* species. The obtained results demonstrated that the recovered *Pseudomonas* isolates were highly resistant to erythromycin (100%), amoxicillin, oxacillin (88.5% for both), ampicillin (65.4%), gentamicin (50%), amikacin (42.3%), tetracycline, nalidixic acid (34.6% for both), clindamycin (30.8%), kanamycin (23.1%), and ciprofloxacin (19.2%). while the recovered isolates were highly sensitive to meropenem (92.3%), cefepime (92.3%), cefazoline (84,6%), imipenem (80,8%), and Sulphamethoxazole (76.9%) (Table 4). Similarly, it was recorded that *Pseudomonas* spp., resist amoxicillin, oxacillin and ampicillin (Abd El Tawab *et al*., 2016). Unlikely, macrolides (erythromycin) had inhibitory effects on *P. aeruginosa* that causes chronic lung diseases, such diffuse panbronchiolitis and cystic fibrosis (Navon-Venezia *et al*., 2005). *Pseudomonas* spp., have resistance twas reported to be resistant to penicillins (amoxicillin and ampicillin) (Pirnay *et al*., 2005).

In this study, *Pseudomonas fluerescens* had MAR (1, 0.75, 0.62, 0.56, 0.37, 0.31, 0.25, 0.18, and 0.06), *P. aeruginosa* had MAR values (0.93, 0.87, 0.5, 0.37, 0.25, and 0.18) (Table 5). This result was in agreement with Odjadjare *et al*. (2012) who recorded MAR values for *P. fragi* at (0.68, 0.25, and 0.06), and *P. Cepacia* (0.56, and 0.18).

The recovered *P. aeruginosa* isolates from both Pagrus and *Saurus* contained virulence genes; las B, *ExoS*, and *pilB* with an incidence percentage of (100%), (71.4%), and (28.6%) (Table 6). It was noted *exoS* has a great ability to cause mutations. The presence of *exoS* is found in 58% -72% of the isolates and is typically associated with an invasive phenotype (Hauser, 2009). It was reported that four effective protein secreted by type III secretion pathway recognized in *P. aeruginosa*; exoenzyme S, exoenzyme Y, exoenzyme U and exoenzyme T (Engel and Balachandran, 2009). *ExoS* translocated directly into by the contact-dependent type III secretory process, bacteria translocate *exoS* into epithelial cells of the host leading to general inactivation of function of cells, exoproducts destruct lung tissues (Nicas *et al*., 1985). On contrast the absence of all genes encoding the T3SS toxins (*exoS*, *exoT*, *exoY*, and *exoU*) was recorded (Roy *et al*., 2010). Another virulence gene is *lasB* (elastases) pseudolysin which was considered as a protease (Kuang *et al*., 2011). Likely, *P. aeruginosa* was isolated from fish and its virulence genes (*exoS*, and *lasB*) were detected (Shahrokhi *et al*., 2022). The *pilB* (pili gene) that is responsible for

Table 4. Antimicrobial susceptibility of *Pseudomonas* species (n=26).

Antimicrobial agent	S		I		R	
	NO	%	NO	%	NO	%
Erythromycin (E)	-	-	-	-	26	100
Amoxicillin (AMX)	-		3	11.5	23	88.5
Oxacillin (OX)	2	7.7	1	3.8	23	88.5
Ampicillin (AM)	5	19.2	4	15.4	17	65.4
Gentamicin (G)	10	38.5	3	11.5	13	50
Amikacin (AK)	14	53.8	1	3.8	11	42.3
Tetracycline (T)	15	57.7	2	7.7	9	34.6
Nalidixic acid (NA)	15	57.7	2	7.7	9	34.6
Clindamycin (CL)	18	69.2	-	-	8	30.8
Kanamycin (K)	19	73.1	1	3.8	6	23.1
Ciprofloxacin (CP)	18	69.2	3	11.5	5	19.2
Sulphamethoxazol (SXT)	20	76.9	2	7.7	4	15.4
Imipenem (IPM)	21	80.8	2	7.7	3	11.5
Cefazolin (CZ)	22	84.6	1	3.8	3	11.5
Cefepime (FEP)	24	92.3	-	-	2	7.7
Meropenem (M)	24	92.3	1	3.8	1	3.8

Table 5. Antimicrobial resistance of *Pseudomonas* species

NO	Key	*Pseudomonas* Spp.	Antimicrobial resistance profile	MAR index
1	15	*P. fluorescens*	E, AMX, OX, AM, G, AK, T, NA, CL, K, CP, SXT, IPM, CZ, FEP, M	1
2	7	*P. fluorescens*	E, AMX, OX, AM, G, AK,T, NA, CL, K, CP, SXT	0.75
3	26	*P. fluorescens*	E, AMX, OX, AM, G, AK, T, NA, CL, K	0.63
4	10	*P. fluorescens*	E, AMX, OX, AM, G, AK, T, NA, CL	0.56
5	24	*P. fluorescens*	E, AMX, OX, AM, G, AK	0.38
6	2	*P. fluorescens*	E, AMX, OX, AM, G	0.31
7	4	*P. fluorescens*	E, AMX, OX, AM, G	0.31
8	13	*P. fluorescens*	E, AMX, OX, AM	0.25
9	22	*P. fluorescens*	E, AMX, OX, AM	0.25
10	25	*P. fluorescens*	E, AMX, OX	0.19
11	14	*P. fluorescens*	E	0.06
12	23	*P. aeruginosa*	E, AMX, OX, AM, G, AK, T, NA, CL, K, CP, SXT, IPM, CZ, FEP	0.94
13	18	*P. aeruginosa*	E, AMX, OX, AM, G, AK, T, NA, CL, K, CP, SXT, IPM, CZ	0.88
14	1	*P. aeruginosa*	E, AMX, OX, AM, G, AK, T, NA	0.5
15	8	*P. aeruginosa*	E, AMX, OX, AM, G, AK	0.38
16	21	*P. aeruginosa*	E, AMX, OX, AM	0.25
17	6	*P. aeruginosa*	E, AMX, OX	0.19
18	20	*P. aeruginosa*	E, AMX, OX	0.19
19	12	*P. fragi*	E, AMX, OX, AM, G, AK, T, NA, CL, K, CP	0.69
20	17	*P. fragi*	E, AMX, OX, AM	0.25
21	5	*P. fragi*	E	0.06
22	16	*P. Cepacia*	E, AMX, OX, AM, G, AK, T, NA, CL	0.56
23	19	*P. Cepacia*	E, AMX, OX	0.19
24	11	*P. lundensis*	E, AMX, OX	0.19
25	3	*P. stutzeri*	E, AMX, OX	0.19
26	9	*P. alcaligenes*	E	0.06
			Average 0.391	

Multiple Antibiotic Resistance (MAR) index for each strain was determined according to the formula stipulated by (Singh *et al*., 2010) as follow: MAR index= No. of resistance (Isolates classified as intermediate were considered sensitive for MAR index) / Total No. of tested antibiotics.
E: Erythromycin; AMX: Amoxicillin; OX: Oxacillin; AM: Ampicillin; G: Gentamicin; AK: Amikacin; T: tetracycline: NA: Nalidixic acid; CL: Clindamycin; L: Levofloxacin; CP: Ciprofloxacin; SXT: Sulphamethoxazol; IMP: Imipenem; CZ: Cefazolin; EP: Cefepime; M: Meropenem

Table 6. Incidence of virulence factors of *Pseudomonas aeruginosa* isolated from the examined samples of (n= 7 strains).

Virulence genes	No. of examined strains	No	%
LasB (*Elastase B gene)*	10	7	100
ExoS (Exoenzyme S gene)		5	71.4
pilB (Pili gene)		2	28.6

colonization of *P. aeruginosa* to specific receptors of the epithelium of the injured tissues (Todar, 2009). *P. aeruginosa* was also isolated from fresh fish and its virulence genes were detected, *lasB*, *exoS*, and *pilB* at 71.4%, 67.3%, and 38.8% (Benie *et al*., 2016).

CONCLUSION

The obtained results of the present study revealed that Pagrus and *Saurus* fish species are regarded as potential sources of *Pseudomonas* spp. In particular, multidrug resistant *P. aeruginosa* harboring virulence associated genes was isolated from the two fish species at variable rates. Therefore, strict hygienic measures should be adopted during all steps of handling of the fish starting from catching till release to the consumers

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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