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PHENOTYPIC, SEROTYPIC AND GENETIC CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBILITY DETERMINATION OF *Vibrio anguillarum*, ISOLATED FROM CULTURED SEA BASS (*Dicentrarchus labrax* L., 1758) IN THE SOUTHEAST BLACK SEA, TURKEY

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SUMMARY

In this study, identification of *Vibrio* isolates from infected sea bass (*Dicentrarchus labrax* L., 1758) in the Black Sea was performed by using conventional methods, and API 20E test kits. All isolates were confirmed by PCR assays specific to the 16S rRNA gene of bacterium. Lam agglutination test was carried out on all vibrio isolates by using raised rabbit serum against *Vibrio anguillarum* O1. According to the result of agglutination, biochemical and PCR tests isolated bacteria determined as *V. anguillarum*. API 20E profile for *V. anguillarum* isolates was usually determined as 3207526. The sequenced *Vibrio* isolates were found to be similar to *V. anguillarum* strain at the rate of 98-100% in GenBank under different accession numbers. In addition, in the treatment for vibriosis, it was intended to detect the most effective agents. Results of the testing susceptibility to antibiotics showed that *V. anguillarum* isolates were resistant to 100% ampicillin, 63.6% erythromycin, 59.1% sulfamethoxazole, 54.6% streptomycin, 45.5% sulfamethoxazole-trimethoprim, and 40.9% oxytetracycline, but all strains except two *V. anguillarum* were found susceptible to 9.1% other antibiotics (oxolinic acid, enrofloxacin and florfenicol). According to the results of the susceptibility test, florfenicol and enrofloxacin were suggested as the most effective chemotherapeutics for vibriosis treatment.

KEYWORDS:
Dicentrarchus labrax, *vibrio anguillarum,* slide agglutination, API 20E, PCR.

INTRODUCTION

Vibriosis causes some of the most important diseases of marine fish and it is characterized by hemorrhagic septicemia. The disease also affects farmed bivalve mollusks and crustaceans [1]. All marine fish are probably susceptible to at least one species. *Vibrio anguillarum* is the most common pathogenic *Vibrio* strain in fish. *V. anguillarum* is a Gram-negative, facultative rod-shaped bacterium [2]. The results of 5S rRNA phylogenetic data of *V. anguillarum* were reclassified as *Listonella*, *Listonella anguillarum* [3]. *V. anguillarum* (=*L. anguillarum*) is a bacterium recently classified under the family Vibrionaceae, the Proteobacteria group, and the Gamma subdivision [2]. However, the name change has not been widely accepted, consequently, the organism is still regarded as *V. anguillarum* [2]. There are a total of 23 O-serotypes of *V. anguillarum* isolates, but only serotype O1, O2, are considered to be the most virulent serotypes and serotype O3 is, to a less extent, associated with significant fish mortalities in farmed and wild fish throughout the world [2, 4]. However, it is still not clear that which of the virulent serotypes has the highest pathogenic potential for fish [5]. Many workers have been used the API 20E system for the identification of fish-pathogenic bacteria [4, 6, 7]. *V. anguillarum* can grow in water with concentrations of NaCl in the range of 0.5%-7%. The optimum concentration is about 1%. When the pathogen is exposed to the sterilized aged lake water, it loses its culture ability without losing respiratory activity [8].

In this study, identification of vibrio species from infected farmed sea bass (*Dicentrarchus labrax* L., 1758) in the Black Sea was performed using API 20E test kits. All identified strains were subsequently confirmed by PCR using the gram negative bacteria specific 16S rRNA universal primers. In addition, for the treatment of vibriosis, it was intended to detect the most effective antimicrobials.

MATERIAL AND METHODS

Fish sampling. Specimens of the sea bass (*Dicentrarchus labrax*) were collected from the 5 different fish farms where disease outbreaks prevail (a fish farm in Persembe in the Ordu Province, three fish farms in Arsin, Darıca and Yomra in the Trabzon Province, a fish farm in Ardesen in the Rize Province) in the Southeast Black Sea, Turkey, between 2004 and 2014. All data of isolates are given in Table 1.

Isolation and identification of bacteria isolates. In the study, twenty two *V. anguillarum* strains isolated from diseased fish were used for material. These strains were isolated from live sea bass showing symptoms of vibriosis sent to the laboratory from different sea farms. Samples from kidney and spleen of infected sea bass showed that typical disease symptoms were streaked with the help of a loop onto the surface of tryptic soya agar (TSA, Merck) with 1.5% sodium chloride (salt). The plates were incubated in the cooled incubator at 20±2°C for 24 and 48 hours to obtain visible bacterial growth. Colonies were purified by spreading on thiosulphate citrate bile salts sucrose agar (TCBS, Merck). Pure yellow colonies on TCBS agar was transferred to TSA supplemented with 1.5 % salt. All strains were stored in TSB added 1.5 % salt with 20% glycerol at -80ºC until used [9].

Phenotypic characterization of bacterial isolates. Cryopreserved stocks of bacteria isolates were revived and cultured in 2 ml TSB at 20°C. All isolates were grown on tryptic soya agar for conventional biochemical tests. Biochemical characterization of all strains was carried out by the following tests: Motility test, Gram staining, catalase, oxidase activity, OF test, growth test on 5% sheep blood agar and resistance to O/129 vibriostatic agent (10 and 150µg; Oxoid). Tolerance to salt was determined by the addition of salt to 1% pepton medium with percentage of 0% and 7% and cultures were examined for growth after 7 days at 25°C. All isolates were inoculated in API 20E kits by adjusting to a turbidity matching a 0.5 McFarland standard in 1.5% sterile saline water. The API 20E kits were incubated at 25°C for 96 h [7, 10, 11].

Serologic characterization. Slide agglutination test was carried out on all isolates by using raised rabbit serum against *V. anguillarum* O1 (ATTC43305) according to Toranzo et al. [12]. Pure colonies derived from the strains were inoculated on TSA added with 1.5 % salt and incubated at 25±1°C for 48 h. They were checked by a slide agglutination test using rabbit anti-*V. anguillarum* serotype O1 serum. A dense suspension of *V. anguillarum* colonies was made in 5 µl of isotonic saline to separate glass slides. Then, 10µl of O1 antiserum was added to one of the suspensions and observed for agglutination. A negative control slide was checked for the absence of auto-agglutination [12, 13, 14].

16S rRNA sequence analysis. To prepare DNA templates for PCR assays, the strains were inoculated into 2 ml TSB containing 2% salt for 18 h at 25±1°C with shaking incubator and transferred to 1 ml centrifuge tubes, and then centrifuged at 12000×g for 3 minutes (min). After decanting the supernatant, the pellet was re-suspended in 500µl of sterile deionized water. Bacterial cells were lysed by boiling for 13 min in thermo shaker incubator and centrifuged at 13000×g for 10 min after cooling for 5 min. After the debris was removed by centrifugation and supernatants were stored in a deep freezer at -20°C, a 1-µl of supernatant was used as template for all PCRs. In the PCR program a first denaturation step at 94°C for 3 min was included, followed by 34 amplification cycles consisting of 94°C for 30 seconds (s), annealing of primers at 47°C for 40 s, and 72°C for 1 min. A final extension step of 5 min at 72°C was also included in the PCR program. The PCR products were then electrophoresed on 1% agarose gel containing 0.5 µg/ml ethidium bromide and visualized with UV light [13, 15, 16]. To identify bacteria that caused vibriosis of sea bass, the universal primers (27 F 5' *AGA GTT TGA TCC TGG CTC AG*-3', 1492 R 5' *GTT TAC CTT GTT ACG ACT T*-3') specific for 16S rRNA gene of eubacteria were used [9]. These primers were then used to yield a 1465-bp 16 S rRNA gene product by PCR. These reactions were performed in a thermal cycler. The 1465-bp PCR product was purified with a NucleoSpin PCR purification kit and PCR products were sent to Macrogen for sequencing. The results of the sequence information were used for homology searches by the BLAST (http://www.ncbi.nlm.nih.gov).

Antimicrobial sensitivity. Antimicrobial susceptibility tests of the *V. anguillarum* isolates were determined by the standard disk diffusion method on Müller-Hilton agar (Merck) plates, by using the nine antibiotics. The plates were incubated at 25°C for 20 h. The following antibiotic disks (Oxoid) were used: oxytetracycline, oxolinic acid, sulfamethoxazole, ampicillin, florfenicol, streptomycin, enrofloxacin, erythromycin and sulfamethoxazole-trimethoprim. The antibiotic disks were placed on the Müller-Hilton agar by using a disc dispenser. Reference strain *Escherichia coli* ATCC 25922 was used as quality a control in the antimicrobial susceptibility tests in Table 2 [13].

RESULTS AND DISCUSSION

During the infection period, water temperature was measured between 18.5 °C and 27.8 °C from June to October peaking in August, besides pH and salinity were found as 7.74 and 17 ppt, respectively. Mortality rates varied from 20-30% in different cages. This disease caused significant economical loses in cultured sea bass in the eastern Black Sea Region. Clinical symptoms of infected fish were erratic swimming, bilateral exophthalmia and hemorrhage in eyes, swelling in the abdomen, common reddish necrotic lesion, ulcer in the body surface, and erythema at the base of the fins, around the vent, and within the mouth. Some clinical symptoms of the infected sea bass are shown ulserative skin lesions and loose scales, exophthalmos in eye, hemorrhaging and erythema at base of fins, focal hemorrhaging and petechial on skin, gills and operculum (Fig. 1).

Internal organs showed typical findings such as enlarged spleen, pale gills, liver and kidney. The gut and rectum were distended and filled with clear yellow viscous fluid. A total of 22 *V. anguillarum* were isolated from the 200 fish samples (Table 1).

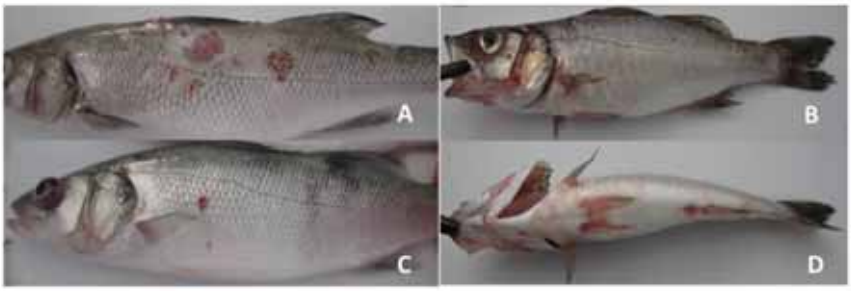

FIGURE 1
Ulserative skin lesions and loose scales (A), focal hemorrhaging and petechial on skin, gills and operculum (B), exophthalmos in eye (C), hemorrhaging and erythema at base of fins (D).

TABLE 1
Epidemiological properties of *V. anguillarum* serotype O1 strains isolated from sea bass.

İsolate no	Farmed place	Isolated organs	Isolation dates	GenBank accession no	Similarity ratio (%)
R327	Ordu / Perşembe	Kidney	20.06.2004	CP006699	98
R352	Ordu /Perşembe	Spleen	06.07.2005	KJ028214	99
R353	Trabzon / Yomra	Kidney	01.10.2006	DQ068933	99
R354	Trabzon / Yomra	Spleen	10.09.2007	DQ068933	100
R655	Trabzon/ Yomra	Kidney	28.05.2008	CP002284	100
R698	Trabzon/ Darıca	Kidney	25.07.2009	LK021130	99
R935	Rize/Ardeşen	Kidney	04.07.2010	KF150786	99
R936	Rize/Ardeşen	Spleen	25.07.2010	CP006699	99
R952	Rize/Ardeşen	Kidney	14.06.2011	LK021130	99
R953	Rize/Ardeşen	Spleen	18.08.2011	KF150786	98
R954	Rize/Ardeşen	Kidney	07.07.2012	CP006699	99
R971	Trabzon/Yomra	Kidney	03.06.2013	LK021130	99
R972	Trabzon/Arsin	Spleen	27.06.2013	LK021130	99
R973	Trabzon/Yomra	Kidney	03.06.2013	CP006699	100
R974	Trabzon/Darıca	Spleen	15.07.2013	KF150786	99
R975	Trabzon/Darıca	Kidney	09.08.2013	CP006699	99
R976	Ordu/Perşembe	Kidney	05.07.2014	LK021130	100
R977	Trabzon/Arsin	Kidney	12.07.2014	KF150786	100
R978	Ordu/Perşembe	Kidney	17.07.2014	CP006699	99
R979	Trabzon/Yomra	Kidney	20.07.2014	LK021130	99
R980	Trabzon/Yomra	Spleen	27.08.2014	KF150786	99
R981	Trabzon/Arsin	Kidney	31.08.2014	CP006699	99

TABLE 2
The comparison with the results of other researchers of the morphologic, biochemical and API 20E test results belong to *Vibrio anguillarum*.

Morphologic and Biochemical Properties	Isolates No																											
	R327	R352	R353	R354	R655	R698	R935	R936	R952	R953	R954	R971	R972	R973	R974	R975	R976	R977	R978	R979	R980	R981	Ref.-1	Ref.-2	Ref.-3	Ref.-4	Ref.-5	Ref.-6	Ref.-7
Gram stain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility by flagella	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in % 0 NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	N	-	V	N	-
Growth in % 7 NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	N	N	-	V	+	-
Effect to O/129 150µg	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	N	N	+	+	+	+
Effect to O/129 10µg	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	N	N	+	+	+	+
Acid from O/F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	N	N	+	+	+	+
Hemolysis on BA	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	N	N	N	N	N	N	N
ONPG	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	V	+
ADH	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	+	V	+	+	+	+
LDH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ODC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CIT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	+	+	V	V	+
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
URE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TDA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	V	V	+	V	V	-
VP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	V	+	+	+	+
GEL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	+	+	+	+	+
GLU	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MAN	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
INO	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	V	V	+
SOR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	V	+	+	+	+
RHA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SAC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	V	+	+	+	+
MEL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AMY	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	V	+	V	V	-
ARA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	V	V	+	V	V	+

Ref.: References, Ref.-1: Kent, (1982); Ref.-2: Maugeri et al., (1983); Ref.-3: Grisez et al., (1991); Ref.-4: Austin and Austin, 1999; Ref.-5: Tanrıkul et al., 2005; Ref.-6: Demircan and Candan, 2006; Ref.-7: Tanrıkul, 2007. +: Positive, -: Negative, V; Variable, N: not stated.

Bacterial strains were founded to be moving, gram-negative, positive of catalase and oxidase testes and fermentative of OF test. The API 20E gave positive results for β-galactosidase, arginine dihydrolase, citrate utilization, indol production, voges-proskuaer reaction and gelatin, but negative results for lysine, ornithine decarboxylase, hydrogen sulphide production, urease production and tryptophan deaminase. Carbohydrate utilization tests showed positive reactions for the acid production from sugar and the others except rhamnose, mellibiose and amygdaline. All the isolates showed results of phenotypic, biochemical and API 20E tests and they were compared with the results of other researchers' API 20E test results regarding to *V. anguillarum* isolates (Table 2).

All the tested *V. anguillarum* isolates were sensitive to the vibriostatic agent, O/129 (10µg and 150µg). All of the *V. anguillarum* isolates were growth in 1% pepton medium containing 7% sodium chloride but not growing 0% salt. All of the *V. anguillarum* isolates showed β-hemolysis onto 5% sheep blood agar plates after 24 h at 25°C. Colony characteristics on the TSA containing 2 % salt have been described as round, cream-colored, entire, raised and shiny colonies. According to the result of agglutination and biochemical tests, isolated bacteria were determined as *V. anguillarum*. API 20E profile for *V. anguillarum* isolates was usually determined as 3207526.

On a lam agglutination tests with anti *V. anguillarum* O1 serum showed that all isolates were serotype O1. All strains identified as *V. anguillarum* with biochemical and agglutination methods were subsequently confirmed by PCR using the universal primers 16S rRNA gene. According to results of the molecular diagnosis, the *Vibrio* strains isolated from sea bass were determined to be similar to *V. anguillarum* at 98-100% compared in GenBank under different accession numbers (Table 1).

In the study, antimicrobial susceptibility test breakpoints were carried out by using nine different antimicrobial agents. The method is in accordance with Clinical Laboratory Standards Institute (CLSI, 2014). The results were interpreted as described in the Clinical Laboratory Standards Institute guidelines for the family Enterobacteriaceae and Gram negative bacteria in human and veterinary medicine, including those used in aquaculture [18].

All of strains were resistant to three or more of nine antimicrobial drugs (Table 3). The highest incidence of resistance was to ampicillin (100%) followed by erythromycin (63.6%), sulfamethoxazole (59.1%), streptomycin (54.6%), sulfamethoxazole-trimethoprim (45.5%), oxytetracycline (40.9%), but all isolates were less resistant to oxolinic acid (9.1%), enrofloxacin (9.1%) and florfenicol (9.1%) (Fig. 2).

Vibriosis, due to *V. anguillarum,* is one of the most important bacterial diseases of fish throughout the world. The *Vibrionaceae* is an important and ubiquitous group of bacteria in marine and estuarine environments and these bacteria are associated with fish and other poikilothermic animals [2, 19, 20]. Among the 40 different vibrio species recorded from wild and cultured fish, nine species i.e., *Vibrio alginolyticus*, *Vibrio anguillarum*, *V. ordalii*, *V. damsela*, *V. pelagius*, *Vibrio harveyi*, *V. splendidus*, *V. salmonicida*, *V. vulnificus* were reported as pathogens infecting the marine fish [6, 21, 22]. *V. anguillarum* was isolated from different marine species by Toranzo and Barja [1].

In this study, all of the vibrio isolates were motile, oxidase and catalase positive, Gram-negative that reduced nitrate to nitrite, grew in TCBS agar medium and was sensitive to the vibriostatic agent O/129 as reported by West and Colwell, 1984 [23]. The diseased fish were noted to have petechiae at the base of the fins and on the skin. Internally, our findings are similar to those of other studies [2, 7, 24, 25]. Colony morphology on the TSA containing 2 % salt has been similarly reported by other researchers [20, 24, 26]. Tanrikul et al. (2005) reported that API 20E strips were used for the identification of *V. anguillarum* O1 which was isolated from cultured sea bass (*D. labrax* L., 1758) in Turkey [27].

TABLE 3
Antimicrobial susceptibility test breakpoints used in the study and sensitivity profile of *V. anguillarum* isolates according to antibiogram test results.

Antimicrobial agents and disc concentration	Diameter of zone of inhibition (mm)			Antimicrobial sensitivity (%)		
	R	IM	S	R	IM	S
T (30 µg)	≤ 14	15-18	≥ 19	9 (40.9%)	6 (27.3%)	7 (31.8%)
OA (2 µg)	≤ 10	11-12	≥ 13	2 (9.1%)	0	20 (90.9%)
SMZ (100 µg)	≤ 12	13-16	≥ 17	13 (59.1%)	3 (13.6%)	6 (27.3%)
AM (10 µg)	≤ 13	14-16	≥ 17	22 (100%)	0	0
FFC (30 µg)	≤ 14	15-18	≥ 19	2 (9.1%)	0	20 (90.9%)
S (10 µg)	≤ 11	12-14	≥ 15	12 (54.6%)	7 (31.8%)	3 (13.6%)
ENR (5 µg)	≤ 16	17-20	≥ 21	2 (9.1%)	0	20 (90.9%)
E (15 µg)	≤ 11	14-22	≥ 23	14 (63.6%)	6 (27.3%)	2 (9.1%)
SXT (25 µg)	≤ 10	11-15	≥ 16	10 (45.5%)	4 (18.1%)	8 (36. 4%)

AM: Ampicillin, E: Erythromycin, ENR: Enrofloxacin, FFC: Florfenicol, OA: Oxolinic acid,
T: Oxytetracycline, S: Streptomycin, SMZ: Sulfamethoxazole, STX: Sulfamethoxazole-Trimethoprim.
R: Resistance**,** IM: Intermediate, S: Sensitive.

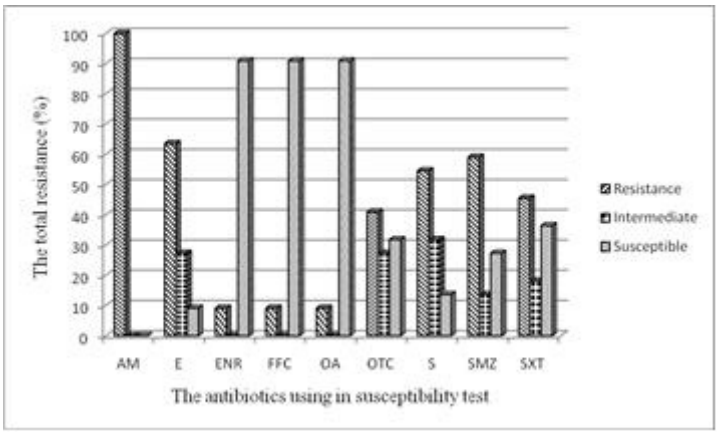

FIGURE 2
Antibiotic susceptibility profiles of *V. anguillarum* isolates.

Phenotypic characteristics of *V. anguillarum* isolates were found significantly similar in comparison with other reference study results showed in Table 2. In addition, according to the API 20E test results, the identified *V. anguillarum* isolates were found to be the similar strain as those identified in other studies [6, 17, 27, 28].

The decisive diagnosis was accomplished by PCR analysis, using universal primers specific to 16S rRNA gene of eubacteria. PCR amplified only 16S rRNA gene of isolates which was subsequently confirmed by sequencing of PCR product. Pure culture of *V. anguillarum* was isolated and confirmed by biochemical tests, lam agglutination test and sequencing of 16S rRNA gene of bacteria.

In order to cope with vibriosis, prophylactic or therapeutic antibiotics are used in fish farms. Antimicrobial compounds including oxytetracycline, sulfamethoxazole-trimethoprim, sulfamethoxazole, oxolinic acid, florfenicol and enrofloxacin have been proved to be useful in the bacterial fish diseases management [6]. However, the extensive use of antibiotics results in an increase in drug-resistance of bacteria in aquatic environments. The microbial biodiversity consisting of beneficial microbes will be affected partially by the normal micro flora of the fish. Prior to using an antibiotic, it is essential to perform susceptibility tests so as to reduce the indiscriminate use of antibiotics. Oxytetracycline and sulfamethoxazole-trimethoprim are used to control vibriosis outbreaks in the sampling marine cultured fish farms.

Although resistance frequency of oxytetracycline and sulfamethoxazole-trimethoprim are higher than oxolinic acid, enrofloxacin and florfenicol; oxytetracycline and sulfamethoxazole-trimethoprim are commonly used to treat bacterial infections of fish in the sampling region because it is cheaper than the others. This treatment protocol is really effective to control the vibriosis outbreaks according to the feedback of the fish farms in the region. Sulfamethoxazole is the most commonly used antibiotics in fish farms in Turkey. Therefore, 100% of the bacteria are resistant to sulfamethoxazole [29].

In this study, the results of susceptibility tests indicated that all the *V. anguillarum* isolates were susceptible to broad spectrum antibiotics like florfenicol, enrofloxacin, oxolinic acid, oxytetracycline by flowed sulfonamides. Several vibrio isolates have acquired resistance to the most commonly employed antibiotics (e.g., oxytetracycline and sulfamethoxazole-trimethoprim and sulfamethoxazole) in sea bass rearing. Antimicrobial test results were found similar resistances to ampicillin, streptomycin and sulfamethoxazole-trimethoprim [30]. It was reported that the resistances of bacteria against the antibiotics were gradually raised by applying oxytetracycline [17]. Consequently, random application of these antimicrobials has led to the generation of resistant strains of vibrios. Vaccination has been successfully used by intra peritoneal injection to control *V. anguillarum* infections in fish**.**

CONCLUSIONS

Consequently, random application of the antimicrobial agents has led to a generation resistant against antibiotics in *Vibrio anguillarum* strains. A judicious exploitation of antibiotics for treatment of diseases in fish farms should be followed to struggle these drugs resistance in pathogenic gram negative bacteria. In this study, infected sea bass were treated with florphenicol (30 mg Kg/day 10 days orally) and mortalities were completely controlled at the end of teatment period. It is thought to be a need on vaccine studies to protect from vibriosis (*V. anguillarum*) that cause

serious economic losses from sea bass farms in Black sea region, in the future years.

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