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Winter Kills in Farmed European Seabass (*Dicentrarchus labrax*): Co- infected with *Shewanella putrefaciens* and *Aeromonas veronii*

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ABSTRACT

Aeromonas and *Shewanella* species are opportunistic pathogens and often cause serious diseases in farmed fish. In the present study, persistent natural mortality was observed in El Manzala intensive marine fish farm stocked with European seabass (*Dicentrarchus labrax*), Damietta, Egypt during the course of winterkills from early December to mid-January. The temperature of the well and fish ponds during episodic die-offs ranged from 13 to 17°C. The diseased European seabass exhibited ulcer, fin hemorrhage, tail fin rot, red and swollen inflamed vents, abdominal distension and hemorrhage, with approximately 30% cumulative mortality. Parasitological examination of diseased fish revealed only mild infestation with *Trichodina* species in the gills. Furthermore, bacteriological examination of diseased fish resulted in the identification of *Shewanella putrefaciens* and *Aeromonas veronii* isolates. These clinical isolates were presumptively identified on the basis of phenotypic characteristics and API 20NE biochemical profiles, followed by *16S rRNA* gene sequencing and BLAST alignments. Additionally, the analysis of the neighbor-joining phylogenetic tree supported the above-mentioned identification. RNA polymerase B subunit (*rpoB*) was considered a proper tight taxonomic tool to differentiate between *Aeromonas* species. Therefore, *Aeromonas* isolates were confirmed as *Aeromonas veronii* based on *rpoB* housekeeping gene. In addition, antimicrobial susceptibility testing revealed that *Shewanella putrefaciens* isolates were resistant to oxytetracycline, doxycycline, trimethoprim/sulfamethoxazole and ampicillin, while *Aeromonas veronii* were susceptible to oxytetracycline, doxycycline and trimethoprim/ sulfamethoxazole. Both isolates were susceptible to florfenicol which was chosen to control these bacterial co-infections in farmed European seabass. This is the first report of *Shewanella putrefaciens* and *Aeromonas veronii* natural co-infections in intensively stocked European seabass.

1. INTRODUCTION

The European seabass (*Dicentrarchus labrax* L.) is an economically important marine species, particularly in the Mediterranean aquaculture **(Cardia & Lovatelli, 2007)**. Total European seabass world production in 2019 was 236,215 Tonnes. Turkey is the biggest producer accounting for 52.21% of the world European seabass production,

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followed by Greece accounting for 15.67%, then Egypt with 11.52% and Spain for 9.6% **(FAO, 2021)**.

Bacterial pathogens are the most frequently encountered infectious diseases that threaten aquaculture industry and thereby leading to the heavy losses in the cultured European seabass production in the Mediterranean area **(Toranzo *et al.*, 2005; Korun *et al.*, 2009; Abdel-Aziz *et al.*, 2013; Smyrli *et al.*, 2017; Abou-Okada *et al.*, 2021b)**. Winter mortality has been documented in a large number of freshwater, estuarine and marine water fishes populations **(Sood *et al.*, 2019; Takegaki and Takeshita, 2020; URKU, 2021)**. Episodic mass mortality of marine fish during the course of low winter temperatures, is now commonly referred to as 'winterkills' **(Hurst, 2007; Mohammed and Peatman, 2018;)**. Winter Ulcer Syndrome is a syndrome associated with skin ulcers that occurs in marine water at low temperature **(Lunder *et al.*, 1995)**. Co-infections are very common in nature and described when fish are infected by two or more genetically different pathogens either by simultaneous or secondary infections. Co-infections can alter the severity of fish diseases and causing the appearance of sudden fish outbreaks **(Kotob *et al.*, 2017; Abou-Okada *et al.*, 2021a).**

Shewanella putrefaciens (earlier was named *Pseudomonas putrefaciens*) is an opportunistic pathogen for the fish, and the bacterium could cause losses under stress conditions **(Koziñska & Pekala, 2004)**. *Shewanella putrefaciens* (*S. putrefaciens*) commonly inhabits marine environments and has been isolated from marine fish and sediment **(Lee et *al.*, 1977; Saeed *et al.*, 1987, 1990)**. It was first isolated from the farmed European seabass (*Dicentrarchus labrax*) as the causative agent of mortalities in Turkey **(Korun *et al.*, 2009)**. The pathogenicity of *S. putrefaciens* has been previously detected in the marine rabbitfish, *Siganus rivulatus* from the Red Sea **(Saeed *et al.*, 1987)** and in the farmed European seabass, *Dicentrarchus labrax* from the Mediterranean Sea **(Korun *et al.*, 2009)**.

Aeromonas veronii (*A. veronii*) is an emerging pathogen that increasingly gaining importance as a serious pathogen for the aquaculture industry. *Aeromonas veronii* has become extremely problematic in the culture of the European seabass (*Dicentrarchus labrax*) in the Mediterranean area **(Smyrli *et al.*, 2017, 2019)**. *Aeromonas veronii* has been reported to affect a fish with different sizes (<50 g to >200 g) with cumulative mortality reaching more than 50% if not treated early with antimicrobials **(Smyrli *et al.*, 2017)**.

Most pathogens that impact aquaculture are deemed opportunistic. *Shewanella putrefaciens* and *Aeromonas veronii* are opportunistic fish pathogens and members of fish intestinal bacterial flora **(Walczak *et al.*, 2017)**. In intensive aquaculture, environmental stress factors generate an optimal setting for the initiation of *S. putrefaciens* and *A. veronii* infections with consequent jeopardy to the fish immune system, leading to disease outbreaks and severe economic losses **(Korun *et al.*, 2009; Mohammed & Peatman, 2018)**.

The main goal of this study was to determine the etiologies of winter kills affecting the European seabass (*Dicentrarchus labrax*) reared in intensive marine fish farms and assess potential antibiotic therapies for the treatment of these infections.

2. MATERIALS AND METHODS

Fish sampling and clinical examination

Winter mortalities were observed among the intensively stocked European seabass (*Dicentrarchus labrax*) marine fish farm, Damietta, Egypt. Moribund fish exhibited signs of abnormal swimming behavior, skin hemorrhages and ulceration. To investigate the cause of winter fish kills, twenty-five diseased European seabass (average weight: 55 ± 10 g) were collected and transported immediately in ice-cooled boxes to the Aquatic Animal Medicine and Management Department. Clinical examination and necropsy of fish samples were performed according to the methods of **Noga (2010)**.

Water quality parameters

Representative water samples from underground well and concrete ponds were collected according to the standard methods of **APHA (2017)**. Chemical water parameters were measured on spot using multiparameter meter with multiple probes (HI-9829, Hanna Instruments Inc., Romania). Briefly, dissolved oxygen (DO, ppm), water temperature (°C), salinity (ppt), pH, Total ammonia nitrogen (TAN, ppm), nitrite nitrogen (NO_2-N, ppm), nitrate-nitrogen (NO_3-N, ppm) and total iron (ppm) were measured according to manufacturer's protocols. The amount of un-ionized toxic ammonia (NH_3) was determined by mathematical calculation according to **Emerson *et al.* (1975).**

Parasitological examination

Skin, fins, gills, muscles, liver, spleen, kidney and intestine of the collected fish were carefully examined by naked eye and handheld lens for macroscopic parasites and then dissected under a dissecting microscope for microscopic parasites. Furthermore, wet-mount and tissue squash preparations from the abovementioned tissues were examined microscopically for the presence of external and internal parasites.

Bacterial isolation and characterization

Under complete aseptic conditions, loopfuls from skin ulcers, hemorrhagic fins, necrotic muscle, gills, spleen, liver and kidney were streaked onto Tryptic soy agar supplemented with 2% NaCl (TSA, Lab M, UK), blood agar (Oxoid) supplemented with (5% sheep blood, 2% NaCl), motility agar (TSB + 0.3% agar + 2% NaCl), TCBS agar (Oxoid). Inoculated plates were incubated for 48–96 h at 5°C, 22°C, 28°C and 37°C. Pure colonies were re-streaked onto TSA + 2% NaCl for phenotypic and biochemical identification. Presumptive identification was accomplished using different phenotypic and biochemical tests (APIID Test Strips®, APIWEB™, Biomérieux, USA) following the manufacture instructions. Moreover, bacterial colonies were inoculated into peptone water supplemented with 0%, 2%, 3%, 4%, 6%, 8% and 10% NaCl to distinguish between bacterial isolates based on the trait of NaCl tolerance (salt tolerance).

Presumptive identified Pure cultures were stored at −20°C in tryptic soy broth (TSB) (Lab M) supplemented with 2% NaCl (Lab M) and 16% glycerol (Sigma-Aldrich) for further molecular characterization.

Genotypic characterization of bacterial isolates

DNA extraction, PCR, 16S rRNA sequencing and phylogenetic analyses

The genomic DNA was extracted from the pure cultures of presumptive identified bacterial isolates using a GF-1 Bacterial DNA Extraction Kit (Vivantis Technologies, Malaysia) following the manufacturer's instructions. DNA was eluted with 50 µL elution buffer, the DNA concentration (ng/ µL) was quantified with a Thermo Scientific Nano-Drop^{TM}1000 spectrophotometer (Thermo Fisher Scientific Inc., USA) and then stored at -20°C. The 16S rRNA gene was amplified by PCR using the 16S universal Eubacterial primers Forward: (5-AGAGTTTGATCCTGGCTCAG-3) and Reverse: (5-GGTTACCTTGTTACGACTT-3) as described previously **(Weisburg *et al.*, 1991)**.

PCRs were performed in a final volume of 25 µL, containing 1 µL of DNA template (50 ng/ µL), 12.5 µL of 2x DreamTaq® Green Master Mix (Thermo Fisher Scientific) and 0.5 µL (10 nmol $L^{-1)}$ of each primer and 10.5 µL of RNase free water. The PCR amplifications were performed using a MyCycler™ thermal cycler (Bio-Rad, USA) with the following cycling conditions, initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 56°C for 30 s, extension at 72°C for 30 s, with a final extension at 72°C for 5 min and then indefinite holding at 4°C. The template-free reactions were included in the PCR setup as non-template control (NTC). The amplified products were analyzed by electrophoresis (120 V, 400 mA and 60 min) on a 1.2% (W/V) agarose gel.

The PCR products were purified with a QIAquick PCR purification kit (Qiagen, USA) and purified amplicons were then submitted for Sanger sequencing with an ABI 3730XL DNA sequencer (Biosystems™, USA). The obtained sequences were searched against National Center for Biotechnology Information (NCBI) GenBank database using BLASTn tools.

The phylogenetic analyses were conducted using the Neighbor-Joining method **(Saitou and Nei, 1987).** The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches **(Felsenstein, 1985)**. The evolutionary distances were computed using the p-distance method **(Nei and Kumar, 2000)** and were in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA 11 **(Tamura *et al.* 2021)**.

Further molecular identification of Aeromonas veronii by rpoB gene

The *rpoB* gene was amplified by PCR using *Aeromonas veronii rpoB* primers, A-Ver F(b1): (5-CGTGCCGGCTTTGAAGTC-3) and A-Ver F(b1): (5-GATCACGTACTTGCCTTCTTCAATA-3) with amplicon size of 224 bp as described by **Persson *et al.* (2015)**. PCR was performed in a final volume of 25 µL, containing 1 µL of DNA template (50 ng/ µL), 12.5 µL of 2x DreamTaq® Green Master Mix (Thermo

Fisher Scientific) and 0.5 µL (10 nmol L^{-1}) of each primer and 10.5 µL of RNase free water. The PCR amplifications were performed using a MyCycler™ thermal cycler (Bio-Rad, USA) with the following cycling conditions, initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, extension at 72°C for 45 s, with a final extension at 72°C for 5 min and then indefinite holding at 4°C. The template-free reactions were included in the PCR setup as non-template control (NTC). The amplified products were analyzed by electrophoresis (120 V, 400 mA and 60 min) on a 1.2% (W/V) agarose gel. The bands were visualized using standard UV Transilluminator (Cleaver Scientific Ltd., UK) to check for the presence and purity of specific single band (224 bp) compared with NTC.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of *Aeromonas veronii* and *Shewanella putrefaciens* were performed using the standard disc diffusion method according to the methods described by **Jorgensen and Turnidge (2007).** Bacterial inocula at a concentration of 1.5 $\times 10^8$ CFU/ml (0.5 McFarland standard) were prepared and streaked on Mueller-Hinton agar plates and blood agar plates. The following antimicrobial discs as fellow: 30 µg oxytetracycline (OT 30), 30 µg florfenicol (FFC 30), 1.25 + 23.75 µg trimethoprim/sulfamethoxazole (SXT 25), 30 µg flumequine (UB 30), 2 µg oxalinic acid (OA 2), 10 µg ampicillin (AMP 10), 15 µg erythromycin (E 15) and 30 µg doxycycline (DO 30) were placed on the surface of agar plates using automatic dispenser. The diameters of inhibition zones (in mm) were measured and recorded after 24 and 48 h of incubation at 28°C. Interpretation of resistant (R), moderate susceptible (M) and susceptible (S) phenotypes was in accordance with previously published guidelines **(Clinical and Laboratory Standards Institute [CLSI], 2016).**

RESULTS

3.1. Clinical examination of fish

Persistent naturally occurring winter mortalities were observed in El manzala intensive marine fish farm, Damietta, Egypt, resulting in severe losses among stocked European seabass. The diseased European seabass displayed abnormal swimming behavior, lethargy, anorexia and skin darkening. The clinical examination of diseased fish revealed detached scales **(Figure 1A)**, skin and eye hemorrhages **(Figure 1B)**, tail fin hemorrhage **(Figure 1C)**, tail fin erosion **(Figure 1E)**, tail fin rot **(Figure 1D, 2A, 2D)**, skin abrasion **(Figure 2D)** and ulceration **(Figure 2A)**, muscle ulceration **(Figure 2B)**, red inflamed vents and abdominal distension **(Figure 2C).** On the other hand, the abdominal cavity is filled with serosanguinous fluids in addition to pale liver and enlarged spleen. The daily mortalities in the concrete-based intensive fish farm were more than 150 fish and these mortalities were persistent over one-month (start from 10th December to 15th January) during low water temperatures. The fish cumulative mortalities were approximately 30% during these episodic die-offs.

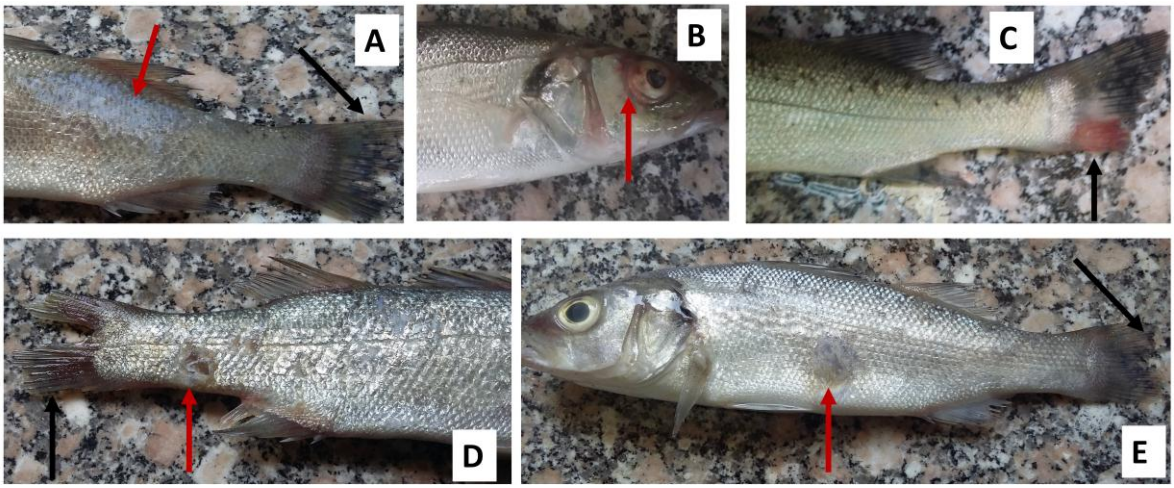

Figure 1. Naturally diseased European seabass *(Dicentrarchus labrax)* showed; **(A)**: detached scales (red arrow) and tail fin rot (black arrow); **(B)**: hemorrhagic eye (red arrow); **(C)**: tail fin hemorrhage and erosion (black arrow); **(D)**: skin erosion and ulceration (red arrow) and tail fin rot (black arrow) and **(E)**: skin erosion (red arrow) and tail fin rot (black arrow).

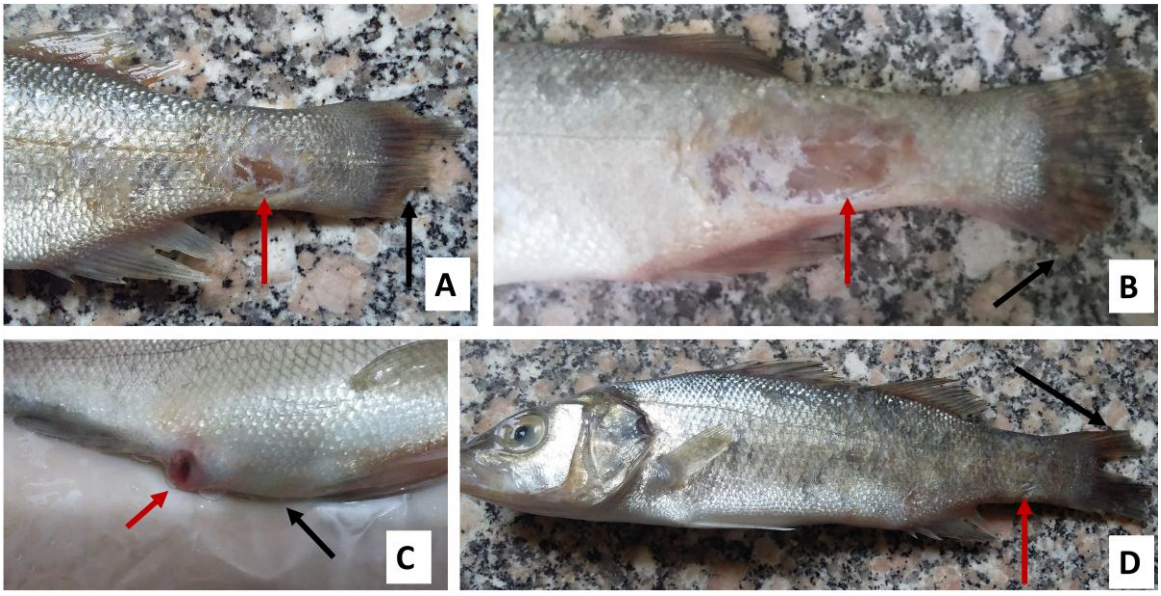

Figure 2. Naturally diseased European seabass (*Dicentrarchus labrax)* showed; **(A, B)**: detached scales, severe skin erosion and ulceration in caudal peduncle area (red arrow) and tail fin rot (black arrow); **(C)**: inflamed vent (red arrow) and distended abdomen (black arrow) and **(D)**: skin erosion (red arrow) and tail fin rot (black arrow).

3.2. Water quality parameters

The analysis of water samples from the underground well revealed low water temperature ranged from 13 to 16°C, water salinity (15 ppt), dissolved oxygen (3 mg/L), pH (6.7), total ammonia nitrogen (TAN, 0.6 mg/L), un-ionized ammonia (NH_3, 0.002 mg/L), nitrite nitrogen (0.08 mg/L), nitrate nitrogen (10 mg/L) and total iron (1.1 mg/L). Furthermore, the analysis of water samples from the fish ponds revealed low water temperature ranged from 15 to 17°C, water salinity (15.5 ppt), dissolved oxygen (6 mg/L), pH (7.1), total ammonia nitrogen (TAN, 2.5 mg/L), un-ionized ammonia (NH_3, 0.009 mg/L), nitrite nitrogen (0.1 mg/L), nitrate nitrogen (15 mg/L) and total iron (1.4 mg/L). Additionally, water had high level of organic matter and filamentous green algae **(Fig. 3**).

Figure 3. Water quality of concrete fish pond revealed **(A)**: poor water quality with high organic matters and filamentous green algae on water and paddles; **(B)**: large mass of filamentous green algae with high organic matters.

3.3. Parasitological examination

Upon macroscopic and microscopic examination of wet mount smears of skin, fins, gills and muscles, only a mild infestation with *Trichodina* species was detected in gills of diseased European seabass. Neither monogeneans nor crustacean parasites were seen in the skin, fin and gill smears. Moreover, no other parasites were seen in tissue squash preparations.

3.4. Phenotypic characterization of bacterial isolates

Eight bacterial isolates were retrieved from the kidney, skin ulcer, muscle, and spleen of diseased European seabass. All isolates were Gram-negative, motile, oxidase and catalase-positive bacilli. Initial characterization of the eight Gram-negative isolates by hemolysis pattern on sheep blood agar identified three isolates with β hemolytic patterns and five isolates didn't produce hemolytic activity **(Table 1)**. Presumptive identification of the isolates with the API-20NE identified three isolates with the API-20NE profile (7176754, 7176654 and 7176714 as *Aeromonas sobria* (94.9%) / *A. caviae* / *A. hydrophila* (5.1%)) and the other five isolates with the API-20NE profile (1410144, 1410054, 1410145, 1410354 and 1411154, as *Shewanella putrefaciens* (99.9%)). The morphological and biochemical characteristics of the eight isolates which identified as *Aeromonas* species (3 isolates) and *S. putrefaciens* (five isolates) were summarized in **Table 1**.

Table 1. The morphological and biochemical characteristics of *Aeromonas veronii* and *Shewanella putrefaciens* isolates from naturally diseased European seabass (*Dicentrarchus labrax*) during winter fish kills.

Test	***Aeromonas veronii*** **(n = 3)**	***Shewanella putrefaciens*** **(n = 5)**
Growth on TSA + 2% NaCl	2-3 mm creamy colonies	1-2 mm light brownish colonies
Growth on sheep blood agar	β hemolysis	No hemolysis
Growth on TCBS	2-3 mm yellow colonies	1 mm blue colonies
Sucrose fermentation	+	-
Growth at 5°C	+	+
Growth at 22°C	+	+
Growth at 28°C	+	+
Growth at 37°C	+	+
Gram staining	Gram negative bacilli	Gram negative bacilli
Motility	Motile	Motile
Oxidase	+	+
Catalase	+	+
TSI	A/A	K/K with H_2S
H_2S production	-	+
Indole production	+	-
Vibriostatic agent (O/129) (10 μg)	Resistant	Resistant
Vibriostatic agent (150 μg)	Resistant	Resistant
Growth at 0% NaCl	+	+
Growth at 2% NaCl	+	+
Growth at 4% NaCl	+	+
Growth at 6% NaCl	-	+
Growth at 8% NaCl	-	+
Growth at 10% NaCl	-	-
Nitrate reduction	+	+
Tryptophane production	+	-
Glucose fermentation	+	-
Arginine Dihydrolase	+	-
Urea hydrolysis	-	-
Esculin hydrolysis	-	+
Gelatin hydrolysis	+	+
PNG (β-Glucosidase)	+	-
Glucose assimilation	+	-
Arabinose assimilation	-	V
Mannose assimilation	+	-
Mannitol assimilation	+	-
N-acetyl glucosamine assimilation	V	+
Maltose assimilation	+	V
Potassium gluconate assimilation	+	V
Capric acid assimilation	+	V
Adipic acid assimilation	-	-
Malate assimilation	V	+
Citrate assimilation	-	-
Phenyl acetic acid assimilation	-	-

+, 100% positive isolates; -, 100% negative isolates; V, variable, TSI, triple sugar iron; A/A, acid slant/acid butt; K/K, alkaline slant/alkaline butt.

3.5. Genotypic characterization of bacterial isolates

3.5.1. 16S rRNA sequencing and phylogenetic analyses

Sequencing of 16S rRNA gene from presumptive identified *Aeromonas* species and *Shewanella putrefaciens* isolates with application of BLAST search of NCBI revealed that the sequences weren't identical and therefore, the two sequences were submitted to GenBank under the accession number OP456599 and OP456625. The blast search of first sequence (OP456599) revealed maximum similarity (>99%, E value of 0.00) with *Aeromonas veronii* (Accession numbers: KP115700, MN603659, CP034967 and MN603645) and 99% identity with *Aeromonas veronii bv veronii* strain ATCC 35624 (Accession number: X60414). In addition, the blast search for second sequence (OP456625) revealed maximum similarity (>99%, E value of 0.00) with *Shewanella putrefaciens* (Accession numbers: AJ000213, KU851956, DQ307729 and KX611361) and 98% identity with *Shewanella putrefaciens* ATCC 8071(Accession number X82133).

Phylogenetic tree established on the 16S rRNA sequence was used to understand the relationship between *Aeromonas veronii* and *Shewanella putrefaciens* isolates in relation to representative *Aeromonas* and *Shewanella* species with *Pseudomonas*, *Vibrio* and *Streptococcus* species as outgroups **(Fig. 4)**. In the neighbor-joining (NJ) phylogenetic tree, *Aeromonas veronii* isolate (OP456599) formed a distinct clade with known species of *Aeromonas veronii* **(Fig. 4)**, whereas *Shewanella putrefaciens* isolate (OP456625) is clearly grouped with a cluster of known species of *Shewanella putrefaciens* **(Fig. 4)**.

2.5.2. Further molecular identification of *Aeromonas veronii* by *rpoB* gene

Further molecular identification of *Aeromonas* isolates (n = 2) by using *rpoB* gene was considered a proper tool than the 16S rRNA gene sequencing for *Aeromonas* species identification. Therefore, the two isolates were identified as *Aeromonas veronii* based on the results of conventional PCR using *Aeromonas veronii rpoB* gene **(Fig. 5)**.

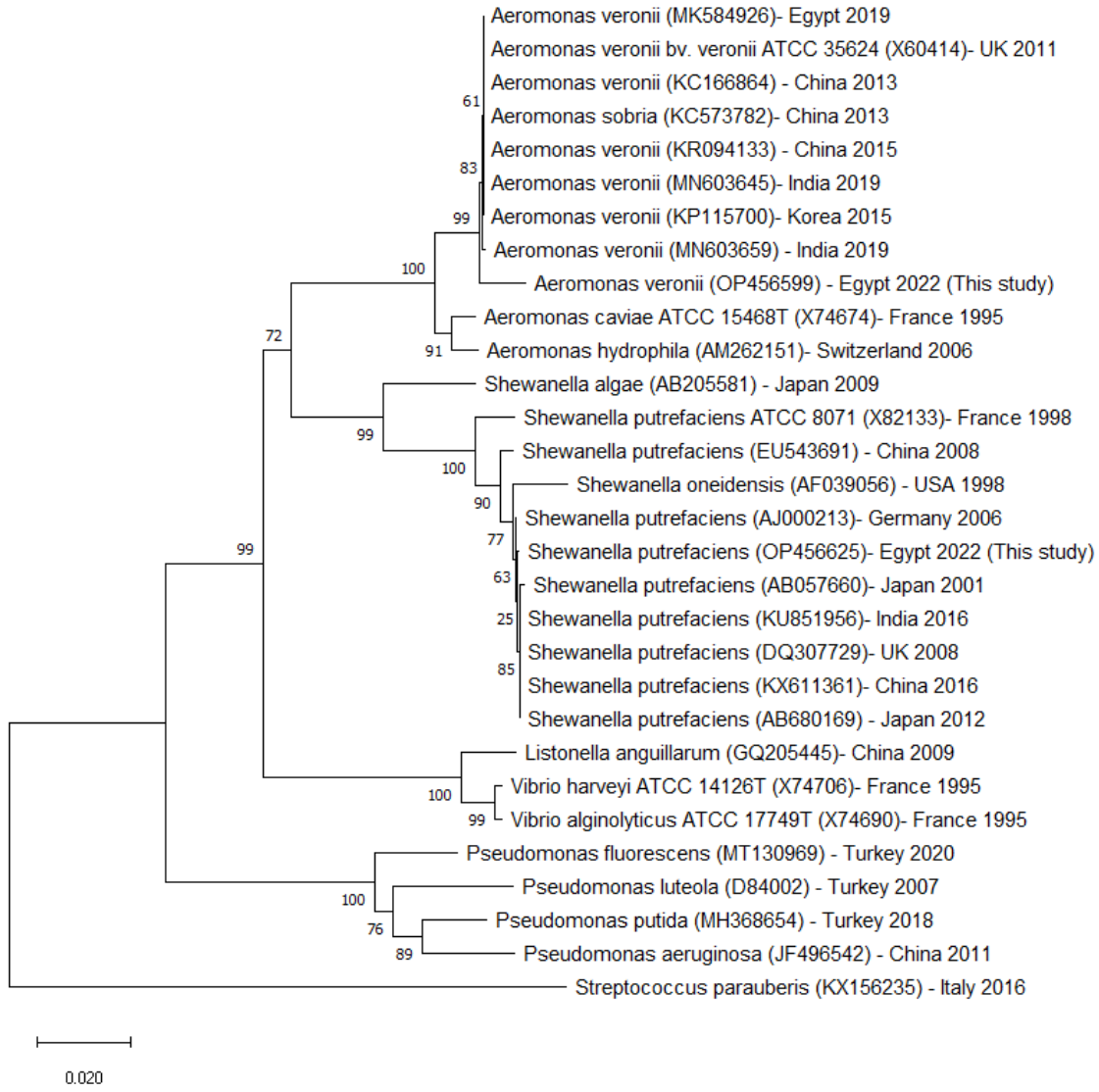

Figure 4. Neighbor-joining phylogenetic trees based on 16S rRNA gene sequences. The percentage of replicate trees in which the associated taxa clustered together based on 1,000 bootstrap replicates are shown adjacent to branches. The evolutionary distances were computed using the p-distance method. Sequence accession numbers are in parentheses. *Aeromonas veronii* isolate (OP456599) formed a distinct clade with *Aeromonas veronii*. *Shewanella putrefaciens* isolate (OP456625) is clearly grouped with a cluster of known species of *Shewanella putrefaciens. Pseudomonas, Vibrio* and *Streptococcus* species was used as outgroups.

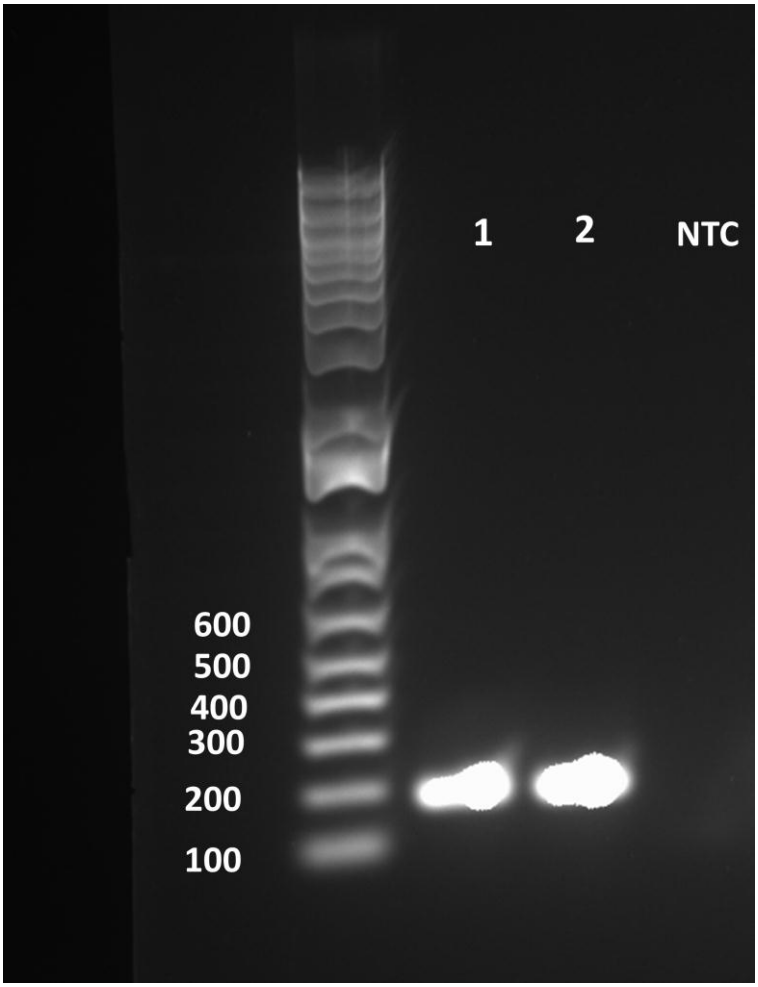

Figure 5. Two clinical isolates analyzed by conventional PCR for further identification of *Aeromonas* species. *Aeromonas veronii rpoB* primer set was designed to be specific for *Aeromonas veronii* and to have a 224 bp amplicon size. Lanes 1 and 2, *Aeromonas veronii*. NTC served as non-template control (control negative).

2.6. Antimicrobial susceptibility testing

Aeromonas veronii isolates (n = 3) were sensitive to oxytetracycline and doxycycline. On the other hand, *Shewanella putrefaciens* isolates (n = 5) were resistant to oxytetracycline and doxycycline. *Aeromonas veronii* and *Shewanella putrefaciens* were sensitive to flumequine and florfenicol **(Table 2)**. Therefore, florfenicol was chosen to control these bacterial co-infections in European seabass intensive fish farm based on the results of *in vitro* antimicrobial susceptibility testing and availability of florfenicol as feed premix (Aquaflor® 50%) **(Table 2)**.

Table 2. Antimicrobial susceptibility testing of *Aeromonas veronii* and *Shewanella putrefaciens* isolates from naturally diseased European seabass (*Dicentrarchus labrax*) during winter fish kills.

Antimicrobials	Disk Content (mg)	*Aeromonas veronii* (n = 3)	*Shewanella putrefaciens* (n = 5)
Ampicillin	10	R	R
Erythromycin	15	R	M
Oxalinic acid	2	M	M
Flumequine	30	S	S
Trimethoprim/sulfamethoxazole	25	S	R
Oxytetracycline	30	S	R
Doxycycline	30	S	R
Florfenicol	30	S	S

S, susceptible; M, moderately susceptible; R, resistant.

4. DISCUSSION

To our knowledge, this is the first study describe the bacterial co-infections (*Shewanella putrefaciens* and *Aeromonas veronii*) affecting farmed European seabass (*Dicentrarchus labrax*) in the Mediterranean area during the episode of winterkills. Disease-related mortality constitutes the most substantial cause of economic losses in fish farming. In the present study, persistent naturally occurring winter mortalities were observed in El manzala intensive marine fish farm, Damietta, Egypt over one month during the course of episodic die offs with cumulative mortalities approximately 30%. Clinical signs of diseased European seabass were investigated **(Figs. 1 and 2)**. European seabass infected with *S. putrefaciens* and *A. hydrophila* revealed epidermal lesions progressively extended to musculature (muscle ulceration), hemorrhagic caudal fins, fin rot, red inflamed vents and abdominal distension **(Korun *et al.,* 2009; Smyrli *et al.,* 2017; Tansel Tanrikul and Dinçtürk, 2021)**. Similar clinical signs have been previously reported in rabbitfish, *Siganus rivulatus* **(Saeed *et al.,* 1987)**, common carp, *Cyprinus carpio* and rainbow trout, *Oncorhynchus mykiss* **(Kozińska and Pękala, 2004)**, freshwater loach, *Misgurnus anguillicaudatus* **(Qin *et al.,* 2014)**, eels, *Anguilla anguilla* **(Esteve *et al.,* 2017)**, channel catfish, *Ictalurus punctatus* **(Mohammed and Peatman, 2018)** and Nile tilapia, *Oreochromis niloticus* **(El-Barbary, 2017)**.

Shewanella putrefaciens and *Aeromonas veronii* are opportunistic fish pathogens **(Walczak *et al.,* 2017).** The fluctuation in water temperature related to climate change might be advantageous for the invasion and establishment of opportunistic pathogens **(Vezzulli *et al.,* 2010)** and could be implicated in emerging pathologies in marine ecosystems. Intensive fish farming systems were particularly vulnerable to infection due to change in water temperature, salinity, high stocking densities and other environmental stressors related to production cycles **(Murray and Peeler, 2005).** Adverse environmental conditions could initiate stress-induced infection with *S. putrefaciens* and

A. veronii leading to emerging disease outbreaks (**Korun *et al.*, 2009; Mohammed and Peatman, 2018).** Aeromonads have been detected in European seabass (*Dicentrarchus labrax*), salmon (*Salmo salar*), turbot (*Psetta maxima*) and rainbow trout (*Oncorhynchus mykiss*) in the low salinity environment of the Black Sea **(Öztürk and Altınok, 2014)**. However, *Aeromonas veronii* are opportunistic fish pathogens, **Smyrli *et al.* (2017)** assumed that the episodic die offs of European seabass were triggered by an establishing disease rather than stress-induced infections.

The phenotypic characterization of bacterial isolates demonstrated that five isolates had phenotypic characteristics corresponded to *Shewanella putrefaciens* and three isolates had phenotypic characteristics corresponded to *Aeromonas* species **(Table 1)**. The presumptive identification of five *S. putrefaciens* isolates revealed different numeric API 20NE profiles (1410144, 1410054, 1410145, 1410354 and 1411154) with 99.9% identity with *S. putrefaciens* that corresponded with previous studies (**Korun *et al.*, 2009; Pękala *et al.*, 2015; El-Barbary, 2017;).** On the other hand, the presumptive identification of three *Aeromonas* species isolates revealed *Aeromonas sobria* (94.9%) / *A. caviae* / *A. hydrophila* (5.1%) with API 20NE profiles (7176754, 7176654 and 7176714) corresponded to previous studies **(Smyrli *et al.*, 2017; Mohammed and Peatman, 2018)**. The typical phenotypic properties of *Aeromonas veronii* was resistance to ampicillin, positive reaction to oxidase, catalase and indole, negative reactions to urease, esculin, L-arabinose and H_2S **(Abbott *et al.*, 2003; Uzun and Ogut, 2015; Smyrli *et al.*, 2017).** Although both strains grew well on TSA supplemented with 0%, 2% and 4% NaCl, the optimum growth was observed on TSA supplemented with 0 % and 2% NaCl. **Saeed *et al.* (1987**) reported that *S. putrefaciens* isolated from the diseased rabbit fish was not able to grow in 0% and 1.0% NaCl but the isolate grew well in 9% NaCl and showed weak growth in 10% NaCl. *Shewanella putrefaciens* isolated from marine water fish had different phenotypic characteristics compared with isolates from freshwater fish **(Kozińska and Pękala, 2004; Korun *et al.*, 2009)**. **Smyrli *et al.* (2017)** reported that *A. veronii* grew well on lower salinity media (0.5–2%) NaCl.

Considering the constraints of the API 20NE and API 20E systems, in particular with bacteria of the genera *Aeromonas* and *Vibrio*, these commercial kits profiles were used only for identification to the genus level **(Austin, 2011; Santos *et al.*, 1993)**. 16S rRNA is one of the most commonly used molecular biological detection methods for identifying bacteria at species level **(Busse *et al.*, 1996)**. In the present study, *Shewanella putrefaciens* and *Aeromonas veronii* were identified at the species level using 16S rRNA gene sequencing and BLAST alignments. The neighbor-joining phylogenetic analyses supported the abovementioned identification as *Aeromonas veronii* isolate (OP456599) clustered with other known *Aeromonas veronii*., while *Shewanella putrefaciens* isolate (OP456625) was clearly grouped with a cluster of known *Shewanella putrefaciens* **(Figure 4)**. 16S rRNA gene sequencing may not be an optimal target due insufficient interspecies sequence variation and heterogeneity among *Aeromonas* species **(Martinez-**

Murcia *et al.*, 1992; Morandi *et al.*, 2005; Küpfer *et al.*, 2006). The housekeeping genes include DNA gyrase B subunit (*gyrB*), RNA polymerase B subunit (*rpoB*) and RNA polymerase D subunit (*rpoD*) were able to differentiate this tight taxonomic group of *Aeromonas* species (**Yáñez *et al.*, 2003; Lamy *et al.*, 2010; Martinez-Murcia *et al.*, 2011)**. Further molecular identification of *A. veronii* using RNA polymerase B subunit (*rpoB)* gene was considered a proper tight taxonomic identification of *Aeromonas* species. Therefore, *Aeromonas* isolates were confirmed as *Aeromonas veronii* based on the results of conventional PCR using *Aeromonas veronii rpoB* gene **(Figure 5)**.

Shewanella putrefaciens and *A. veronii* had a variety of hosts and can live in aquatic animals and in the environment, which negatively affect fish farming industry and public health. Bacterial resistance to antibiotics affects the health of humans, animals and the environment (One health approach) **(Wu *et al.*, 2019)**. Extensive use of antibiotics in veterinary medicine has caused accelerated antibiotic resistance. Antibiotic resistance is a natural feature of diverse microbial ecosystems, as most antibiotics are natural products or derivatives of natural products **(Crofts *et al.*, 2017)**. In the present study, *A. veronii* isolates were sensitive to flumequine, oxytetracycline, doxycycline and florfenicol. Additionally, *S. putrefaciens* were sensitive to flumequine and florfenicol. All tested isolates were resistant to ampicillin. *Shewanella putrefaciens* isolates were resistant to oxytetracycline and doxycycline **(Table 2)**. Previous studies showed that *A. veronii* was resistant to ampicillin and amoxicillin **(Wang *et al.*, 2021)**. *Shewanella putrefaciens* strain isolated from diseased European seabass (*Dicentrarchus labrax*), Turkey showed resistance to compound sulphonamides, novobiocin, tetracycline and trimethoprim **(Korun *et al.*, 2009)**. In contrast, *S. putrefaciens* strains isolated from common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*), Poland showed sensitivity toward flumequine, oxytetracycline, ciprofloxacin and enrofloxacin (**Kozińska and Pękala, 2004)**. On the other hand, *S. putrefaciens* isolated from cultured Nile, *Oreochromis niloticus*, Egypt was sensitive to ciprofloxacin, gentamycin, oxytetracycline and sulfamethoxazole (**El-Barbary, 2017)**. *Aeromonas veronii* isolated from European seabass (*Dicentrarchus labrax*), Turkey was sensitive to florfenicol, tetracycline, oxytetracycline and flumequine **(Smyrli *et al.*, 2017)**. *Shewanella putrefaciens* isolated from channel catfish (*Ictalurus punctatus*) was sensitive to florfenicol, while moderately sensitive to oxytetracycline **(Mohammed and Peatman, 2018)**.

5. CONCLUSION

This is the first study to report *S. putrefaciens* and *A. veronii* co-infections in naturally infected European seabass, Egypt during the course of winterkills in intensive marine fish farm. *Shewanella putrefaciens* isolates were resistant to oxytetracycline, doxycycline and trimethoprim/sulfamethoxazole, while *A. veronii* isolates were resistant to ampicillin and erythromycin. Florfenicol was chosen to control these bacterial co-infections based on the results of *in vitro* antimicrobial susceptibility testing.

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