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Bull Vet Inst Pulawy 57, 173-177, 2013
DOI: 10.2478/bvip-2013-0032

ANTIBIOTIC RESISTANCE AND PLASMID PROFILE OF *VIBRIO ALGINOLYTICUS* STRAINS ISOLATED FROM CULTURED EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*, L.)

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Received: November 7, 2012 *Accepted: May 3, 2013*

Abstract

In the study, antimicrobial resistance and plasmid profile of *Vibrio alginolyticus* strains isolated from diseased European sea bass (*Dicentrarchus labrax*, L.) collected from a commercial farm located on the Agean Region coast of Turkey, were investigated. The agent was identified by classical biochemical tests and the API 20NE system. Bacterial strains were tested for resistance to different antimicrobials by disc agar diffusion method and minimal inhibitory concentration (MIC) at two different incubation temperatures (22 ± 2°C and 28 ± 2°C for 24 to 28 h), and screened for plasmid DNA by agarose gel electrophoresis. It was found that 15 strains were susceptible to kanamycin but they revealed resistance to ampicillin, bacitracin and streptomycin at 22°C. The strains were resistant to bacitracin and streptomycin but 14 strains were found to be intermediately resistant. One strain was resistant to ampicillin at 28°C; two strains showed sensitivity to kanamycin, whereas 12 strains were intermediately resistant, and one strain was resistant to the same antibiotic at 28°C. The strains demonstrared susceptibility to low concentrations of chloramphenicol, kanamycin, and trimethoprim (MICs of ≤ 8 µg/mL) at 22°C. The examined strains were found to be susceptible to chloramphenicol (MICs of ≤8 µg/mL) and trimethoprim (MICs of ≤8 µg/ mL) at 28°C. The strains harboured 2-3 plasmids, with sizes ranging from 68 to 126 kb.

Key words: *Dicentrarchus labrax*, *Vibrio alginolyticus*, antibiotic resistance, plasmid.

European sea bass (*Dicentrarchus labrax*, L.) is one of cultured marine fish with high economic value in the Mediterranean industry including Greece, Spain, Tunis, and Turkey (4, 5). Although this species has a great economic importance, a number of pathogenic microorganisms affect the culture of sea bass and thereby leads to the heaviest losses in the production of the species (6). One of the major bacterial diseases affecting sea bass farming is vibriosis, which leads to significant economic losses (3). The most common fish pathogenic *Vibrio* species is *Listonella* (*Vibrio*) *anguillarum*; however *V. alginolyticus* has been commonly involved in epizootic outbreaks in marine fish species (21, 22). Disease outbreaks are an important constraint to the development of aquaculture (10). Using antibiotics to treat bacterial infections and incorporation of subtherapeutic doses of antibiotics into feeds for cultured organism resulted in a global increase in antibiotic resistance among pathogenic bacteria *via* resistant plasmids (18, 20). Plasmids are found in both Gram-negative and Gram-positive bacterial species (1). Plasmid carriage by strains of different *Vibrio* species such as *L.* (*V.*) *anguillarum*, *V. fluvialis*, and *V. furnissii* has also been reported (18, 19). The aim of the study was to investigate and evaluate antimicrobial resistance and presence of plasmids in *Vibrio alginolyticus* strains isolated from an outbreak of vibriosis occurring in a commercial marine fish farm in the Aegean Region of Turkey.

Material and Methods

Sampling and characterization of bacterial strains. Fish samples (weighing 350-400 g) indicating various clinical signs like haemorrhages on fins, operculum, jaws, exophthalmia and corneal opacity, pale liver and kidney, spleen enlargement, and ascites were taken from a commercial fish farm in the Aegean Region of Turkey. The water temperature was 27 ± 2°C. Bacterial samples from the liver, spleen, kidney and blood were cultured on brain hearth infusion agar and tryptic soy agar supplemented with 1.5% (w/v) NaCl. All the inoculated media were incubated at 24 ± 2°C for

24 to 48 h. The most abundant colonies were selected and obtained in pure culture for characterization and identification. Bacterial strains isolated from affected sea bass were subjected to standard tests using the procedures described in *Bergey's Manual of Systematic Bacteriology* (11). Gram-staining, motility, morphology, cytochrome-oxidase and catalase tests, O/F glucose test, susceptibility to the vibriostatic compound O/129 (10 µg and 150 µg), and growth on thiosulphate-citrate-bile-salt-sucrose agar supplemented with 1.5% (w/v) NaCl (TCBSS) were the main tests applied to identify the bacterial strains. Afterwards, the bacterial strains were characterised by some tests for phenotypic characterisation described by Alsina and Blanch (2), and Noguerola and Blanch (17). A commercial miniaturised API 20NE system (BioMerieux) was also used according to the instructions of the manufacturer. *V. alginolyticus* (ATCC 33840) was also included in the study as a reference strain.

Antibiotic resistance assays. *In vitro* susceptibility tests of the bacterial isolates were carried out using the disc agar diffusion method according to CLSI (M42-A) (7). *Escherichia coli* ATCC 25922 was used as the quality control strain. The tested bacteria taken from 24-28 h culture (inoculated from a single colony) were suspended in nutrient broth supplemented with 1.5% NaCl. Before the bacterial suspension was spread onto Mueller-Hinton agar with 1.5% NaCl (MHAS), turbidity of the suspension was adjusted to a final optical density of 0.5 McFarland unit (1.5×10^8 CFU/mL). The bacterial isolates were tested against fifteen commercially prepared antibiotic disc, which comprised ampicillin (AMP, 10 µg), bacitracin (BCD, 0.04 µg), chloramphenicol (C30, 30 µg), compound sulfonamids (S300, 300 µg), erythromycin (E15, 15 µg), flumequine (UB 30, 30 µg), furazolidone (FR 15, 15 µg), kanamycin (K30, 30 µg), nalidixic acid (NA30, 30 µg), oxolinic acid (OA2, 2 µg), oxytetracycline (OT30, 30 µg), streptomycin (S10, 10 µg), sulfamethoxazole (RL25, 25 µg), tetracycline (TE30, 30 µg), and trimethoprim (W5, 5 µg). The tests were carried out at two different temperatures: agar plates were incubated at 22 ± 2°C and 28 ± 2°C for 24 to 28 h. All disc diffusions were performed in duplicates. The diameter of clear zone of bacterial growth surrounding the disc was measured, recorded, and averaged. The data were analysed and both strains isolated from the diseased sea bass and *E. coli* ATCC 25922 were defined a susceptible, intermediate resistant or resistant according to the CLSI (M42-A) (7) and CLSI (8).

For minimal inhibitory concentration (MIC) of the strains, chloramphenicol, erythromycin, kanamycin, oxytetracycline, streptomycin, and trimethoprim were used. The MICs of the different antibiotics were estimated by broth dilution technique (microdilution) using Mueller-Hinton broth (1% NaCl) supplemented with Mg^{++} stock solution (8.36 g $MgCl_2.6H_2O$, 100 mL H_2O) (cation adjusted Mueller-Hinton broth, CAMHB) according to CLSI (M49-A) (9). *E. coli* ATCC 25922 was used as internal standard in all tests. The tests were carried out as duplicate at the two different temperatures (22 ± 2°C and 28 ± 2°C) (9). Then, the wells were observed for growth and the initial screening MIC value was recorded. The MIC was defined as the lowest concentration at which visible growth was inhibited and the isolated strains as well as *E. coli* ATCC 25922 were defined as susceptible, intermediately resistant or resistant according to the CLSI (8), CLSI (M49-A) (9) and NCCLS *Enterobacteriaceae* breakpoints (16).

Plasmid DNA extraction. Bacterial strains harvested from a single colony were grown in a nutrient broth supplemented with 1.5% NaCl in the presence of antibiotic overnight at 28°C in an orbital shaker at 200 RPM. Suspensions (ca 20 mL) were centrifuged to obtain bacterial pellet. The pellets were resuspended in 10 mL lysis buffer and plasmids were extracted using QIAGEN Plasmid Mini Kit according to the procedure described by the manufacturer. This procedure is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to an anion exchange resin. Extracted plasmid DNA was analysed using a 0.7% agarose gel electrophoresis technique to visualise the plasmid DNA. The DNA (ca 2 µg) was digested using restriction endonucleases (*Aat* I, *Bam* HI, *Cla* I, *EcoR* I, *Hae* III, *Hind* III, *Hinf* I, *Msp* I, *Rsa* I, and *Vsp* I). Digested products were seperated in 2% High Range DNA ladder (10171-48502 bp) (Fermentas). Products were stained in ethidium bromide, destained in water, and photographed using UVPro programme.

Results

All strains were Gram-negative rod shape, motile by polar flagella, cytochrome oxidase, and catalase positive, demonstrating the ability to ferment glucose, and sensitivity to the vibriostatic agents (10 µg and 150 µg). For such reasons, 15 strains isolated from the affected fish tentatively were identified as the genus *Vibrio*. The profile numbers of API 20NE Strep of the strains and reference bacteria *V. alginolyticus* (ATCC 33840) are 3476744.

According to the test, the results obtained at 22 ± 2°C, demonstrate that all isolates tested were resistant to ampicillin, bacitracin, as well as streptomycin (Table 1). However, the strains were found to be susceptible to chloramphenicol, flumequine, furazolidone, kanamycin, nalidixic acid, tetracycline, and trimethoprim. Fourteen isolates showed intermediate resistance to oxytetracycline and one isolate was sensitive (35 mm ≤) to this antibiotic. Fourteen isolates were intermediately resistant to erythromycine, but one strain showed sensitivity to this antibiotic. Eleven strains showed sensitivity to sulfamethoxazole but the remaining strains were resistant to it. According to the test, the results obtained at 28 ± 2°C, demonstrate that all isolates were resistant to bacitracin and streptomycin (Table 1). However, the strains were found susceptible to eigth different antibiotics that included chloramphenicol, compound sulfonamids, flumequine, furazolidone, nalidixic acid, sulfamethoxazole, tetracycline, and trimethoprim. Inhibition zone at two different incubation temperatures revealed that the strains were resistant to bacitracin and

streptomycin. Furthermore, 15 strains were resistant against compound sulfonamids and four strains were non-susceptible against sulfamethoxazole at 22 ± 2°C. It was noted that these strains were susceptible to the same antibiotics at 28 ± 2°C. *E. coli* ATCC 25922 was resistant to bacitracin and was susceptible to chloramphenicol, flumequine, kanamycin, nalidixic acid, streptomycin, tetracycline, and trimethoprim both at 22 ± 2°C and 28 ± 2°C (Table 2). The reference strain revealed susceptibility to sulfamethoxazole at 22 ± 2°C, however was resistant to the same antibiotic at 28 ± 2°C. All strains were susceptible to low concentrations of chloramphenicol, kanamycin, and trimethoprim at 22 ± 2°C. Thirteen strains showed resistance to erythromycin (MICs≥8 µg/mL), whereas two strains were found to be intermediately resistant to the same antibiotic. Seven strains were resistant to oxytetracycline (MICs≥16 µg/mL); however, the remaining strains showed intermediate resistance to this antibiotic. MICs of four antibiotics (chloramphenicol, kanamycin, streptomycin, and trimethoprim) for *E. coli* ATCC 25922 were not detected as no bacterial growth was observed. However, the bacterium showed a resistance to erythromycin and oxytetracycline (MICs≥8 µg/mL, MICs of≤2 µg/mL, respectively) at 22 ± 2°C. All strains were susceptible to chloramphenicol and trimethoprim at 28 ± 2°C. Six strains showed resistance to oxytetracycline (MICs≥16 µg/mL); however, it was found that one strain was susceptible to this antibiotic (MICs≤4 µg/mL), while the remaining strains were intermediately resistant to this antibiotic. MICs of streptomycin for *E. coli* ATCC 25922 at 28 ± 2°C were not detected but the bacterium showed resistance to erythromycin and oxytetracycline (MICs≥8 µg/ml, ≥8 µg/mL, respectively). The bacterium was susceptible to chloramphenicol (MICs≤ µg/mL), kanamycin (MICs≤16 µg/mL), and trimethoprim (MICs≤ 8 µg/mL). When comparing MICs results of the strains for antibiotics tested at two different incubation temperatures, all strains were sensitive to chloramphenicol, kanamycin, and trimethoprim at 22 ± 2°C. Nevertheless, the strains showed an intermediate resistance to kanamycin at 28 ± 2°C. Thirteen strains were resistant and two of them were found to be intermediately resistant to erythromycin at 22 ± 2°C but all strains showed resistant to the same antibiotic at 28 ± 2°C. Bacterial growth in the positive control wells of plates tested at both incubation temperatures was observed.

Table 1
Antimicrobial test results of *V. alginolyticus* strains (n=15) at 22 ± 2°C and 28 ± 2°C

22 ± 2°C				28 ± 2°C			
Antimicrobial	Disc diffusion			Antimicrobial	Disc diffusion		
	S[a]	IR	R		S	IR	R
AMP			15	AMP		14	1
BCD			15	BCD			15
E15	1	14		E15	1	14	
UB30	15			UB15	15		
FR15	15			FR15	15		
K30	15			K30	2	12	1
C30	15			C30	15		
NA30	15			NA30	15		
OA2	10	5		OA2		15	
OT30	1	14		OT30		15	
S10			15	S10			15
RL25	11		4	RL25	15		
S300	1		14	S300	15		
TE30	15			TE30	15		
W5	15			W5	15		

[a]S - sensitive, IR - intermediate resistant, R - resistant

Table 2
Antimicrobial test results of *E. coli* ATCC 25922 (n=1) at 22 ± 2°C and 28 ± 2°C

22 ± 2°C				28 ± 2°C			
Antimicrobial	Disc diffusion			Antimicrobial	Disc diffusion		
	S[a]	IR	R		S	IR	R
AMP		1					1
BCD			1	BCD			1
E15		1		E15		1	
FR15	1			FR15	1		
UB30	1			UB30	1		
K30	1			K30	1		
C30	1			C30	1		
NA30	1			NA30	1		
OA2		1		OA2		1	
OT30			1	OT30			1
S10			1	S10		1	
RL25	1			RL25			1
S300	1			S300	1		
TE30	1			TE30	1		
W5	1			W5	1		

[a]Symbols as in Table 1

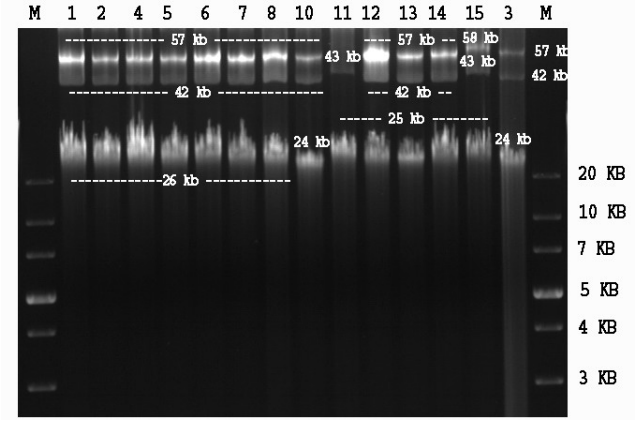


Fig. 1. Profile of plasmids isolated from bacterial strains. M: marker

Table 3
Plasmid sizes of strains used in the study

Strain number	Plasmid sizes (kb)			
	Plasmid 1	Plasmid 2	Plasmid 3	Total (kb)
1	57	42	26	125
2	57	42	26	125
3	57	42	24	123
4	57	42	26	125
5	57	42	26	125
6	57	42	26	125
7	57	42	26	125
8	57	42	26	125
10	57	42	24	123
11	43	25		68
12	57	42	25	124
13	57	42	25	124
14	57	42	25	124
15	58	43	25	126

When comparing MICs results of *E. coli* ATCC 25922 for antibiotics tested at the two different incubation temperatures, it was observed that the bacterium did not grow at 22 ± 2°C in wells containing CAMHB with chloramphenicol, kanamycin, and streptomycin. As a controversy to this finding, the bacterium was sensitive to kanamycin and trimethoprim at 28 ± 2°C, but it did not grow in well containing CAMHB with streptomycin at 28 ± 2°C. The bacterium was resistant to trimethoprim at 22 ± 2°C; however, it was susceptible to this antibiotic at 28 ± 2°C.

Plasmids extracted were seperated on 0.7% agarose gels for 6 h and 12 h. Longer electrophoresis (12 h) showed that nicked, circular, linear covalently closed supercoiled and supercoiled plasmids were present in each strains indicating the successful purification of plasmid DNA. Most of the plasmids in longer electrophoresis diffused and it was difficult to present different forms of the plasmids in a gel image. Therefore, Fig. 1 contains a gel image taken at 6 h of electrophoresis. Numbers of plasmids isolated from the strains were 2 and 3 depending on the isolates. It was found that isolates numbers 1-8, 10, 12, 15 contained three plasmids while isolates numbered 11 had two plasmids. Strain 9 was not included in the study because of subculturing problems. Size and number of plasmids from each isolate are shown in Table 3. It was determined that the plasmids were of three different sizes (small, intermediate, and large). Small plasmids were approximately 24, 25, and 26 kb, intermediate plasmids were 42 and 43 kb, sizes of large plasmid were 57 and 58 kb. Strains 1, 2, and 4-8 had three plasmids and size of total 125 kb. Strain 10 had also three plasmids but the total size was 123 kb. Similarly, strain 15 had three plasmids and its total plasmid size was different from strain 10 (126 kb). Other strains (12-14) had three plasmids and their total size of plasmids was found to be 124 kb. Strain 11 had two plasmids and its total plasmid size was 64 kb. Restriction enzyme digestion studies were used to differentiate plasmids extracted from different isolates. Plasmids from isolates 11 and 12 differed from each other and the rest of the plasmids extracted from other isolates. Digestion patterns of restriction enzyme *EcoR* I also confirmed that plasmids from isolates 11 and 12 were different from each other and the rest of the plasmids. Restriction enzyme *Hind* III digested more frequently in some plasmid DNA sites and these small DNA fragments moved away from agarose gel in a short time. Restriction digestion patterns of *Hind* III also confirmed the findings of *Msp* I and *EcoR* I restriction enzymes. When electrophoresis analysis of plasmid DNA digested by restriction enzyme *Bam* HI was examined, at least one of the plasmids from isolate 11 was found to be different from the others.

Discussion

Bacteriological analysis of the infected sea bass resulted in isolation of 15 *Vibrio alginolyticus* strains. The strains were identified as *V. alginolyticus* according to schemes of Alsina and Blanch (2), Farmer *et al.* (11), and Noguerola and Blanch (17). The studied strains were ONPG-positive and lysine decarboxylase negative. When the incubation zone results for 22 ± 2°C and 28 ± 2°C were compared, the strains showed resistance to ampicillin, bacitracin, and streptomycin at 22 ± 2°C but they were found to be resistant to bacitracin and streptomycin; however, 14 strains showed intermediate resistance, and one strain was resistant to ampicillin at 28 ± 2°C. Although the strains were susceptible to kanamycin at 22 ± 2°C, 12 strains were intermediately resistant and one strain was resistant to the same antibiotic at 28 ± 2°C. These results matched with the results published by Zanetti *et al.* (23). Miller *et al.* (14) reported that erythromycin, being active for Gram-positive bacteria, was effective against *E. coli* and *Aeromonas salmonicida* subsp. *salmonicida* under *in vitro* conditions at 22 ± 2°C and 28 ± 2°C. However, 14 *V. alginolyticus* strains in the study were intermediately resistant to erythromycin and one strain was sensitive to the same antibiotic at 22 ± 2°C and 28 ± 2°C. The MICs of *V. alginolyticus* and *E. coli* ATCC 25922 were difficult to compare with published results because the MIC determination tests were done under different

conditions and with different antimicrobial agents from those used in other studies; however, the MICs obtained from the study were similar to those presented by Laganà *et al*. (12) and Mohney *et al.* (15). In the study, eight *V. alginolyticus* strains were sensitive to oxytetracycline and their MIC values were 4 µg/mL. These results were similar to the findings of Laganà *et al*. (12). The remaining strains showed intermediately resistance to this antibiotic with MIC values 8 µg/mL at 22 ± 2°C. However, while one strain was sensitive to oxytetracycline, eight strains showed an intermediate resistance to this antibiotic and six strains were resistant to the same antibiotic at 28 ± 2°C. Bacterial resistance is usually associated with the presence of plasmids and the ability for transconjugation. Generally, the trans-conjugated plasmids possess high molecular weight (13). Fourteen *V. alginolyticus* strains in the study have been found to harbour 2-3 plasmids, with sizes ranging from 68 to 126 kb. These results remain with the agreement with the results of Li *et al.* (13). The authors reported that the *V. alginolyticus* strains harboured 1-4 plasmids, with sizes ranging from 18-123 kb. According to the latest available data about *Vibrio alginolyticus* in Turkey, this is the first study describing plasmid isolation and effects of the different incubation temperatures of antimicrobial agents on *V. alginolyticus* strains recovered from diseased European sea bass.

Acknowledgments: This work was supported by Akdeniz University Scientific Projects Unit under Project No. 2007.01.0121.003. It was a part of the project.

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