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Antibiotic resistance of *Vibrio* species isolated from *Sparus aurata* reared in Italian mariculture

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SUMMARY

Extensive use of antimicrobial agents in finfish farming and the consequent selective pressure lead to the acquisition of antibiotic resistance in aquaculture environment bacteria. *Vibrio* genus represents one of the main pathogens affecting gilthead sea bream. The development of antibiotic resistance by *Vibrio* represents a potential threat to human health by exchange of resistant genes to human pathogens through food chain. The objective of the present study was to conduct a multisite survey on the antibiotic resistance of *Vibrio* spp. isolated from gilthead sea bream reared in Italian mariculture. *Vibrio* spp. strains were isolated from skin, gills, muscles and intestinal content of 240 gilthead sea bream. A random selection of 150 strains was sequenced for species identification. Resistance against 15 antimicrobial agents was tested by the broth microdilution method. *Vibrio harveyi* and *Vibrio alginolyticus* accounted for 36.7% and 33.3% of the isolates respectively. 96% of the strains showed multiple resistance to the tested drugs, with two strains, *Vibrio aestuarianus* and *Vibrio harveyi* resistant to 10 and 9 antibiotics, respectively. Ampicillin, amoxicillin, erythromycin and sulfadiazine showed low efficacy against *Vibrio* spp. Rational use of antimicrobial agents and surveillance on antibiotic administration may reduce the acquisition of resistance by microorganisms of aquatic ecosystems.

KEY WORDS: Antibiotic resistance, *Vibrio* spp., Aquaculture, *Sparus aurata.*

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INTRODUCTION

In the last thirty years the world demand for seafood products has increased, driving the growth of finfish and shellfish mariculture. Among European countries Italy ranks in the top five for annual production with about 6% of the total aquaculture production (FAO 2012). Gilthead sea bream (*Sparus aurata*) is one of the most important finfish for marine aquaculture and is commonly reared in the Mediterranean basin with Greece, Turkey, Spain and Italy representing the main producing countries (EC 2012). Large-scale aquaculture is characterized by intensive cultivation methods with high stocking density leading to problems related to poor hygienic conditions (Diana *et al.,* 2013). As a consequence, the incidence of some fish diseases caused by bacterial infections is increasing in aquaculture production. *Vibrio* spp. strains are widespread in the marine environment, especially in tropical and temperate waters, and they represent the major bacterial pathogens affecting fish farming in the Mediterranean Sea (Pujalte *et al.,* 2003).

The term *vibriosis* is commonly used to refer to bacterial diseases affecting wild and farmed fish caused by members of the *Vibrionaceae* family. The genus *Vibrio* includes some species that behave as opportunistic pathogens and can be involved both in cultured gilthead sea bream outbreaks (Balebona *et al.,* 1998) and in cases

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of human disease (Austin and Zhang 2006; Austin 2010). The significance of *vibriosis* as public health risk is associated with the increased exposure due to some cultural traditions of eating raw or minimally processed fishery products especially in many Western countries (WHO 2004).

Antibiotics in aquaculture are largely used for therapeutic and prophylactic purposes (Bjørklund *et al.,* 1991; Primavera *et al.,* 1993; Hirsch *et al.,* 1999; Jerbi *et al.,* 2011). Despite their widespread use, there is still little availability of registered drugs for aquaculture with only 7 antimicrobial drugs approved in Europe (Furones and Rodgers, 2009). Worldwide a great variation exists in antibiotic regulation and enforcement causing differences in the levels of consumer protection with regard to antibiotic residues. European Regulation EC 37/2010 and its amendment establish maximum residue limits in foodstuffs of animal origin, including fish.

Regulating the extent of antimicrobial usage is aimed to guarantee the safety and efficacy of the antibiotics used to treat animal diseases and to protect consumer health. A consequence of the indiscriminate use of antibiotics in aquaculture is the promotion of antimicrobial resistance in the bacterial population (Alderman & Hastings, 1998; Teuber, 2001). Antibiotic-resistant bacteria may represent a potential threat to human health due to direct transmission through the food chain (Duran and Marshall 2005) or by transferring the acquired antimicrobial resistance to human pathogens by mobile genetic elements (Angulo, 2000; Serrano, 2005; Guglielmetti *et al.,* 2009). Although several investigations have been conducted in different countries regarding antibiotic resistance in *Vibrio* spp. isolated from aquaculture (Dang *et al.,* 2006; Akinbowale *et al.,* 2007; Laganà *et al.,* 2011; Rebouças *et al.,* 2011; Raissy *et al.,* 2012), little research focused on *Vibrio* spp. isolated from gilthead sea bream (Snoussi *et al.,* 2006).

The present study aimed to investigate the susceptibility of *Vibrio* spp. strains isolated from *Sparus aurata* reared in Italian mariculture farms, in order to provide useful information to implement surveillance programmes on antibiotic resistance.

MATERIALS AND METHODS

Fish sampling and microbiological analysis

Gilthead sea bream with no clinical signs of bacterial infections were obtained from mariculture farms located in three different Italian regions (Sardinia, Tuscany and Sicily). From each region two farms characterized by intensive fish rearing system in sea cages were selected. The sea-water salinity was 33‰ and the temperature ranged between 16°C and 22°C. Each farm was visited twice at 4-month intervals and, during each visit, 20 randomly selected *Sparus aurata* at commercial size (about 250 g) were caught and slaughtered by immersion under fusing ice. After collection, fish samples were placed in expanded polystyrene boxes, covered with a plastic film, transported refrigerated to the laboratory and processed for microbiological analysis within 3 hours. From each fish, skin, gills, muscles and intestinal content were aseptically collected in order to detect *Vibrio* spp. Samples were diluted with sterile Phosphate Buffered Saline (PBS, Oxoid, Basingstoke, UK) and incubated at 30°C for 24 hours. After incubation, 0.1 mL were inoculated onto Thiosulphate Citrate Bile Salts Sucrose Agar plates (TCBS Colera Medium, Oxoid, Basingstoke, UK) supplemented with 1.5% (w/v) of NaCl and then incubated at 30°C for 48 hours. The colonies with typical aspect were subcultured on Trypticase Soy Agar (TSA, Oxoid, Basingstoke, UK) and submitted to the following physiological and biochemical identification tests: Gram staining, cytochrome-oxidase, motility, H_2S production, growth on TSA with NaCl (3%) and susceptibility to the vibriostatic agent O-129 (2,4-diamino-6,7-diisopropylpteridine). After presumptive identification, strains were stored at -80°C for further analysis.

Vibrio spp. identification and statistical analysis

Total genomic DNA was extracted using the boiling method. Briefly, 1 mL of broth culture was centrifuged at 10,000 x *g* for 5 min and the pellet resuspended in 500 µL of sterile water. Samples were boiled for 10 min and centrifuged again at 10,000 x *g* for 5 min. Two-hundred µL of the supernatant were picked and stored at -20°C. DNA concentration was estimated spectrophotometrically (Shimadzu,

Duisburg, Germany) before use. Detection of *rpoA* gene, a conserved region of *Vibrio* genus, was carried out according to Dalmasso *et al.* (2009). PCR reactions were performed using a Thermal Cycler GeneAmp PCR 9700 (Applied Biosystems, Carlsbad, USA). Five µL of each amplicon were loaded into 2% agarose gel (Bio-Rad Laboratories, Hercules, USA) and submitted to linear electrophoresis at 90 volts for 60 min. After staining with ethidium bromide, gels were visualized by a UV-transilluminator (Gel Doc XR, Bio-Rad Laboratories, Hercules, USA) and analyzed with Quantity One software (Bio-Rad Laboratories, Hercules, USA). A 100 bp DNA Ladder (Invitrogen Ltd, Paisley, UK) was used as a reference marker. A selection of *Vibrio* spp. strains was submitted to sequencing for species identification. From each region 50 strains were selected. Within each region the strains were selected as representative of fish farm, sampling time, fish and matrices within fish. In order to avoid clonality, selection criteria excluded strains originated from the same sample. Bacteria species were identified by sequencing of the *rpoA* gene fragment. Amplified products were purified by means of Exo-Sap treatment according to the manufacturer's recommendations (USB Europe, Staufen, Germany). Forward and reverse sequencing reactions were performed using the ABIPrism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 (Applied Biosystems, Foster City, CA, USA). The extension products were purified with DyeEx 2.0 Spin kit (Qiagen, Valencia, CA, USA) and resolved by capillary electrophoresis using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The electropherograms were analyzed using Chromas 2.22 software (Technelysium, Epoch Life Science Inc) and the sequences were submitted to the BLAST similarity search software on the National Center for Biotechnology Information (NCBI) website. The effects of location (Sardinia, Tuscany and Sicily), sampling time (first visit and second visit) and fish matrix (skin, gills, muscle and intestinal content) on *Vibrio* spp. isolation were investigated using logistic regression analysis. Differences between regions in the prevalence of *Vibrio* spp. recovered from the different fish matrices were investigated using the chi-Square test of independence. Statistical analysis was performed using Statgraphics software (Centurion XVI, StatPoint Technologies, Warrenton, VA, USA).

Antibiotic susceptibility

After species identification, Minimum Inhibitory Concentrations (MICs) of 15 antibiotics were performed by broth microdilution method (CLSI 2007, 2010). Antibiotics were selected among the most commonly used in aquaculture and human therapy: ampicillin (AM), amoxicillin (AMX), cephalothin (CF), cloramphenicol (CL), erythromycin (E), florfenicol (FF), flumequine (FM), gentamicin (GM), kanamycin (K), oxolinic acid (OXA), oxytetracycline (OT), streptomycin (S), sulfadiazine (SZ), tetracycline (TE) and trimethoprim (TMP). Antibiotic powders (Sigma-Aldrich, St. Louis, USA) were weighed, dissolved in appropriate solvents to obtain stock solutions (2,560 µg/mL) and frozen at -80°C. The preparation of each microtitre plate was carried out with 12 twofold serial dilutions of each antibiotic stock solution (Work Station - Micro Star, Hamilton, Bonaduz GR, Switzerland) using deionized sterile water with the exception of AMP and AMX which were diluted in phosphate buffer (pH 6.0, 0.1 mol/L). Final antibiotic concentrations ranging between 0.06 µg/mL and 128 µg/mL (0.12-512 µg/mL for SZ antibiotic) were obtained. Isolates were subcultured twice on Brain Heart Infusion agar (BHI, Oxoid, Basingstoke, UK). After overnight incubation at 37°C, two or more colonies were picked from BHI agar plates and transferred into sterile saline solution (0.85% w/v) obtaining a 0.5 McFarland turbidity suspension (Densimat, bioMérieux, Lyon, France). In order to adjust the concentration to about 10^6 cfu/mL bacterial suspensions were diluted in Cation-Adjusted Mueller Hinton Broth (CAMHB, Oxoid, Basingstoke, UK) supplemented with NaCl (1% w/v). Fifty microlitres of the final suspension were dispensed onto microtitre wells containing 50 µL of each antimicrobial agent dilutions. The reference strain *E. coli* ATCC 25922 was used as quality control. Then each microplate was incubated under aerobic conditions at 35°C for 18-20 hours. Antibiotic resistance was determined comparing the MIC of each antibiotic with the breakpoint values (CLSI 2005, 2007). The MICs' range and mode,

MIC_{50} and MIC_{90} of each antimicrobial agent were also determined.

RESULTS

Prevalence and identification of Vibrio spp.
Overall, 240 *Sparus aurata* were collected and *Vibrio* spp. occurred in 141 (58.7%) skin samples, 165 (68.7%) gill samples, in 23 (9.6%) muscle samples and 99 (41.2%) intestinal content. The logistic regression showed that the recovery of *Vibrio* spp. was significantly affected by region ($P<0.001$), sampling time ($P<0.001$) and fish matrix ($P<0.001$). The test of independence showed a significant association between the recovery of *Vibrio* spp. from a given matrix and the location (Table 1). Sequencing conducted on the selection of 150 strains (50 from each region) identified 17 different *Vibrio* species. The most represented were *V. harveyi* and *V. alginolyticus* with 55 and 50 strains, respectively (Table 2).

Antimicrobial susceptibility
The susceptibility of 150 *Vibrio* strains was assessed against 15 different antibiotics. Over 90% of the tested strains showed susceptibility to OXA, CL, GM, K, OT and TE. Multiple resistance was observed in 144 strains (96.0%) with two strains, identified as *V. aestuarianus* and *V. harveyi*, resistant to 10 and 9 antibiotics, respectively. In strains with multiple resistance the most frequent antibiotic combination was AM, AMX, E and SZ (Table 2). Table 3 reports the MIC_{50}, MIC_{90}, mode and range of MICs, the number of the susceptible, intermediate and resistant strains according to the breakpoints proposed by NCCLS and CLSI.

DISCUSSION

Gene sequencing conducted on a selection of 150 *Vibrio* spp. strains isolated from *Sparus aurata* showed that the most represented species were *Vibrio harveyi* (36.7%) and *V. alginolyticus* (33.3%).
In our study, the prevalence for these two species was greater compared with another survey conducted in Spain, reporting 13.6% and 21.4% of V. *harveyi* and *V. alginolyticus*, respectively (Zorrilla *et al.,* 2003). *V. harveyi* is considered one of the main pathogens of marine fish in aquaculture (Cano-Gomez *et al.*, 2009), though sporadic cases of infection have been reported also in humans (Pavia *et al.,* 1989; Wilkins *et al.,* 2007). Instead, *V. alginolyticus* is an opportunistic pathogen for gilthead sea bream (Balebona *et al.,* 1998; Zorrilla *et al.,* 2003; Austin and

TABLE 1 - *Comparison of the prevalence of Vibrio spp. isolated from Sparus aurata in 3 different Italian regions by sampling time and fish matrix.*

Sampling	*Matrix*	*Location*						
		Sardinia		*Sicily*		*Tuscany*		
		n	*%*	*n*	*%*	*n*	*%*	*P value*
First	Skin	21/40	52.5	24/40	60.0	6/40	15.0	<0.001
	Gills	30/40	75.0	38/40	95.0	16/40	40.0	<0.001
	Muscle	10/40	25.0	0/40	0.0	0/40	0.0	<0.001
	Intestinal content	26/40	65.0	18/40	45.0	5/40	12.5	<0.001
Second	Skin	40/40	100	38/40	95.0	12/40	30.0	<0.001
	Gills	40/40	100	16/40	40.0	25/40	62.5	<0.001
	Muscle	12/40	30.0	0/40	0.0	1/40	2.5	<0.001
	Intestinal content	23/40	57.5	18/40	45.0	9/40	22.5	ns

TABLE 2 - *Antimicrobial resistance of 150 Vibrio strains isolated from Sparus aurata.*

Species	*n. (%)*	*OXA*	*AMX*	*AM*	*CF*	*CL*	*E*	*FM*	*FF*	*GM*	*K*	*OT*	*S*	*SZ*	*TE*	*TMP*
V. harvey	55 (36.7%)	2	48	48	8	1	44	5	3	1	1	1	8	42	1	15
V. alginolyticus	50 (33.3%)	8	41	40	28		18	10		1	2	1	10	37		8
V. diabolicus	11 (7.3%)		10	9	9		3		1	1				10		1
V. mytili	9 (6%)		1	1			5					1		8	1	
V. parahaemolyticus	4 (2.7%)			1	1		1						1	4		
V. ordalii/anguillarum	3 (2%)				1		1							3		
V. casei	3 (2%)				2		3							3		
V. litoralis	2 (1.3%)		1				2							1		
V. aestuarianus	2 (1.3%)	1	1	1	1		2	1	1				1	2		1
V. harveyi/owensii	2 (1.3%)	1			2		2	1						2		2
V. tasmaniensis	2 (1.3%)		2	2	2		2		2				1	2		2
V. orientalis	2 (1.3%)	1				1	2	1	1		1			2		2
V. mediterranei	1 (0.7%)															
V. communis /owensii	1 (0.7%)		1	1										1		
V. orientalis/hepatarius	1 (0.7%)		1	1	1		1	1	1					1		1
V. gigantis	1 (0.7%)		1	1	1		1			1			1	1		1
V. vulnificus	1 (0.7%)	1						1						1		
Total	150	14	107	105	56	2	87	20	9	4	4	3	22	120	2	33

Oxolinic acid (OXA), ampicillin (AM), amoxicillin (AMX), cephalothin (CF), cloramphenicol (CL), erythromycin (E), florfenicol (FF), flumequine (FM), gentamicin (GM), kanamycin (K), oxytetracycline (OT), streptomycin (S), sulfadiazine (SZ), tetracycline (TE) and trimethoprim (TMP).

Austin, 2007) and humans (Horii *et al.,* 2005). Although *V. parahaemolyticus* and *V. vulnificus* represent other important human pathogens, in the present study they were isolated with low prevalence, accounting for 2.7% and 0.7%, respectively. In Europe, the use of antimicrobial agents is regulated differently from country to country (Daniel, 2002), being either very strict or under-regulated.

High costs are necessary to license antimicrobial agents, therefore there is still little availability of products authorized in Europe for use in aquaculture (Schnick *et al.,* 1997; Daniel, 2002). The active substances approved in Italy for aquaculture are: amoxicillin, flumequine, oxytetracycline, chlortetracycline, and sulfadiazine-trimethoprim. This picture might increase the indiscriminate and extra-label use of antibiotics leading to the acquisition of resistance against these molecules by microorganisms (WHO/FAO/OIE 2006).

Possible hazards associated with drug abuse in fish farming are the presence of residues in food and the development of antibiotic resistance in the bacterial population. *Vibrio* spp. showed different susceptibility against cephalosporin and penicillins.

Resistance to cephalothin was observed in 37.3% of the strains, while over 70% of the strains were resistant to ampicillin and amoxicillin. Our results are consistent with previous studies reporting β-lactamic resistance rang-

TABLE 3 - *MICs (µg/mL) and antimicrobial susceptibility of Vibrio spp. strains isolated from Sparus aurata.*

Antibiotic	*Breakpoint*	*MIC_{50}*	*MIC_{90}*	*Moda*	*Range*	*S (%)*	*I (%)*	*R (%)*
OXA	<4->8[a]	0.25	4	0.25	0.06-32	136 (90.7)	-	14 (9.3)
AMX	<8->32[b]	>128	>128	>128	0.06->128	40 (26.7)	3 (2.0)	107 (71.3)
AM	<8->32[b]	>128	>128	>128	0.06->128	34 (22.7)	11 (7.3)	105 (70.0)
CF	<8->32[b]	16	>128	8-16	0.06->128	61 (40.7)	33 (22.0)	56 (37.3)
CL	<8->32[b]	0.5	8	0.5	0.06-64	141 (94.0)	7 (4.7)	2 (1.3)
E	<0.5->8[c]	8	128	8	0.06->128	8 (5.3)	55 (36.7)	87 (58.0)
FM	<2->4[a]	0.5	4	0.5	0.06-128	130 (86.7)	-	20 (13.3)
FF	<2->8[a]	0.5	4	0.5	0.06-32	127 (84.7)	14 (9.3)	9 (6.0)
GM	<4->16[b]	2	4	2	0.06->128	136 (90.7)	10 (6.7)	4 (2.7)
K	<16->64[d]	8	16	8	0.25->128	140 (93.3)	6 (4.0)	4 (2.7)
OT	<4->16[b]	1	4	1	0.12-128	137 (91.3)	10 (6.7)	3 (2.0)
S	<6->25[d]	8	32	4-8	0.25->128	55 (36.7)	73 (48.7)	22 (14.7)
SZ	<256->512[d]	>512	>512	>512	0.12->512	30 (20.0)	-	120 (80.0)
TE	<4->16[b]	0.5	2	0,5	0.06-64	146 (97.3)	2 (1.3)	2 (1.3)
TMP	<8->16[d]	4	64	4	0.06-128	117 (78.0)	-	33 (22.0)

Oxolinic acid (OX), ampicillin (AM), amoxicillin (AMX), cephalothin (CF), cloramphenicol (CL), erythromycin (E), florfenicol (FF), flumequine (FM), gentamicin (GM), kanamycin (K), oxytetracycline (OT), streptomycin (S), sulfadiazine (SZ), tetracycline (TE) and trimethoprim (TMP). [a]=M3-A3 (CLSI 2007); [b]=M45-P (CLSI, 2005); [c]=M31A (NCCLS, 1999); [d]=M100-S22 (CLSI, 2012).

ing between 14.0% and 100% (Zorrilla *et al.,* 2003; Snoussi e*t al.,* 2006; Laganà *et al.,* 2011; Snoussi *et al.,* 2011). The high rate of resistance to β-lactamics could be explained with the susceptibility of these antibiotics to β-lactamase expressed by *Vibrio* spp. (Roque *et al.,* 2001). *Vibrio* spp. showed a low resistance against oxolinic acid (9.3%) and flumequine (13.3%), two registered quinolone antibiotics widely used in aquaculture (WHO/FAO/OIE 2006). The literature reports a wide range of resistance for these drugs ranging from 8.7% to 60.0% (Akinbowale *et al.,* 2006; Laganà *et al.,* 2011; Snoussi *et al.,* 2011). In our study, more than 50.0% of the strains resistant to these antibiotics were *V. alginolyticus*, with 16.0% of the isolates resistant to oxolinic acid and 20.0% to flumequine. The relationship between quinolone resistance and *V. alginolyticus* was previously reported (Snoussi *et al.,* 2008). The tetracyclines are a group of antibiotics widely used in human and veterinary practices for the treatment of vibriosis because of their broad spectrum activity, low toxicity and their cost-effectiveness (Morris, 2003; Silva *et al.,* 2010). Although oxytetracycline is the most common antibiotic licensed in Europe and used in aquaculture, only 2% of the isolates showed resistance. Similar efficacy was also showed by tetracycline with 1.3% of resistant strains. Several studies support the great susceptibility of *Vibrio* spp. to tetracycline observed in this study (Zorrilla *et al.,* 2003; *Akinbowale et al.,* 2006; Vignesh *et al.,* 2012). Although erythromycin is not included in the list of antibiotics registered for treatment of mariculture finfish, maximum residue limits are established by Regulation EC 37/2010. In the macrolides class, erythromycin demonstrated little efficacy with 80% of resistant strains. Similar findings were also observed in *Vibrio* strains isolated in Tunisian and Malaysian aquaculture (Snoussi *et al.,* 2008; Snoussi *et al.,* 2011; Lajnef *et al.,* 2012). Resistance is to be expected since Gram-negative bacteria are char-

acterized by intrinsic resistance to macrolides mediated by efflux mechanisms (Poole, 2005). The combination of sulfadiazine-trimethoprim antibiotics is among the few drugs authorized for aquaculture in Italy. In the present study these antibiotics were individually tested. Sulfadiazine demonstrated low efficacy against *Vibrio* spp., with 80.0% of strains resulting resistant. This is higher than the data obtained by Zheng *et al.* (2011) who observed resistance in 48.6% of the isolates. Conversely, resistance to trimethoprim was found in 22.0% of the isolates, lower than the rates reported by Lajnef *et al.* (2012) and Jun Li *et al.* (1999), 68.0% and 76.0% respectively. Overall, aminoglycoside antibiotics showed a high efficacy with resistance ranging from 2.7% for kanamycin and gentamycin to 14.7% for streptomycin. However, streptomycin was characterized by many intermediate strains (48.7%), thus indicating that the drug is associated with an uncertain *in vitro* therapeutic effect. Though not registered, fenicol antibiotics, cloramphenicol and florfenicol, proved effective against *Vibrio* spp.

The present study demonstrated that gilthead sea bream reared in mariculture farms are a potential source of *Vibrio* spp. The broad use of antibiotics in aquaculture can contribute to an increase in microorganism resistance. *Vibrio harveyi* and *Vibrio alginolyticus* were the most represented species isolated from *Sparus aurata* in Italy and showed wide resistance against the most commonly used antibiotics. The fish farm environment may represent a potential reservoir of resistant bacteria that can be transmitted to humans through fish consumption. Resistance was observed both for licensed and unlicensed drugs for aquaculture in Italy. This could be the result of the intrinsic resistance of microorganisms, horizontal gene transfer or antibiotic pressure. Therefore it is essential to urge food business operators to use antimicrobial agents more rationally and to improve the surveillance on antibiotic use in the fish farming sector.

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