



# The phenotypic and genetic characteristics of *Pseudomonas anguilliseptica* strains associated with mortalities in farmed sea bream and sea bass

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## Abstract

To investigate the presence of *Pseudomonas anguilliseptica*, one hundred specimens of sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) were collected alongside water samples during episodes of widespread fish mortality in marine fish farms located in the northern region of Egypt. This study documented the clinical and postmortem manifestations observed in moribund sea bream and sea bass, thus providing evidence for the occurrence of a septicemic-hemorrhagic bacterial disease. Fourteen strains of *P. anguilliseptica* were isolated and characterized from both sea bream and sea bass specimens. The conventional bacteriology methods were employed to retrieve the causative bacterial agent and subsequently evaluate its phenotypic traits. Moreover, sequencing of the 16 S rRNA was conducted to characterize the identified microorganism. Furthermore, multilocus sequence analysis (MLSA) was employed to confirm the identity of *P. anguilliseptica* and elucidate the phylogenetic interrelationship among various strains. Regardless of their source or the fish species from which the strains were obtained, these isolates showed a high level of phenotypic homogeneity. MLSA displayed a genetic homogeneity among isolates despite their different geographic origins. Antibiogram revealed the sensitivity of some *P. anguilliseptica* strains for antibiotics (florfenicol, trimethoprim, sulfamethoxazole, cefotaxime, and ciprofloxacin). Isolated strains were harboring some antibiotic-resistant genes, with the most prevalent being *tetA* gene, followed by *ermB* gene. Water physico-chemical parameters (low temperature and salinity fluctuation) were convenient for bacterial growth. In addition, *P. anguilliseptica* strains could resist several antibiotics and harbored antibiotic-resistant genes, resulting in difficulties in fish treatment.

**Keywords** *Sparus aurata* · *Dicentrarchus labrax* · *Pseudomonas anguilliseptica* · Antibiotic-resistant gene · Ammonia · Antibiogram

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## Introduction

*Pseudomonas* species, a group of gram-negative bacteria, are widely distributed in aquatic environments and are known to be associated with stress-related diseases in farmed fish, particularly in intensive culture systems. These bacteria have been identified as the primary cause of fish diseases, leading to significant economic losses, with reported mortality rates reaching 100% in valuable fish species such as rainbow trout (*Oncorhynchus mykiss*), sea bream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*), and ayu (*Plecoglossus altivelis*) (López et al. 2012; Derome et al. 2016). The most commonly isolated species include *Pseudomonas luteola*, *P. anguilliseptica*, *P. baetica*, *P. fluorescens*, *P. aeruginosa*, *P. koreensis*, *P. plecoglossicida*, *P. pseudoalcaligenes*, *P. putida*, and *P. chlororaphis* (Austin and Austin 2016; Sherif et al. 2021).

*P. anguilliseptica*, a bacterium commonly found in aquatic environments, is characterized as a Gram-negative microorganism. It exhibits fastidious characteristics, displaying slow growth and weak reactions when cultured on bacterial media commonly employed in microbiology laboratories (Daly 1999; López-Romalde et al. 2003). *P. anguilliseptica* is an opportunistic microorganism responsible for causing a disease commonly referred to as red spot disease or “sekiten-byo” in Japan. This pathogen was initially documented in pond-farmed Japanese eel (*Anguilla japonica*) (Wakabayashi and Egusa 1972). Additionally, it is considered a prevalent infectious pathogen among farmed marine and brackish water fishes (Daly 1999). Outbreaks of *P. anguilliseptica* in farmed gilt-head seabream have been reported in several Mediterranean countries, including France, Portugal, and Spain. These outbreaks have resulted in a disease known as hemorrhagic septicemia, associated with high mortality rates and significant financial losses for the aquaculture industry (Doménech et al. 1999; Romalde et al. 2001; Ali et al. 2022). Similar occurrences have also been reported in other fish species such as turbot (*Scophthalmus maximus*), black sea bream (*Acanthopagrus schlegeli*), European eel (*Anguilla anguilla*), ayu (*P. altivelis*), sea trout (*Salmo trutta*), rainbow trout (*O. mykiss*), Atlantic salmon (*Salmo salar*), striped jack (*Pseudocaranx dentex*), whitefish (*Coregonus* sp.), Baltic herring (*Clupea harengus membras*), and orange-spotted grouper (*Epinephelus coioides*) (Al-Marzouk 1999; Austin and Austin 1999; Daly 1999; López-Romalde et al. 2002).

Identifying these bacterial species using microbiological analyses is challenging due to slow growth, low metabolic activity, and variability observed in their morphology and biochemical characteristics among different isolates. Consequently, molecular techniques accurately identify and distinguish *P. anguilliseptica* from other closely related genera (Mulet et al. 2012; Palleroni 2015). These techniques include 16 S rRNA sequencing for genus identification and multilocus sequence analysis (MLSA) for species classification (Mulet et al. 2012; Gomila et al. 2015; Abdelsalam et al. 2022). The multilocus sequence analysis (MLSA) technique was developed to assess molecular variations in multiple protein-coding housekeeping genes to compare closely related bacterial species (Mjølnerød et al. 2021). Multiple species of bacteria were subjected to standardization using MLSA, and the genetic variations derived from conserved housekeeping genes were revealed in the phylogenetic relationship. The MLSA technique is the most reliable method for studying the epidemiology, geographical distribution, and characterization of *Pseudomonas* spp. It can distinguish between different pathogenic strains of this pathogen (Sánchez et al. 2014; Gomila et al. 2015). The MLSA technique, which involves partial sequencing of four housekeeping genes (*rpoB*, *rpoD*, *gyrB*, and *aroE*), effectively identifies *Pseudomonas* spp. This technique has provided valuable insights into the phylogenetic relationships among different *Pseudomonas* strains and enabled discrimination between them (Mulet et al. 2012; Gomila et al. 2015).

Recently, antibiotic-resistant bacteria were frequently isolated from aquatic animals (Naik et al. 2018), aquatic water, and sediment (Pham et al. 2018; Shah et al. 2012). In Egypt, aquatic farmers and paramedics routinely use different antibiotics without a veterinary consultant during disease outbreaks, resulting in emerging antibiotic-resistant bacteria (WHO 2006; Sherif et al. 2021, 2022).

Over the past year, multiple reports of fish mortalities have occurred in farms that specifically reared seabass and seabream.

The objective of the current study was to conduct phenotypic and genetic characterizations of *P. anguilliseptica* strains isolated from moribund seabream and sea bass. Furthermore, the study aimed to investigate the weak response to antibiotic treatment. In addition, the quality of fish farm water associated with this particular bacterium was examined.

## Materials and methods

### Fish farms and samples collection

Specimens of moribund sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) were obtained from a collective of four fish farms located in the governorates of Dameitta (comprising three farms) and Kafrelsheikh (comprising one farm). These particular fish farms encountered high mortality rates during the winter season in November 2022. One hundred moribund fish were concurrently collected from affected fish ponds with water samples in triplicates, as described by Sherif et al. (2023) and Eldessouki et al. (2023). Water samples were collected at a depth of 0.5 m using aseptic techniques to guarantee the absence of contamination in the collected samples. The investigation was conducted to determine the presence of bacteria in fish samples. Bacterial cultures were obtained from the internal organs, specifically the kidney, spleen, and hepatopancreas tissues, using the protocols described by Woo and Bruno (2014) and Sherif and Kassab (2023).

### Bacterial analyses

#### Bacterial isolation

Pooled specimens of livers, kidneys, and spleens, each fish species per farm considered one sample, were streaked on various agar mediums. Subsequently, these samples were incubated at 25 °C for 24 to 72 h (Wakabayashi and Egusa 1972). The agar media used consisted of tryptic soy agar (TSA) supplemented with 3% sodium chloride and 5% sheep blood agar. The prevailed colonies were selected and purified by subculturing onto TSA under the same conditions. Isolates were confirmed by selective isolation on Salmonella-Shigella agar SS agar. Gram staining, oxidase test, catalase activity, lactose fermentation on MacConkey agar, and motility testing using motility mediums were performed for the preliminary identification of bacterial isolates. The stock cultures were maintained at -70 °C in tryptic soy broth (Difco Laboratories), supplemented with 1% sodium chloride and 15% glycerol. The biochemical analyses were conducted in triplicate using API20 E following the guidelines provided by the manufacturer (BioMerieux, Marcy l' Etoile, France) and the recommendations presented by Madigan and Martinko (2005). In this work, the biosafety protocols adhered to the Pathogen Safety Data Sheet for Infectious Substances-*P. Anguilliseptica*, as the Pathogen Regulation Directorate of the Public Health Agency of Canada (2022) outlines.

## Molecular identification

**DNA extraction** Genomic DNA from the pure 14 isolates of *Pseudomonas* spp. was extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany, Cat. no. 513) following QIAGEN's recommended protocol. The extracted DNA was eluted using the kit's 100 µl elution buffer. The concentration and integrity of DNA were determined using the Nanodrop spectrophotometer following the manufacturer guidelines at 260/280 nm (ND-1000 UV-VIS, Thermo Scientific, USA). The extracted DNA was stored at  $-20^{\circ}\text{C}$  for subsequent PCR amplifications and sequencing.

**PCR amplifications** The identity of *Pseudomonas* spp. isolates was initially confirmed by amplifying and sequencing the 16 S rRNA gene using the universal primer pair 9 F and 1541R (Table 1), following the established protocol described by Osborne et al. (2005). For more in-depth phylogenetic analysis, four housekeeping genes—*gyrB* (703 bp), *rpoB* (529 bp), *rpoD* (766 bp), and *aroE* (653 bp)—were individually PCR amplified from each *Pseudomonas* isolate using multilocus sequence typing (MLST). The MLST primers were adopted from Mjølnerød et al. (2021). Additionally, bacterial strains were screened for resistance to tetracycline, erythromycin, sulfonamide, and quinolone by detecting the presence of resistance genes *tetA*, *qnrS*, *sulI*, and *ermB*, respectively, using PCR as described by Randall et al. (2004). All primers used in this study are documented in Table 1.

In brief, PCR amplifications were conducted in 25 µl total reaction volumes containing 4 µl DNA template (50 ng), 12.5 µl MyTaq Red Mix (Bioline), 1 µl of each primer (20 pmol), and 6.5 µl nuclease-free water. Each gene (*16 S rRNA*, *gyrB*, *rpoB*, *rpoD*, *aroE*, *sulI*, *tetA*, *qnrS*, and *ermB*) was individually amplified from every *Pseudomonas* isolate. Thermocycling conditions began with initial denaturation at  $95^{\circ}\text{C}$  for 4 min followed by 35 cycles of denaturation at  $95^{\circ}\text{C}$  for 1 min, annealing for 1 min, and extension at  $72^{\circ}\text{C}$  for 1 min. Annealing temperatures were adjusted based on the target gene, specifically  $52^{\circ}\text{C}$  for *16 S rRNA*,  $56^{\circ}\text{C}$  for the four MLST genes *gyrB*, *rpoB*, *rpoD*, and *aroE*,  $55^{\circ}\text{C}$  for *tetA*,  $68^{\circ}\text{C}$  for *sulI*,  $53^{\circ}\text{C}$  for *qnrS*, and  $62^{\circ}\text{C}$  for *ermB*. A final extension step at  $72^{\circ}\text{C}$  for 7 min completed the PCR. Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) per the manufacturer's instructions. Purified products were Sanger sequenced in both forward and reverse directions using the ABI 3730xl DNA Analyzer (Applied Biosystems). Raw sequence reads were trimmed, edited, and assembled into contigs using BioEdit version 7.2.5 (Hall 1999).

For species identification, the assembled 16 S rRNA sequences were aligned against other 16 S rRNA sequences of *Pseudomonas* spp. using the online BLAST tool of NCBI. The obtained 14 sequences were deposited in the database of GenBank under the following accession numbers starting from ON926995 to ON927008 (Tables 2 and 3). The identity of *Pseudomonas* isolates was confirmed at the species level when the BLAST similarity reached  $\geq 99\%$  to the relevant 16 S rRNA sequences available in the GenBank database, as illustrated by Drancourt et al. (2000). The multiple sequence alignments were carried out using the MEGA program to match the interspecies and intrastain similarities index (Kumar et al. 2018). The neighbor-joining phylogenetic analysis of the current 16 S rRNA sequences was performed against 14 typing strains including, *P. anguilliseptica* (3 isolates), *P. resinovorans* (1 isolate), *P. aeruginosa* (1 isolate), *P. asplenii* (1 isolate), *P. monteilii* (1 isolate), *P. tolaasii* (1 isolate), *P. fluorescens* (2 isolates), *P. chlororaphis* (2 isolates), and *P. putida* (2 isolates).

**Table 1** The primers used in this study

Name of gene	Sequencing of primers	Annealing temp (°C)	Reference
<i>16 S rRNA</i>	9 F: 5'-GAGTTGATCMITGGCTCAG-3' 1541 R: 5'-GGYTACCTTGTACGACTT -3'	58	Osborne et al. (2005)
DNA gyrase beta subunit ( <i>gyrB</i> )	329 F: 5'-ACAGCTACAAGTTTCCGGC-3' 1089 R: 5'-CTTGGCCCAITTCCTGCTCGA-3'	56	Mjølnertødt et al. (2021)
RNA polymerase beta subunit ( <i>rpoB</i> )	3307 F: 5'-TGTGGTCTCGGTGATCATGC-3' 3878 R: 5'-GAACCTGGCCCTTACCACCC-3'	56	
RNA polymerase, sigma factor ( <i>rpoD</i> )	294 F: 5'-GACCCAGTGGCATGTACAT-3' 1204 R: 5'-ATGCCGACGGTTGATGTCCIT-3'	56	
Shikimate dehydrogenase ( <i>aroE</i> )	42 F: 5'-CAAAGTCGCCGCTGATTCATC-3' 761 R: 5'-GTTTCGACACGATGCCAG-3'	56	
Sulphonamide ( <i>SulI</i> )	F: CGG CGT GGG CTA CCT GAA CG R: GCC GAT CGC GTG AAG TTC CG	68	
Tetracycline ( <i>TetA</i> )	F: GCT ACA TCC TGC TTG CCT TC R: CAT AGA TCG CCG TGA AGA GG	55	
Quinolone ( <i>QnrS</i> )	F: ACG ACA TTC GTC AAC TGC AA R: TAA ATT GGC ACC CTG TAG GC	53	Robicsek et al. (2006)
Erythromycin ( <i>ermB</i> )	F: TGG TAT TCC AAA TGC GTA ATG R: CTG TGG TAT GGC GGG TAA GT	62	

**Table 2** Bacterial growth in different incubation temperatures and salinities

No.	Bacteria strain	Incubation temperature (°C)				Salinity (g/L)			
		8	25	37	42	0	1	3	5
1	NDA0201	+	+	-	-	-	W	+	+
2	NDA0202	+	+	-	-	-	W	+	+
3	NDA0203	+	+	-	-	-	W	+	+
4	NDA0204	+	+	-	-	-	W	+	+
5	NDA0205	+	+	-	-	-	W	+	+
6	NDA0206	+	+	-	-	-	W	+	+
7	NDA0207	+	+	-	-	-	W	+	+
8	NDA0208	w	+	-	-	-	W	+	+
9	NDA0209	w	+	-	-	-	W	+	+
10	NKF0301	w	+	-	-	-	-	+	W
11	NKF0302	w	+	-	-	-	-	+	W
12	NKF0303	w	+	-	-	-	-	+	W
13	NKF0304	w	+	-	-	-	-	+	W
14	NKF0305	w	+	-	-	-	-	+	W

-, negative; +, positive; w, weak

**Multilocus sequence analysis** The raw sequences of *gyrB*, *rpoB*, *rpoD*, and *aroE* genes were trimmed and edited using BioEdit software ver. 7.2.5 (Hall 1999). The assembled sequences were submitted to the GenBank database under the following accession numbers: *gyrB* (OQ694532- OQ694545), *rpoB* (OQ726020- OQ726033), *rpoD* (ON981406- ON981419), and *aroE* (OQ744508- OQ744521). The final sequences were imported and concatenated in Microsoft Excel. The phylogenetic analysis of the concatenated sequences of housekeeping genes was constructed by neighbor-joining methods of the MEGA 11 with the pairwise deletion option (Kumar et al. 2018). The node supports were estimated using nonparametric bootstrapping with 1000 replicates and Kimura's two-parameter model was applied.

### Antibiogram of *Pseudomonas anguilliseptica* strains

In line with an earlier study (CLSI 2010) and using the disc diffusion method, bacterial strains were examined for their sensitivity to some antibiotics on Mueller-Hinton agar (Oxoid™). The antimicrobial agents utilized in the assessment were tetracycline (TetA) 30 µg, ciprofloxacin (Cip) 5 µg, trimethoprim/sulfamethoxazole (SXT) 1.25/23.75 µg, erythromycin (E, 15 µg), florfenicol 30 µg, gentamicin 10 µg, amoxicillin 10 µg, kanamycin 30 µg, cefotaxime 30 µg, ampicillin (Amp) 10 µg, and streptomycin 30 µg. The test was conducted in triplicates, and isolates of *P. anguilliseptica* were subcultured into tryptic soy broth, then incubated overnight at 30 °C, and placed onto Mueller-Hinton agar plates using a cotton swab. Following incubation for 24 h at 30 °C, the diameter of the inhibition zones was measured, and the results were interpreted based on the Clinical Laboratory Standards Institute criteria. The equation determined the multidrug resistance (MDR) index:

$$\text{MDR} = X/Y$$

**Table 3** *Pseudomonas anguilliseptica* isolated in this study

No.	Bacteria	Strain	Host	Location	16 S rRNA	aroE	gyrB	rpoB	rpoD	Reference
1	<i>P. anguilliseptica</i>	NDA0201	Sea bass	Damietta/farm1	ON926995	QQ871614	QQ871628	QQ871642	QQ871656	This study
2	<i>P. anguilliseptica</i>	NDA0202	Sea bream	Damietta/farm1	ON926996	QQ871615	QQ871629	QQ871643	QQ871657	This study
3	<i>P. anguilliseptica</i>	NDA0203	Sea bream	Damietta/farm2	ON926997	QQ871616	QQ871630	QQ871644	QQ871658	This study
4	<i>P. anguilliseptica</i>	NDA0204	Sea bass	Damietta/farm2	ON926998	QQ871617	QQ871631	QQ871645	QQ871659	This study
5	<i>P. anguilliseptica</i>	NDA0205	Sea bream	Damietta/farm2	ON926999	QQ871618	QQ871632	QQ871646	QQ871660	This study
6	<i>P. anguilliseptica</i>	NDA0206	Sea bream	Damietta/farm3	ON927000	QQ871619	QQ871633	QQ871647	QQ871661	This study
7	<i>P. anguilliseptica</i>	NDA0207	Sea bream	Damietta/farm3	ON927001	QQ871620	QQ871634	QQ871648	QQ871662	This study
8	<i>P. anguilliseptica</i>	NDA0208	Sea bass	Damietta/farm3	ON927002	QQ871621	QQ871635	QQ871649	QQ871663	This study
9	<i>P. anguilliseptica</i>	NDA0209	Sea bass	Damietta/farm3	ON927003	QQ871622	QQ871636	QQ871650	QQ871664	This study
10	<i>P. anguilliseptica</i>	NKF0301	Sea bass	KafrElshikh/farm1	ON927004	QQ871623	QQ871637	QQ871651	QQ871665	This study
11	<i>P. anguilliseptica</i>	NKF0302	Sea bass	KafrElshikh/farm1	ON927005	QQ871624	QQ871638	QQ871652	QQ871666	This study
12	<i>P. anguilliseptica</i>	NKF0303	Sea bream	KafrElshikh/farm1	ON927006	QQ871625	QQ871639	QQ871653	QQ871667	This study
13	<i>P. anguilliseptica</i>	NKF0304	Sea bream	KafrElshikh/farm1	ON927007	QQ871626	QQ871640	QQ871654	QQ871668	This study
14	<i>P. anguilliseptica</i>	NKF0305	Sea bream	KafrElshikh/farm1	ON927008	QQ871627	QQ871641	QQ871655	QQ871669	This study
15	<i>P. anguilliseptica</i> *	NCIMB1949	European eel	Japan	AB021376	MW685293	MW685134	MW684975	MW684922	Mjølnerød et al. (2021)

\**P. anguilliseptica*\* (NCIMB1949) European eel Japan is a typing isolate Mjølnerød et al. (2021)

$x$  represents the number of antibiotic categories to which the isolates were resistant, while  $y$  represents the assessment of antibiotics. If the obtained MDR index is greater than 0.2, the bacterial strain is resistant to multiple antibiotics (Krumperman 1983).

## Examination of water physicochemical parameters

The temperature and salinity of water samples at farm sites were analyzed using a YSI Environmental (Model EC300), while dissolved oxygen (DO) was analyzed using an Aqualytic (Model OX24), and pH was analyzed using a Thermo Orion (Model 420 A). The samples were collected in 1-L polyethylene bottles and transported on ice to the laboratory, namely, total ammonia nitrogen (TAN), unionized ammonia ( $\text{NH}_3$ ), nitrite ( $\text{NO}_2$ ), and nitrate ( $\text{NO}_3$ ); a UV/visible spectrophotometer (Thermo-Spectronic 300) was used for ammonia compound analyses following the recommendations of Rice and Bridgewater (2012).

## Statistical examination

Different parameters were assessed using the ANOVA test and Duncan's Multiple Range (Duncan 1955) by determining the mean and standard error of the collected data using SPSS software (SPSS 2004). At  $P \leq 0.05$ , values were considered statistically significant.

## Results

### Clinical signs and post-mortem of farmed sea bream and sea bass infected with *P. anguilliseptica*

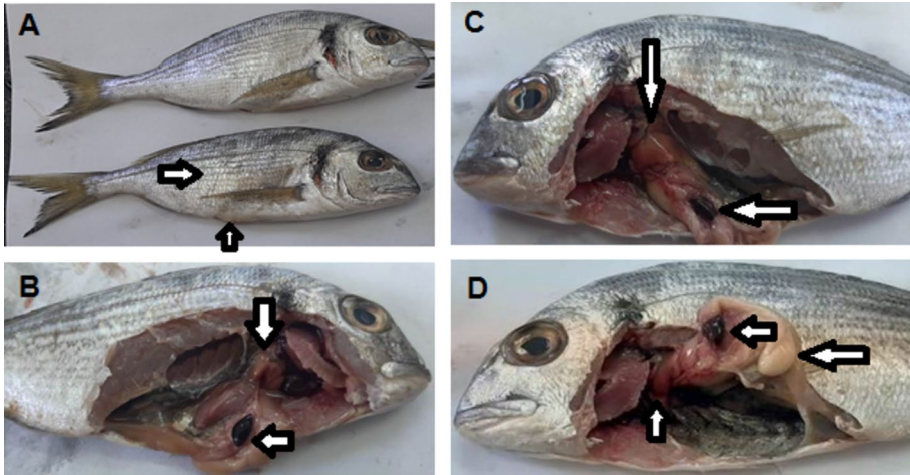
In Fig. 1, the moribund sea bream showed signs of septicemic bacterial infection. These were hemorrhages on the external body surface behind the pectoral fin and abdomen with ulcers under the dorsal fin. Postmortem signs were hemorrhages on the liver surface and splenomegaly (rounded surface).

In Figs. 2 and 3, moribund sea bass showed hemorrhages on the fish skull, eye loss, hemorrhages on the external body surface, and gills with faint red. Post-mortem revealed signs of hemorrhagic diseases: light-brownish liver, hemorrhages on liver and internal surfaces, abdominal fat with splenomegaly, and hemorrhagic-distended kidney.

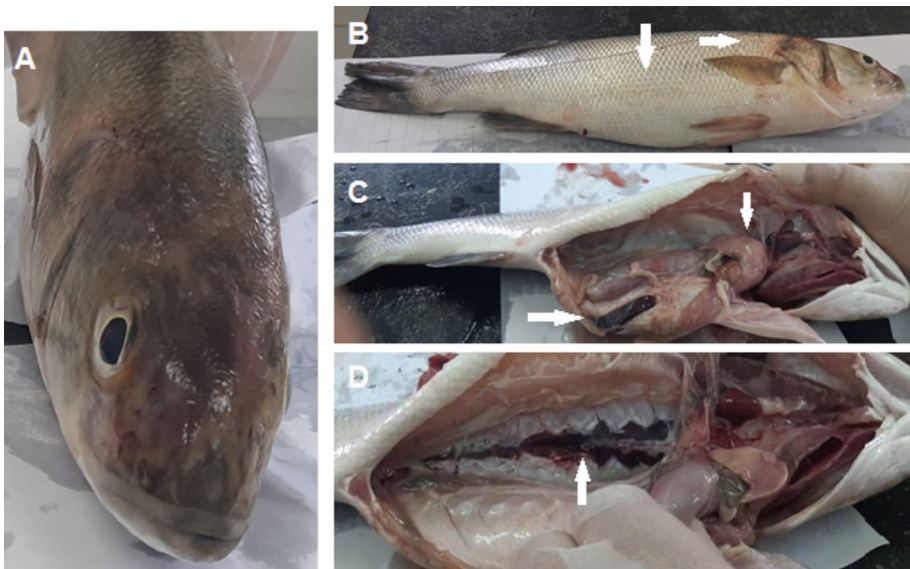
### Bacterial growth on agar plate

The isolated bacteria were negative for gram stain, and by using API 20E, they had the number 220,004, which represented the *Pseudomonas* spp. The bacterial growth was evaluated in different incubation temperatures (8, 25, 37, and 42 °C) and salinity (0, 1, 3, 5 g/L) (Table 2). No growth was detected in all bacterial strains at 37 and 42 °C, while NDA0208 and NDA0209 (both are from Damietta farms) and all bacterial isolates of Kafrelshiekh farms (NKF0301-5) showed weak growth. All *P. anguilliseptica* strains were unable to grow in 0 g/L salinity. Also, all strains isolated from Damietta farms showed weak growth in 1 g/L salinity, while those recovered from Kafrelsheikh did not grow. All strains could grow in 3 and 5 g/L, except those recovered from Kafrelsheikh had weak growth in 5 g/L.





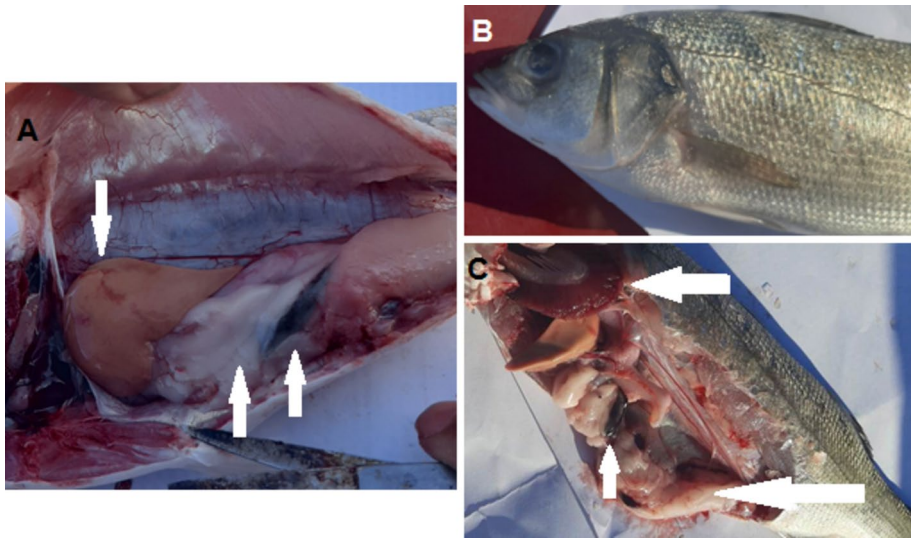
**Fig. 1** Clinical signs and post-mortem of farmed sea bream infected with *P. anguilliseptica*. **A** Arrows pointed to hemorrhages behind the pectoral fin and abdomen with ulcers under dorsal fine, **B** brown-reddish liver and splenomegaly, **C** brown liver with hemorrhages and splenomegaly, **D** the same fish of **C** with clear splenomegaly (rounded) and abdominal fat



**Fig. 2** Clinical signs and post-mortem of farmed sea bass infected with *P. anguilliseptica*. **A** Hemorrhages on fish skull, **B** hemorrhages on abdomen and behind gill cover, **C** light-brownish liver with splenomegaly, and **D** hemorrhagic and distended kidney

### Sequencing of 16 S rRNA and MLST

The amplified 16 S rRNA gene was successfully performed from 14 *Pseudomonas* spp. isolates using the gene-specific primers pair. The purified PCR products were then

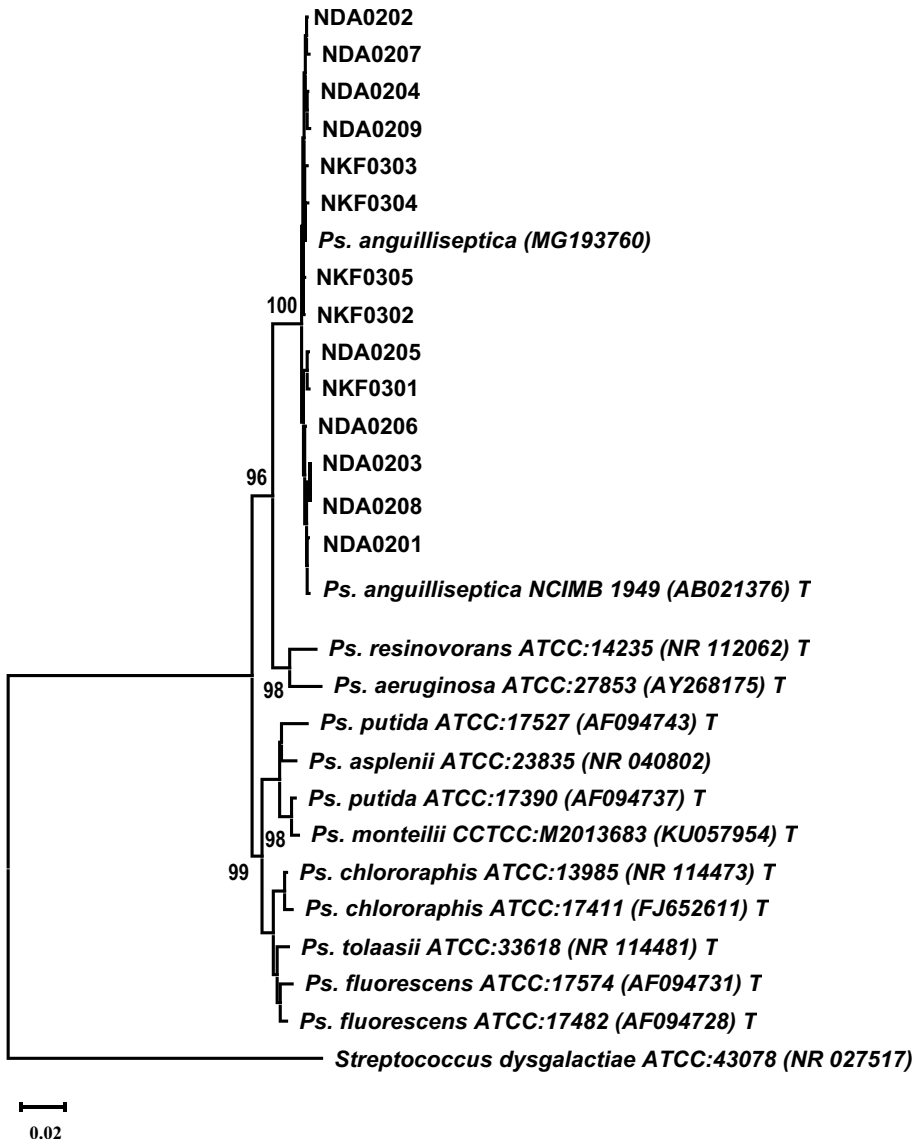


**Fig. 3** Clinical signs and post-mortem of farmed sea bass infected with *P. anguilliseptica*. **A** Arrows pointed to hemorrhages on liver and internal surfaces abdominal fat and splenomegaly, **B** eye lost, **C** gills with faint red color and splenomegaly

sequenced to confirm the identities of *Pseudomonas* spp. at the species level. The successive accession numbers of these sequences (ON926995 to ON927008) were issued by the GenBank staff (Table 3). The BLAST analysis of 16 S rRNA gene sequences proved that all 14 bacterial strains belonged to the genus *Pseudomonas*, and all isolates were identified as *P. anguilliseptica*. The intrastrain nucleotide differences within the isolates of *P. anguilliseptica* varied from 99.80 to 99.39% with three to nine nucleotide differences. The comparative sequence analysis of the current 16 S rRNA sequences (ON926995 to ON927008) revealed a high similarity index ranging between 99.86 and 99.39% with relevant sequences of *P. anguilliseptica* (AB021376<sup>T</sup>, LC071931, LC194236, LC071928, LC071927, and LC071926). The alignment of these accession numbers (ON926995 to ON927008) showed 96.73–95.45% 16 S rRNA similarity to typing isolates of the following *Pseudomonas* spp.: *P. resinovorans* (NR\_112062)<sup>T</sup>, *P. aeruginosa* (AY268175)<sup>T</sup>, *P. asplenii* (NR\_040802)<sup>T</sup>, *P. monteilii* (KU057954)<sup>T</sup>, *P. tolaasii* (NR\_114481)<sup>T</sup>, *P. fluorescens* (AF094731)<sup>T</sup>, *P. chlororaphis* (FJ652608)<sup>T</sup>, and *P. putida* (AF094743)<sup>T</sup>.

In Fig. 4, the phylogenetic analysis of the 16 S rRNA sequences of *P. anguilliseptica* isolates formed two major clades. The first clade was separated into two subclades. The first subclade involved all *P. anguilliseptica*, grouped with the sequence of typing *P. anguilliseptica* (NCIMB 1949) with a substantial 100% bootstrap value. In this study, all isolates of *P. anguilliseptica* were separated from other *Pseudomonas* spp. belonging to *P. resinovorans*, *P. aeruginosa*, *P. asplenii*, *P. monteilii*, *P. tolaasii*, *P. fluorescens*, *P. chlororaphis*, and *P. putida*. The typing strain of *S. dysgalactiae* (ATCC 43,078) served as an outgroup.

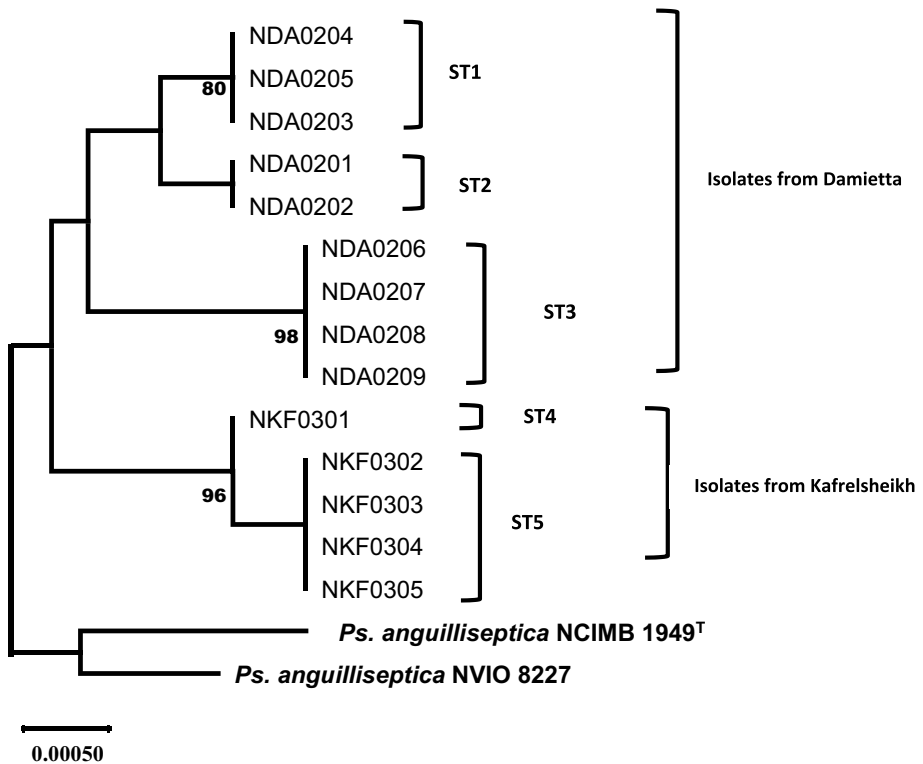
In Table 3, MLSA allowed unambiguous identification of all recovered *Pseudomonas* spp. isolates as *P. anguilliseptica* (14 isolates). All isolates shared 99.62–99.70% concatenated sequence similarity to typing *P. anguilliseptica* (NCIMB 1949) strain. In addition, the alignment of concatenated sequences of these isolates showed high discriminatory power between *P. anguilliseptica* of this study and typical isolates of *P. chlororaphis* *subsp.*



**Fig. 4** The neighbor-joining phylogenetic tree based on the comparative analysis of the 16 S rRNA gene sequences, demonstrating the relationship between *P. anguilliseptica* isolates in this study and closely related *Pseudomonas* spp.

*piscium* ATCC 17,809 (CP027709.1)<sup>T</sup>, *P. fluorescens* ATCC 13,525 (QVNA00000000.1)<sup>T</sup>, *P. aeruginosa* ATCC 27,853 (CP015117.1)<sup>T</sup>, and *P. putida* ATCC 12,633 (CP101910.1)<sup>T</sup>, with similarity index ranging between 80.92 and 79.54%. On the other hand, the comparative sequence alignment of four concatenated housekeeping genes (2363 bp) demonstrated similarity scores ranging between 99.75 and 100% among all retrieved isolates.

In Fig. 5, the phylogenetic tree derived from MLSA based on concatenated sequences of 14 isolates displayed five distinct sequence types (ST1, ST2, ST3, ST4, and ST5)



**Fig. 5** Phylogenetic analysis of MLSA of fourteen *P. anguilliseptica* isolates based on the concatenated *gyrB*, *recA*, *rpoB*, *rpoD*, and *aroE* gene sequences

(Fig. 2). The phylogeny tree of MLSA revealed that all *P. anguilliseptica* isolates belonged to three main clusters. Clustering was correlated with the geographical source of the strains. Clusters 1 and 2 were associated with isolates originating from Damietta farms, while cluster 3 was related to isolates recovered from Kafrelsheikh farms. The first clade grouped ST1 and ST2, and the node supported with a bootstrap value of 73%. The second clade gathered ST3, with a bootstrap value of 98%. The third clade grouped ST4 and ST5 and supported with a bootstrap value of 96%.

### Antibiotic resistance genes and antibiogram of *P. anguilliseptica* strains

Concerning the prevalence of ARG, tetracycline (*tetA*) was the most predominant, followed by erythromycin (*ermB*), while sulfonamide (*sulI*) and quinolone (*qnrS*) did not present in those isolated at Kafrelsheikh farm (Tables 4 and 5).

In Table 4, *P. anguilliseptica* strains were tested against 11 antibiotic types. All *P. anguilliseptica* strains were resistant to the selected antibiotics tetracycline, amoxicillin, ampicillin, and kanamycin, whereas they were sensitive to gentamycin, florfenicol, and trimethoprim plus sulfamethoxazole. Antibiogram of *P. anguilliseptica* strains differed according to the site of collection. A set of tetracycline (TE), ampicillin (AMP), amoxicillin (AML), kanamycin (K), and E was predominant in *P. anguilliseptica* trimethoprim/

**Table 4** Antibiogram of the isolated *P. anguilliseptica* strains

No.	Bacteria strain ( <i>N</i> = 14)	S (%)	IM (%)	R (%)
1	Tetracycline 30 µg	0 (0)	6 (42.86)	14 (100)
2	Trimethoprim 1.25 µg Sulfamethoxazole 23.75 µg	6 (42.86)	5 (35.7)	3 (21.43)
3	Ciprofloxacin 5 µg	2 (14.29)	7 (50)	5 (35.7)
4	Florfenicol 30 µg	9 (64.29)	3 (21.43)	0 (0)
5	Erythromycin 15 µg	0 (0)	5 (35.7)	9 (64.29)
6	Gentamycin 10 µg	10 (71.43)	4 (28.57)	0 (0)
7	Amoxicillin 30 µg	0 (0)	0 (0)	14 (100)
8	Ampicillin 10 µg	0 (0)	0 (0)	14 (100)
9	Kanamycin 30 µg	0 (0)	0 (0)	14 (100)
10	Cefotaxime 30 µg	4 (28.57)	0 (0)	10 (71.43)
11	Streptomycin 30 µg	0 (0)	3 (21.43)	11 (78.57)

*S* sensitive, *IM* intermediate, *R* resistant

sulfamethoxazole (SXT), ciprofloxacin (Cip), and streptomycin (S) not in NKF were presented in those recovered at Damietta farms and absent at Kafrelsheikh farm (Table 5).

### Examination of water physicochemical parameters of fish farms

In Table 6, water temperature ranged between 18.03 and 19.87 °C, showing significant differences suitable for bacterial infection. The water salinity was significantly higher in

**Table 5** Data on multidrug resistant index and genes of the isolated bacterial strains

No.	Bacteria strain	Antibiotics resistant	MDR index	ARG
1	NDA0201	TE, AMP, AML, K, SXT, CIP, S	0.64	sul1, tetA, qnrs,
2	NDA0202	TE, AMP, AML, K, SXT, CIP, S	0.64	sul1, tetA, qnrs,
3	NDA0203	TE, AMP, AML, K, SXT, CIP	0.55	sul1, tetA, qnrs,
4	NDA0204	TE, AMP, AML, K, SXT, CIP, E	0.64	sul1, tetA, qnrs, ermB
5	NDA0205	TE, AMP, AML, K, SXT, CIP, E	0.64	sul1, tetA, qnrs, ermB
6	NDA0206	TE, AMP, AML, K, CTX, E	0.55	tetA, ermB
7	NDA0207	TE, AMP, AML, K, CTX, E	0.55	tetA, ermB
8	NDA0208	TE, AMP, AML, K, CTX, S	0.55	tetA,
9	NDA0209	TE, AMP, AML, K, CTX, E	0.55	tetA, ermB
10	NKF0301	TE, AMP, AML, K, S	0.45	tetA,
11	NKF0302	TE, AMP, AML, K, CTX, E	0.55	tetA, ermB
12	NKF0303	TE, AMP, AML, K, CTX, E	0.55	tetA, ermB
13	NKF0304	TE, AMP, AML, K, CTX, E	0.55	tetA, ermB
14	NKF0305	TE, AMP, AML, K, CTX, E	0.55	tetA, ermB

*MDR* multidrug resistant genes, *TE* tetracycline, *CIP* ciprofloxacin, *SXT* trimethoprim/sulfamethoxazole, *AMP* ampicillin, *E* erythromycin, *K* kanamycin, *AML* amoxicillin, *CTX* cefotaxime, *S* streptomycin, *tetA* tetracycline, *ermB* erythromycin, *sul1* sulfonamide, and *qnrs* quinolone

**Table 6** Water parameters of fish farms under investigation

Items	ND1	ND2	ND3	NK1
Temp (°C)	19.67 <sup>A</sup> ± 0.18	18.03 <sup>C</sup> ± 0.09	19.87 <sup>A</sup> ± 0.12	18.23 <sup>C</sup> ± 0.15
Salinity (g/L)	25 <sup>A</sup> ± 0.12	23.77 <sup>B</sup> ± 0.18	22.8 <sup>C</sup> ± 0.17	19.43 <sup>D</sup> ± 0.32
DO (mg/L)	4.06 <sup>BC</sup> ± 0.05	3.43 <sup>C</sup> ± 0.29	4.34 <sup>AB</sup> ± 0.09	5 <sup>A</sup> ± 0.07
pH	8.17 ± 0.06	8.22 ± 0.07	8.22 ± 0.03	8.19 ± 0.12
TAN (mg/L)	0.47 <sup>B</sup> ± 0.02	0.56 <sup>A</sup> ± 0.03	0.58 <sup>A</sup> ± 0.03	0.45 <sup>B</sup> ± 0.03
NH <sub>3</sub> + NH <sub>4</sub> (mg/L)	0.61 <sup>B</sup> ± 0.02	0.72 <sup>A</sup> ± 0.04	0.74 <sup>A</sup> ± 0.05	0.57 <sup>B</sup> ± 0.04
NH <sub>3</sub> (mg/L)	0.026 <sup>B</sup> ± 0.001	0.03 <sup>AB</sup> ± 0.003	0.037 <sup>A</sup> ± 0.002	0.025 <sup>B</sup> ± 0.005
NO <sub>2</sub> (mg/L)	0.002 ± 0.00	0.005 ± 0.003	0.003 ± 0.001	0.002 ± 0.00
NO <sub>3</sub> (mg/L)	0.71 ± 0.13	0.79 ± 0.2	0.74 ± 0.07	0.72 ± 0.24

ND, north Damietta; NK, north Kafrelsheikh; Temp, temperature; DO, dissolved oxygen; TAN, total ammonia nitrogen; NH<sub>3</sub>, unionized ammonia; NO<sub>2</sub>, nitrite; NO<sub>3</sub>, nitrate

Damietta farm (22.8 to 225 g/L) than Kafrelsheikh farm (19.43 g/L). The level of DO was above 4 mg/L in all farms except farm ND2 at Damietta, which was 3.43 mg/L. The pH levels, which ranged between 8.17 and 8.22, were suitable for fish culture and bacterial growth. Ammonia compounds, both TAN and total ammonium (NH<sub>3</sub> + NH<sub>4</sub>), were significantly higher in ND2 and ND3 compared to investigated farms (0.56 and 0.58; 0.72 and 0.74 mg/L, respectively). With the same trend, NH<sub>3</sub> was significantly higher in ND2 and ND3 (0.03 and 0.037 mg/L, respectively). There were no significant differences in NO<sub>2</sub> and NO<sub>3</sub> in fish farms under investigation.

## Discussion

Clinical and post-mortem signs were recorded in moribund sea bream and sea bass in this study, indicating the occurrence of septicemic-hemorrhagic bacterial disease, which were hemorrhages on the external body surface and internal organs with faint-reddish gills, splenomegaly, and hemorrhagic-distended kidney. In the field studies, general clinical symptoms such as pale skin color, loss of scales, hemorrhages, and ulcers on the body surface, and anemia in the internal organs were observed and showed similarities with previous reports for each particular bacterial fish pathogen (Akayli 2001; Austin and Austin 2016; Roberts 2012).

In this work, microbiological methods of isolation and identification confirm the presence of gram-ve, and identification number 220,004 was obtained using API 20E, indicating the presence *Pseudomonas* spp. unable to identify the bacteria associated with sea bream and sea bass mass mortality. Similarly, false-negative results were detected in the API 20NE (Balboa et al. 2007), with *P. anguilliseptica* (López-Romalde 2005; Canak and

Akayli (2018). Even when Topic Popovic et al. (2007) developed API 20E kits for pathogenic fish bacteria used in field, they recorded misidentification with *P. stutzeri* and *P. anguilliseptica* isolated from moribund gilt-head sea bream. In addition, *P. anguilliseptica* was misidentified as *P. fluorescens/putida* with API 20NE kit Canak and Akayli (2018). So, the API 20NE method could be used to screen and confirm the presence of *Pseudomonas* spp. in sea bream and sea bass.

In this work, no bacterial growth was detected in all bacterial strains at 37 and 42 °C, and no growth was detected at 0 g/L salinity. All strains could grow in 3 and 5 g/L, except those recovered from Kafrelsheikh had weak growth in 5 g/L. Similarly, *Pseudomonas* spp. could tolerate a wide range of water temperature, pH, and salinity and thus could remain in the aquatic environment; however, nutrient sources are scarce (Liu et al. 2015; Palleroni 2015; Moradali et al. 2017). Inconsistency, Duman et al. (2021) found that *Pseudomonas* spp. could grow at NaCl concentrations of 0–45 g/L, whereas higher salinity 60, 70, 80, and 90 g/L NaCl resulted in different growth rates of 20%, 61%, 73%, and 91%, respectively, such as strains of *P. haemolytica*, *P. proteolytica*, *P. lundensis*, and *P. weihenstephanensis*. In addition, it was noted that all *Pseudomonas* spp. could grow at 37 °C incubation temperature, such as *P. mandelii*, *P. meridiana*, *P. migulae*, and *Pseudomonas* spp. tolerated 42 °C, and *P. haemolytica* was heat-tolerant up to 45 °C. It can be easily differentiated *Pseudomonas* sp. from *P. anguilliseptica* based on its ability to grow at 41 °C, which *P. anguilliseptica* cannot (Austin and Austin 1999).

Due to the misidentification of *Pseudomonas* sp. using traditional bacteria methods, ninety isolates were identified using the phylogenetic method with 4-gene multilocus sequence analysis based on the partial sequencing of the 16 S rRNA, *gyrB*, *rpoB*, and *rpoD* genes (Duman et al. 2021).

In this study, sequencing of 16 S rRNA confirmed that all 14 bacterial strains belonged to the genus *Pseudomonas* group. By BLAST analysis of 16 S rRNA, *Pseudomonas* strains were molecularly recognized as *P. anguilliseptica* (14 isolates).

However, relying on the 16 S rRNA could lead to misidentification in some instances (Elgendy et al. 2022). In this study, sequencing of 16 S rRNA allowed the differentiation of *P. anguilliseptica* isolates from other typing *Pseudomonas* spp. isolates of *P. resinovorans*, *P. aeruginosa*, *P. asplenii*, *P. montelii*, *P. tolaasii*, *P. fluorescens*, *P. chlororaphis*, and *P. putida* and showed 96.73–95.45% similarity score index. Mulet et al. (2012) and Gomila et al. (2015) obtained similar results.

On the other hand, the MLSA assay based on four housekeeping genes (*gyrB*, *rpoB*, *rpoD*, and *aroE*) was done to confirm the identification of isolates and to study the phylogenetic relationships in *P. anguilliseptica*. In addition, several studies used these five housekeeping genes (16 S rRNA, *gyrB*, *rpoB*, *rpoD*, and *aroE*) to characterize and epidemiological studies of *Pseudomonas* spp. In this study, the concatenation of four housekeeping genes provided sufficient discriminatory power to separate *P. anguilliseptica* isolates regarding sequence divergences from other *Pseudomonas* spp. A prominent gap between the intraspecies and interspecies MLSA similarities is essential for identifying the isolates at the species level, and a threshold of 97% of MLSA similarity was suggested for species differentiation in the genus *Pseudomonas* (Mulet et al. 2010, 2012). In this study, *P. anguilliseptica* isolates shared the highest MLSA similarity (99.62–99.70%) with the typing *P. anguilliseptica* (NCIMB 1949) and displayed 80.92–79.54% MLSA similarity index with other typing isolates of *Pseudomonas* spp.: *P. chlororaphis* subsp. *piscium* ATCC 17,809 (CP027709.1)<sup>T</sup>, *P. fluorescens* ATCC 13,525 (QVNA00000000.1)<sup>T</sup>, *P. aeruginosa* ATCC 27,853 (CP015117.1)<sup>T</sup>, and *P. putida* ATCC 12,633 (CP101910.1)<sup>T</sup>. Therefore, the MLSA assay of four HKGs (*gyrB*, *rpoB*, *rpoD*, and *aroE*) revealed a considerable performance for

the identification of *P. anguilliseptica*. On the other hand, MLSA is superior for resolving the intrastrain relationship among *P. anguilliseptica* isolates (Mjølnerød et al. 2021).

In this study, *P. anguilliseptica* strains were tested against 11 antibiotic types. All *P. anguilliseptica* strains were resistant to the selected antibiotics tetracycline, amoxicillin, ampicillin, and kanamycin, while they were sensitive to gentamycin, florfenicol, and trimethoprim plus sulfamethoxazole. Antibiogram of *P. anguilliseptica* strains differed according to the fish collection site. The most predominant ARG was *tetA* followed by *ermB* and *sull*. There was a good correlation between the presence of resistance genes and corresponding resistance phenotypes, suggesting resistance genes, when present, were usually expressed (Randall et al. 2004). Accordingly, the antibiotics used in antibiogram tests were available in the local market; they could also be used in fish treatment (Noga 2010). Similarly, Canak and Akayli (2018) found that the isolated *P. anguilliseptica* was sensitive to ciprofloxacin and enrofloxacin, whereas it was resistant to 6 of the 10 antimicrobials. Other reports considered *Pseudomonas* spp. multidrug-resistant bacteria as they were resistant to ampicillin, trimethoprim + sulfamethoxazole, cefotaxime, aztreonam, and nitrofurantoin (Matyar et al. 2008; Devarajan et al. 2017); also, López-Romalde et al. (2003) found that all *P. anguilliseptica* strains were trimethoprim-sulfamethoxazole. In contrast, Haenen and Davids (2001) recorded that *P. anguilliseptica* were sensitive to neomycine, trimethoprim, trimethoprim-sulphadiazine, sulphonamids, oxytetracycline, flumequine, and oxolinic acid. These differences are attributed to use or lack of the use of these antimicrobials.

The intensive culture of sea bream in Egypt resulted in a high infection rate of pathogenic bacteria that grow well in brackish water ponds 23.5 to 26.5 g/L (Eisawy and Wassef 1984). In this work, water temperature ranged between 18.03 and 19.87 °C, while salinity was significantly higher in Damietta (22.8 to 22.5 g/L) than in Kafrelshiekh farm (19.43 g/L); these parameters were suitable for bacterial infection. Similarly, Haenen and Davids (2001) isolated *P. anguilliseptica* at 23–25 °C in diseased European eelers that were maintained in Netherlands. These findings could be due to the decline in antibody production. Accordingly, Yamaguchi et al. (1980) found that fish were immunosuppressed in response to a decline in water temperature and salinity because of a decrease in antibody formation as antibody production was seasonality dependent. In addition, the fluctuation of water temperature suppressed the fish immunity, causing them to become more vulnerable to *P. anguilliseptica* infection (Tort et al. 1996), so mortality ceased by raising the water temperature above 26 °C (Muroga et al. 1973; Haenen and Davids 2001). Accordingly, bacterial disease outbreaks were recorded in fish farms at a water temperature of 16–18 °C (Kim et al. 2010), whereas the disease persisted in sea bream until the water temperature increased to 18–20 °C (Doménech et al. 1999).

The level of NH<sub>3</sub> was stressful for fish in the examined fish farms according to standard values recommended (0.002–0.05 mg/L) by FAO (2023). Ammonia compounds were not statistically correlated with isolated bacteria strains, antibiotic resistance, MDR index, or ARGs. Accordingly, the ammonia compounds accumulated did not significantly affect the bacterial community or ARG composition; they might still cause immunosuppression status in the fish and other metabolic processes. These effects could render the fish more vulnerable to bacterial infections (Sreedevi and Ramasubramanian 2011; Rousselet et al. 2018; Sherif and AbuLeila 2022). Other findings, it was found that *P. anguilliseptica* infection is interrelated with winter disease (WD) syndrome in sea bream (Doménech et al. 1999); this syndrome is a multifactorial disease associated with many factors, mainly deteriorated water quality (high ammonia compound and heavy metals pollution) which could cause immunosuppressed fish (Tort et al. 1998).



## Conclusion

In this study, MLSA was used to type fourteen *P. anguilliseptica* isolates. Bacterial infections were accompanied by low water temperature and fluctuation of water salinity. Despite the stress levels of unionized ammonia (NH<sub>3</sub>) in fish farm water, no statistical correlation was observed with bacterial infection. All *P. anguilliseptica* strains could grow in 3 and 5 g/L, except those recovered from Kafrelsheikh farm, which had weak growth in 5 g/L. Isolates were sensitive to florfenicol, trimethoprim, sulfamethoxazole, cefotaxime, and ciprofloxacin. In addition, tetracycline *tetA* and erythromycin *ermB* genes were the highest-presented antibiotic-resistant genes.

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**Data availability** The datasets and materials used in this study are available in the manuscript.

## Declarations

**Ethics approval** This study was approved by the Ethics Committee of Animal Experiments of the Faculty of Veterinary Medicine, Cairo University.

**Competing interests** The authors declare no competing interests.

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