Susceptibility to antibiotics of *Vibrio* spp. and *Photobacterium damsela* ssp. *piscicida* strains isolated from Italian aquaculture farms

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SUMMARY

The antibiotic resistance patterns of aetiological agents responsible for vibriosis and pasteurellosis were studied to contribute to control the spread of these two bacterial diseases in Mediterranean fish farming. Strains of *Photobacterium damsela* ssp. *piscicida*, *Vibrio fluvialis*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio metschnikovii*, isolated from Italian aquaculture (fish, shellfish and crustaceans) sites, were assayed for their susceptibility to some antibacterial agents currently used in farming practices.

Kirby Bauer and Minimum Inhibitory Concentration (M.I.C.) tests were performed. The bacterial strains showed resistance to ampicillin, carbenicillin, kanamycin, cefalothin, while they were sensitive to chloramphenicol, nitrofurantoin and tobramycin; the sulfadiazine-trimethoprim association was completely ineffective. Conversely, flumequine showed the lowest M.I.C. value (0.97 μg mL^{-1}), suggesting its marked antibiotic effect. Considering that quinolone resistance can be transmitted only by selection of mutations and not by other genetic mechanisms, this study stresses the importance of a more responsible use of this antibacterial drug in aquaculture.

KEY WORDS: Aquaculture, Bacteria, Antibiotic susceptibility, *Vibrio* spp., *Photobacterium damsela* ssp. *piscicida*

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INTRODUCTION

Infectious diseases are recognised to be one of the most frequent and devastating problems in aquaculture. Chemotherapeutic agents, among which antibiotics, are conventionally included in current aquaculture practices and represent an important tool to prevent infectious disease outbreaks and to reduce economic losses associated with illness. The expansion of fish farming has induced an increasing trend in the consumption of antibiotics in many European Union countries (Díaz-Cruz *et al.,* 2003). As a consequence, antibiotic resistance phenomena, with the occurrence and spread of determinants of resistance to antibiotics, have become one of the most serious emerging threats in aquaculture (Sørum, 1999; 2006; Smith, 2008), triggered by the selective pressure on bacterial populations played by the extensive use of antimicrobial drugs, especially those present in waste waters (Baquero and Blazquez, 1997; Barbosa and Levy, 2000). Fish and shellfish farmers are recently facing risks related to the persistence of residues of antimicrobial drugs in seafood products as well as to the selection and spread of resistance to antibiotics both in animal and environmental bacterial populations. Antibiotic-resistant bacteria can represent a potential public health hazard (Levy, 1992), as a consequence of direct transmission to humans through consumption of contaminated food (Alderman and Hastings, 1998; Duran and Marshall, 2005), or indirectly through the transfer of their resistance genes into fish pathogenic bacteria or human pathogens (Smith *et al.,* 1994; Alderman and Hastings, 1998; Angulo, 2000; Sørum 2006; Guglielmetti *et al.,* 2009). Fish farms

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have been thought to act as reservoirs for antibiotic-resistant bacteria, thereby increasing the risk of transfer resistance determinants to human pathogens associated with fish consumption (Miranda and Zemelman, 2002). Serious therapeutic problems have arisen especially following the use of molecules whose class and structure is similar or, in certain cases, identical to those used in human medicine.

Antibiotic resistance phenomena have assumed increasing importance also in European Countries, where the awareness of severe consequences for human and animal health related to antibiotic use has stimulated the implementation of monitoring and intervention plans for surveillance of resistance and antibiotic use. The Common European Directives (2003/99/EC) are one example of regulations dealing with this issue.

While most research focusing on antibiotic resistance concerns bacteria of clinical relevance, only a few studies have addressed environmental isolates, especially some bacterial species (*Vibrio* spp. and *Photobacterium damsela* ssp. *piscicida*) recognised to be significant for fish pathology, being the aetiological agents of the most widespread and devastating diseases in Mediterranean aquaculture such as vibriosis and pasteurellosis. To date, studies on the incidence of antibacterial resistance and the susceptibility of fish bacterial pathogens to antibiotic treatment in Italian production areas are still limited (Ferrini *et al.,* 2008). The aim of our investigation was to determine the antibiotic susceptibility of *Vibrio* spp. and *Photobacterium damsela* ssp. *piscicida* strains isolated from some Italian aquaculture farms and involved in the outbreaks of epizootic diseases, to evaluate their potential responsiveness to the suite of antibiotic treatments most frequently used in aquaculture for prophylactic and therapeutic purposes.

MATERIALS AND METHODS

Bacterial strains

Strains of *Vibrio* spp. and *Photobacterium damsela* ssp. *piscicida* were collected from different aquaculture settlements (fish, shellfish and crustacean farms) located in two different Italian regions (Sicily and Veneto) affected by the outbreak of bacterial diseases. The analytical procedure for the collection of the bacterial strains relied on the spreading of small pieces of the target organs (kidney, spleen) on the surface of plates of the media TCBS (thiosulfate-citrate-bile-sucrose agar, Oxoid, Milan, Italy) and Tryptic Soy Agar (TSA, Oxoid), supplemented with NaCl (final concentration 1.5%), according to the aetiological agent to be determined (*Vibrio* spp. and *P. damsela* ssp. *piscicida*, respectively). Phenotypic identification of *Vibrio* spp. strains was achieved using both API 20 E and NE systems (BioMérieux, Marcy l'Etoile, France) and the biochemical keys reported by Noguerola and Blanch (2008). With respect to *P. damsela* ssp. *piscicida*, although this species is not included in the API 20-E code index, all the strains belonging to this microrganism were identified by the code number 2 005 004, in agreement with Zorrilla *et al.* (1999). The presumed colonies were also confirmed by the agglutination latex test Mono-Pp (Bionor AS, Skien, Norway).

The bacterial strains subject of this study are listed in Table 1, where their origin (animal species, region) is indicated.

Screening for antibacterial susceptibility

All the bacterial isolates were tested for resistance or sensitivity to different antibacterials using the standard disk diffusion method (Kirby Bauer test) and the Minimum Inhibitory Concentration (M.I.C.) test. Kirby Bauer and M.I.C. tests were

TABLE 1 - *List of the examined bacterial strains and of their origin.*

Strain number	*Identification*	*Isolation from: species (region of provenance)*
1	*Photobacterium damsela* ssp. *piscicida*	grey mullet (Sicily)
2	*P. damsela* ssp. *piscicida*	sea bass (Veneto)
3	*P. damsela* ssp. *piscicida*	sea bass (Veneto)
4	*P. damsela* ssp. *piscicida*	sea bass (Veneto)
5	*Vibrio fluvialis*	oyster (Sicily)
6	*V. alginolyticus*	mussel (Sicily)
7	*V. fluvialis*	shrimp (Sicily)
8	*V. fluvialis*	shrimp (Sicily)
9	*V. fluvialis*	oyster (Sicily)
10	*V. parahaemolyticus*	mussel (Sicily)
11	*V. metschnikovii*	mussel (Sicily)

performed after revitalising the bacterial strains in Marine Broth 2216 (Difco, Detroit, MI, USA). In particular, the susceptibility of the *Vibrio* and *P. damsela* ssp. *piscicida* strains to several antibiotics was tested according to the agar disk diffusion method (Bauer *et al.,* 1966). For the disk diffusion assay, bacteria were grown for 48 h on plates of Marine agar 2216 (Difco), harvested and then suspended in 1.5% sterile physiological saline solution adjusted to a 0.5 McFarland (bioMérieux, Marcy l'Etoile, France) turbidity standard, corresponding to 1-2x10^8 CFU mL^{-1}. The inoculum was streaked onto plates of Mueller-Hinton agar using a cotton swab; results were read after 72 h of incubation at 26°C.
Commercially available antibacterial disks, obtained from Oxoid (Basingstoke, Hampshire, United Kingdom) were used to determine the resistance patterns of the isolates against 31 different antibacterials (dose/disk), grouped into the following classes according to their mechanisms of action:

Cell envelope antibiotics:
1) beta-lactams, including *penicillins* [ampicillin (AMP, 10 µg), carbenicillin (CAR, 100 µg), mezlocillin (MEZ 75 µg), piperacillin (PRL 100 µg)], *monobactams* [aztreonam (ATM, 30 µg)] and *cephalosporins* [cefalothin (KF, 30 µg), cefazolin (KZ, 30 µg), cefotaxime (CTX, 30 µg), cefoxitin (FOX 30 µg), ceftazidime (CAZ 30 µg), cefuroxime (CXM 30 µg)],
2) fosfomycin (FOS 30 µg),
3) polymyxin [colistin sulphate (CS, 10 µg)]

Nucleic acid inhibitors:
1) quinolones [cinoxacin (CIN, 100 µg), enoxacin (ENX 10 µg), nalidixic acid (NA, 30 µg), ofloxacin (OFX 5 µg), oxolinic acid (OA, 2 µg), pipemidic acid (PI 20 µg)] and fluoroquinolones [ciprofloxacin (CIP, 5 µg), norfloxacin (NOR 10 µg)]
2) potentiated sulfonamides [sulphamethoxazole + trimethoprim (SXT, 23.75 µg +1.25 µg)]
3) DNA inhibitors [nitrofurantoin (F, 300 µg)]
4) RNA synthesis inhibitors: rifamycins [rifampicin (RD, 30 µg)]

Protein synthesis inhibitors:
1) *aminoglycoside* antibiotics [amikacin (AK, 30 µg), gentamicin (CN, 20 µg), netilmicin (NET, 30 µg), sisomicin (SIS 10 µg), tobramycin (TOB 10 µg)],
2) *tetracyclines* [tetracycline (TE, 30 µg)].

In addition, *phenicol derivatives*, such as chloramphenicol (C, 30 µg) were also assayed.
The diameter of the zone of inhibition around each disk was measured with a precision calliper (Mitutoyo, Andover, UK). Each bacterial species was classified as Resistant (R), Intermediately resistant (I) or Sensitive (S) according to the breakpoints established by Clinical Laboratory Standards Institute (CLSI, 2006).
Moreover, five antibacterial agents (among *penicillin derivatives:* amoxicillin, AMX; among *quinolones:* flumequine, FLU, and oxolinic acid, OA; among *tetracyclines:* oxytetracycline, OXT, and among *potentiated sulfonamides*: sulfadiazine-trimethoprim, S-T), routinely used in Mediterranean aquaculture for the treatment of bacterial fish diseases (Rigos and Troisi 2005), were selected for M.I.C. determination as representatives of different classes of antibacterial drugs, to better depict the behaviour of the examined strains against these molecules.
For M.I.C. determination, stock solutions of each antibiotic compound (provided from Sigma, St. Louis, MO, USA) were prepared in appropriate buffers according to the manufacturer's instructions and serially diluted to reach final antibiotic concentrations ranging from 250 to 0.015 µg mL^{-1}. M.I.C. was determined using a broth macrodilution method in Nutrient Broth (Difco) containing 3% NaCl and seeded with bacterial cultures at a concentration of 1.5x10^8 cells mL^{-1}, according to the standard procedures conventionally applied at the Department of Hygiene, University of Messina.
The highest dilution of the antibiotic at which no growth was visually determined was considered as the M.I.C. value. *Escherichia coli* ATCC 25922 was used as a reference strain to determine the susceptibility of marine strains in the absence of standard interpretative criteria for environmental isolates (Chelossi *et al.,* 2003).

RESULTS

The results of Kirby Bauer test are reported in Figure 1, where the strains were grouped as resistant, intermediate or sensitive according to

their susceptibility to each tested antibiotic. All strains were resistant to AMP; resistance to other β-lactams such as CAR, KF and KZ, as well as to quinolones (CIN) and sulfonamides (SXT) was also observed in a high percentage (from 62.5 to 87.5% of the total) of the tested strains. Resistance was also found in a moderate percentage (from 25 to 50% of the total) of the strains against cell envelope antibiotics (FOS, MEZ, CXM, CS, FOX) and quinolones such as PI. Conversely, only a limited number of strains (12.5 % of the total) showed resistance against NA, OA, ATM, CAZ, PRL, RD and TE. Moderate or high susceptibility was recorded against aminoglycoside (AK, CN, NET, SIS) and quinolones (ENX, OFX, CIP, NOR), as well as against CTX. All the examined strains were sensitive to C, F and TOB.

M.I.C. values obtained for each tested antibiotic agent and bacterial strain are shown in Tables 2-5. M.I.C. values towards AMX (Table 2) ranged from 125 to 7.8 μg mL^{-1} in 7 strains (*V. fluvialis, P. damsela* ssp. *piscicida*), which were considered sensitive to this antibiotic, whereas *V. fluvialis* strain 9 was classified as an intermediate resistant. Three strains, identified as *V. parahaemolyticus, V. metschnikovii, V. alginolyticus*, exhibited resistance. The lowest M.I.C. value (0.015 μg mL^{-1})

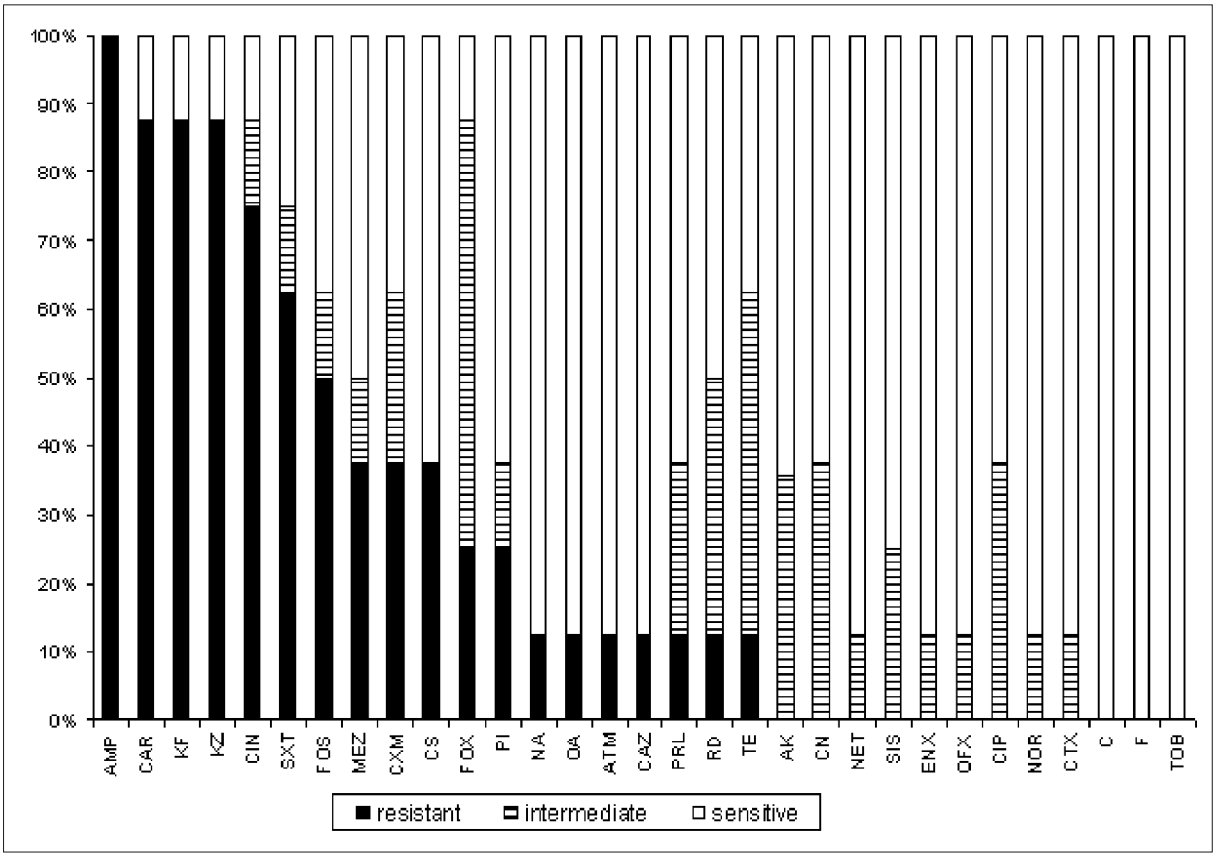

FIGURE 1 - *Kirby-Bauer test performed on Vibrio spp. and Photobacterium damsela spp. piscicida: the percentage of strains resistant, intermediate or sensitive to each assayed antibiotic agent is shown. The following antibiotics were used: amikacin (AK, 30 μg), ampicillin (AMP, 10 μg), aztreonam (ATM, 30 μg), carbenicillin (CAR, 100 μg), cefazolin (KZ, 30 μg), cefotaxime (CTX, 30 μg), cefoxitin (FOX 30 μg), ceftazidime (CAZ 30 μg), cefuroxima (CXM 30 μg), cefalothin (KF, 30 μg), chloramphenicol (C, 30 μg), cinoxacin (CIN, 100 μg), ciprofloxacin (CIP, 5 μg), colistin sulphate (CS, 10 μg), enoxacin (ENX 10 μg), fosfomycin (FOS 30 μg), gentamicin (CN, 20 μg), mezlocillin (MEZ 75 μg), nalidixic acid (NA, 30 μg), netilmicin (NET, 30 μg), nitrofurantoin (F, 300 μg), norfloxacin (NOR 10 μg), ofloxacin (OFX 5 μg), oxolinic acid (OA, 2 μg), pipemidic acid (PI 20 μg), piperacillin (PRL 100 μg), rifampicin (RD, 30 μg), sisomicin (SIS 10 μg), sulfamethoxazole + trimethoprim (SXT, 23.75 μg +1.25 μg), tetracycline (TE, 30 μg), tobramycin (TOB 10 μg).*

was observed in one strain of *P. damsela* ssp. *piscicida* (strain 1), suggesting that AMX was still effective against this microorganism. All the examined strains were sensitive to FLU (Table 3), showing M.I.C. values ranging from 62.5 to 0.97 µg mL^{-1}; the most sensitive strains were *V.*

TABLE 2 - *M.I.C. values obtained in the examined bacterial strains with respect to Amoxicillin.*

Amoxicillin concentration (µg mL^{-1})	*Photobacterium damsela* ssp. *piscicida*				*Vibrio fluvialis*				*V. alginolyticus*	*V. parahaemolyticus*	*V. metschnikovii*
	Strain 1	*Strain 2*	*Strain 3*	*Strain 4*	*Strain 5*	*Strain 7*	*Strain 8*	*Strain 9*	*Strain 6*	*Strain 10*	*Strain 11*
250	0,19	0,37	0,21	0,21	0,33	0,20	0,02	1,04	3,33	4,33	4,50
125	0,20	0,35	0,20	0,14	0,48	0,40	0,01	1,31	3,35	4,35	4,40
62,5	0,17	0,48	0,20	0,14	1,30	0,52	0,45	1,38	3,39	4,38	4,60
31,25	0,20	0,55	0,37	0,25	1,49	0,90	0,79	1,43	3,41	4,38	4,50
15,62	0,19	0,60	0,43	0,34	1,65	1,15	1,40	1,70	3,48	4,42	4,30
7,8	0,18	0,66	0,50	0,51	1,65	1,35	1,58	2,64	3,52	4,42	4,20
3,9	0,17	0,74	0,76	0,65	1,80	1,33	1,69	3,20	3,58	4,48	4,50
1,9	0,23	0,78	0,80	0,88	1,80	1,30	1,68	3,52	3,60	4,50	4,50
0,97	0,18	0,83	0,80	0,96	1,80	1,37	1,66	3,58	3,65	4,52	4,70
0,48	0,20	0,92	0,90	1,15	1,88	1,35	1,65	3,60	3,68	4,52	4,20
0,24	0,10	0,97	0,95	1,24	1,83	1,40	1,65	3,66	3,70	4,60	4,30
0,12	0,25	1,05	1,00	1,28	1,87	1,44	1,75	3,68	3,72	4,68	4,50
0,06	0,35	1,13	1,20	1,30	1,88	1,40	1,78	3,70	3,72	4,75	4,70
0,03	0,45	1,20	1,30	1,36	1,88	1,45	1,80	3,72	3,78	4,80	4,23
0,015	0,10	1,26	1,40	1,45	1,90	1,45	1,87	3,75	3,80	4,80	4,50

TABLE 3 - *M.I.C. values obtained in the examined bacterial strains with respect to Flumequine.*

Flumequine concentration (µg mL^{-1})	*Photobacterium damsela* ssp. *piscicida*				*Vibrio fluvialis*				*V. alginolyticus*	*V. parahaemolyticus*	*V. metschnikovii*
	Strain 1	*Strain 2*	*Strain 3*	*Strain 4*	*Strain 5*	*Strain 7*	*Strain 8*	*Strain 9*	*Strain 6*	*Strain 10*	*Strain 11*
250	0,18	0,30	0,27	0,27	0,30	0,48	0,45	0,28	0,40	0,30	0,30
125	0,35	0,32	0,35	0,32	0,30	0,54	0,48	0,32	0,40	0,35	0,35
62,5	0,50	0,50	0,42	0,38	0,35	0,58	0,50	0,30	0,45	0,32	0,35
31,25	0,90	0,55	0,52	0,45	0,40	0,68	0,50	0,28	0,48	0,34	0,38
15,62	0,98	0,76	0,85	0,48	0,40	0,72	0,52	0,28	0,52	0,32	0,42
7,8	1,27	0,85	0,96	0,56	0,45	0,78	0,52	0,30	0,75	0,30	0,48
3,9	1,42	0,95	1,02	0,77	0,46	0,86	0,58	0,32	0,80	0,37	0,52
1,9	1,50	0,98	1,08	0,80	0,55	1,20	0,80	0,40	2,93	0,45	0,58
0,97	1,52	0,98	1,39	0,80	0,95	1,45	1,20	0,54	3,00	0,58	1,67
0,48	1,52	1,03	1,50	0,82	1,18	1,67	1,28	0,80	3,00	3,00	3,00
0,24	1,54	1,07	3,00	0,85	1,55	2,64	1,44	1,28	3,00	3,00	3,00
0,12	1,55	1,09	3,00	0,88	1,62	3,00	1,50	1,80	3,00	3,00	3,00
0,06	1,56	1,09	3,00	0,95	1,70	3,00	1,75	2,08	3,00	3,00	3,00
0,03	1,56	1,12	3,00	0,98	1,70	3,00	1,80	2,44	3,00	3,00	3,00
0,015	1,56	1,15	3,00	0,98	1,75	3,00	1,97	2,77	3,00	3,00	3,00

metschnikovii, *V. parahaemolyticus* and two strains of *V. fluvialis* (strains 8 and 9).
M.I.C. towards OA (Table 4) ranged from 62.5 to 3.9 μg mL^{-1} in 10 strains, and only one strain (*V. fluvialis,* strain 8) was resistant.
Of the 11 *Vibrio* and *P. damsela* ssp. *piscicida* iso-

TABLE 4 - *M.I.C. values obtained in the examined bacterial strains with respect to Oxolinic acid.*

Oxolinic acid concentration (μg mL^{-1})	*Photobacterium damsela* ssp. *piscicida*				*Vibrio fluvialis*				*V. alginolyticus*	*V. parahaemolyticus*	*V. metschnikovii*
	Strain 1	*Strain 2*	*Strain 3*	*Strain 4*	*Strain 5*	*Strain 7*	*Strain 8*	*Strain 9*	*Strain 6*	*Strain 10*	*Strain 11*
250	0,28	0,28	0,30	0,20	0,32	0,32	0,70	0,38	0,26	0,40	0,52
125	0,32	0,35	0,38	0,28	0,36	0,35	0,75	0,40	0,36	0,44	0,55
62,50	0,34	0,38	0,43	0,35	0,41	0,48	0,78	0,41	0,33	0,48	0,56
31,25	0,33	0,44	0,47	0,42	0,48	0,53	0,80	0,48	0,42	0,52	0,68
15,62	0,33	0,49	0,52	0,48	0,52	0,66	0,83	0,52	0,55	0,56	0,72
7,8	0,39	0,51	0,55	0,52	0,56	0,73	0,85	0,65	0,70	0,67	0,75
3,9	0,50	0,55	1,05	0,56	0,64	0,80	0,90	0,74	0,85	0,70	0,80
1,9	0,56	0,57	1,15	0,67	0,70	1,00	0,94	0,82	1,20	0,76	0,80
0,97	0,89	0,80	1,04	0,80	0,77	1,02	0,96	0,94	1,52	3,00	1,20
0,48	0,94	1,00	1,02	0,90	0,84	1,04	1,35	1,22	1,98	3,00	3,00
0,24	0,80	1,30	1,03	0,95	0,92	1,04	3,00	1,51	3,00	3,00	3,00
0,12	0,94	1,38	1,22	0,94	1,20	1,07	3,00	3,00	3,00	3,00	3,00
0,06	1,00	1,30	1,20	1,00	1,42	1,80	3,00	3,00	3,00	3,00	3,00
0,03	1,00	1,20	1,20	1,08	1,60	2,00	3,00	3,00	3,00	3,00	3,00
0,015	1,00	1,35	1,15	0,99	2,00	3,00	3,00	3,00	3,00	3,00	3,00

TABLE 5 - *M.I.C. values obtained in the examined bacterial strains with respect to Oxytetracycline.*

Oxytetracycline concentration (μg mL^{-1})	*Photobacterium damsela* ssp. *piscicida*				*Vibrio fluvialis*				*V. alginolyticus*	*V. parahaemolyticus*	*V. metschnikovii*
	Strain 1	*Strain 2*	*Strain 3*	*Strain 4*	*Strain 5*	*Strain 7*	*Strain 8*	*Strain 9*	*Strain 6*	*Strain 10*	*Strain 11*
250	0,31	0,50	0,48	0,75	0,25	0,42	0,30	0,40	0,01	0,48	0,18
125	0,20	0,51	0,43	0,70	0,34	0,75	0,30	0,44	0,01	0,50	0,18
62,5	0,40	0,53	0,48	0,64	0,77	0,82	0,45	0,70	0,52	0,50	0,50
31,25	0,44	0,64	0,56	0,70	0,87	1,02	0,86	0,68	0,75	0,80	0,80
15,62	0,43	0,66	0,59	0,70	0,80	1,08	0,92	0,70	0,85	0,84	0,80
7,8	0,47	0,60	0,59	0,75	0,90	1,12	0,97	0,82	1,00	0,90	0,92
3,9	0,50	0,67	0,65	0,80	0,86	1,18	1,03	0,93	1,25	0,97	0,99
1,9	0,50	0,63	0,60	0,80	0,97	1,18	1,30	1,03	1,60	1,18	1,26
0,97	0,60	0,66	0,60	0,80	1,20	1,20	1,56	1,10	3,27	1,70	1,63
0,48	0,60	0,76	0,66	0,75	1,40	1,50	1,73	1,95	3,35	3,20	2,23
0,24	0,68	1,05	0,76	0,99	1,70	1,85	3,35	2,70	3,42	3,25	3,57
0,12	0,73	1,10	0,88	0,92	1,73	1,88	3,40	3,22	3,48	3,38	3,64
0,06	0,90	1,25	0,95	0,88	1,85	1,90	3,48	3,32	3,55	3,46	3,72
0,03	0,90	1,28	1,06	1,16	1,80	1,98	3,56	3,38	3,62	3,55	3,78
0,015	0,90	1,28	1,10	1,18	1,80	2,05	3,60	3,40	3,70	3,60	3,80

lates, ten were sensitive to OXT and their M.I.C. ranged from 250 to 62.5 μg mL^{-1}; only one strain (*P. damsela,* strain 4) was resistant (Tab. 5). Conversely, the association S-T was completely ineffective against all the tested strains (data not shown).

DISCUSSION

This study concerns the determination of the profiles of antibiotic resistance of some bacterial pathogens, *Vibrio* and *P. damsela* ssp. *piscicida*, recognised as causative agents of important diseases in Mediterranean fish farming (Rigos and Troisi, 2005).

The most sensitive fish species to vibriosis and pasteurellosis are seabass, seabream, common dentex and sharp snout seabream, but shellfish and crustaceans are also subjected to vibriosis. Surveillance for antibacterial resistance in aquaculture represents a preliminary step to detect changes in the susceptibility of bacterial pathogens, whose knowledge is needed to evaluate the opportunity of using some specific antibiotics for disease prevention in terms of actual benefits. The limited availability or the side effects of methods (i.e. vaccines) able to control disease outbreaks, still makes it necessary to consider chemotherapy an effective tool against bacterial infections, especially in finfish (Subasinghe, 2009). At present, only a few antibiotics (amoxicillin, flumequine, oxytetracycline, tetracycline, sulfadiazine + trimethoprim) are officially registered in Italy to be used as a premix in the formulation of medicated feed (Manfrin *et al.,* 2009); among these, flumequine and oxytetracycline are the most commonly used (Lalumera *et al.,* 2004). All the *Vibrio* and *P. damsela* ssp. *piscicida* isolated from fish, shellfish and shrimps reared in Sicily and Veneto and tested in this study showed different levels of resistance to the antibiotics used in human medicine as well as to those commonly used in aquaculture. This result agrees with the increasing incidence of bacteria resistant to antimicrobial compounds (McPhearson *et al.,* 1991; Smith *et al.,* 1994; Schmidt *et al.,* 2000; Petersen *et al.,* 2002; Alcaide *et al.,* 2005; Smith, 2008; Sørum, 1999), which is a consequence of the selective pressure exerted by the extensive use of these agents in fish farms.

Antibiotic resistance has been documented in animal pathogens, in commensal bacteria and in disease agents that can spread to man via the food chain (Ferrini, 2004) and can be transmitted through sequential mutations in chromosomal genes (Wang *et al.,* 2001) or through the acquisition of genetic elements such as plasmids, bacteriophages or transposons (Levy and Marshall, 2004; Smith, 2008). Monitoring the spread of antibiotic resistance is particularly important for the risk assessment related to *Vibrio* species, as many of them are reported to cause fish and shellfish diseases (Woo and Kelly, 1995; Austin and Austin, 1999; Nakayama *et al.,* 2006; Tansel Tanrikul, 2007). Moreover, there are incomplete data on minimum inhibitory concentrations (M.I.C.) for common antibacterials used against the major bacterial pathogens of Mediterranean fish species (Rigos and Troisi, 2005). In spite of the limited number of bacterial strains examined in this study, some basic information on the spread of antibiotic resistance within some Italian aquaculture settlements can be achieved. The results obtained in this research showed that 100% of the examined strains were resistant to ampicillin; a high number of them were also resistant to other beta-lactams (i.e. carbenicillin, cefalotin, cefazolin), as well as to quinolones (i.e. cinoxacin and pipemidic acid). Foti *et al.* (2009) reported significant rates of antibiotic resistance to carbenicillin and cefalotin in bacterial isolates from loggerhead sea turtles collected in the central Mediterranean Sea, suggesting the widespread occurrence of bacteria resistant to both antibiotics in this aquatic environment. The majority of *Vibrio* and *P. damsela* ssp. *piscicida* strains examined in the present study were resistant to potentiated sulfonamides, whereas they were sensitive to aminoglycosides and other quinolones, such as enoxacin, ofloxacin, ciprofloxacin, norfloxacin. Moreover, resistance to ceftazidime and tetracycline was detected in a low percentage (12% of total strains) of *Vibrio* spp. only. However, controversial results have been reported in literature regarding the susceptibility of *Vibrio* species to antimicrobials. Halophilic vibrios isolated from seafood in Hong Kong were found to be susceptible to chloramphenicol and tetracycline (French *et al.,* 1989), while *Vibrio* spp. isolated from cultured silver sea bream developed intermediate resistance to the same antibiotics upon exposure

to indiscriminate amounts of antibiotics (Li *et al.,* 1999). Han *et al.* (2007) reported that tetracycline, cefotaxime, ceftazidime, and fluoroquinolones were highly effective against *Vibrio parahaemolyticus* strains recovered from Louisiana Gulf and retail oysters. Comparing our data to other reports from Italian aquaculture, Ferrini *et al.* (2008) reported that 82% of *Vibrio* isolated from fish settings as well as both national and imported seafood showed resistance to ampicillin, followed by sulfamethoxazole, tetracycline and trimethoprim/sulfamethoxazole, against which only a low percentage of the isolates (ranging from 1 to 7% of the total) were resistant. In a previous study on potentially pathogenic vibrios isolated from seafood, Ottaviani *et al.* (2001) also found that those bacteria were sensitive to chloramphenicol (90% of the total), oxolinic acid and trimethoprim-sulfamethoxazole, flumequine, cefotaxime, nalidixic acid and ciprofloxacin; conversely, they were resistant to ampicillin, penicillin, carbenicillin, kanamycin and cefalothin. Bacterial resistance to ampicillin and oxytetracycline was the most frequently detected among *Vibrio* species isolated from fishery products (Akinbowale *et al.,* 2006) as well as from a shrimp hatchery (Hameed *et al.,* 2003) and shrimp farms (Vaseeharan *et al.,* 2005). Conversely, tetracycline and chloramphenicol were ineffective against *Vibrio* spp. isolated from the environment (Zanetti *et al.,* 2001). Resistance against amoxicillin, ampicillin, carbenicillin, cefuroxime, rifampicin and streptomycin was also detected in *Vibrio* spp. isolated from coastal and brackish areas, which were moderately resistant against chloramphenicol, tetracycline, nalidixic acid, trimethoprim, neomycin and amikacin (Manjusha *et al.,* 2005).

Concerning *P. damsela* ssp. *piscicida*, during this study one strain (strain 1) showed the lowest M.I.C. value against amoxicillin; while another (strain 4, isolated from sea bass) was resistant to oxytetracycline, suggesting that these antibiotics were effective and ineffective against this pathogen, respectively. The most widely used therapeutic agents to treat pasteurellosis include amoxicillin, ampicillin, chloramphenicol, erythromycin, florfenicol, flumequine, oxolinic acid, oxytetracycline, nitrofuraxone, sulfadiazine-trimethoprim and tetracycline (Toranzo *et al.,* 1991; Bakopoulos *et al.,* 1995; Sano, 1998). However, Thyssen and Ollevier (2001), investigating the susceptibility to several antimicrobials of *Photobacterium damsela* ssp. *piscicida* isolated from different geographical areas, found that the majority (93%) of the isolates were resistant to erythromycin, and also, in a lower percentage (less than 10%), to amoxicillin, ampicillin, florfenicol and trimethoprim-sulfamethoxazole. In particular, European *P. piscicida* isolates were sensitive to kanamycin, florfenicol and trimethoprim-sulfamethoxazole. Conversely, multiple resistance of Japanese strains of this pathogen to antimicrobial agents such ampicillin, chloramphenicol, tetracycline, kanamycin was documented (Kim and Aoki, 1993; Kawanishi *et al.,* 2006). Bakopoulos *et al.* (1995) reported similar antibiotic sensitivity patterns for Greek, Italian and French bacterial isolates, which were resistant to erythromycin, kanamycin and streptomycin, potentiated sulfonamides.

An interesting result obtained from the present survey concerned the marked antibiotic effect of Flumequine. This synthetic molecule, used exclusively in aquaculture (Cabello, 2006) is a fluoroquinolone derivative which inhibits DNA-gyrase (Drlica and Coughlin, 1989) and has a broad activity spectrum mainly against Gram-negative bacteria. Nevertheless, only limited information for a few bacterial fish pathogens is available for this antibiotic. Previous studies have revealed that flumequine induces satisfactory kinetic properties in some farmed fish species (Rogstad *et al.,* 1993; Martinsen and Horsberg, 1995) and low M.I.Cs against important bacterial fish pathogens (Ledo *et al.,* 1987; Martinsen *et al.,* 1992), therefore this molecule is a promising therapeutic candidate for fish farming. Rigos *et al.* (2003) reported a very low M.I.C. of flumequine (0.15 µg ml^{-1} against *V. damsela*) under marine conditions, suggesting that this drug is very effective for controlling pasteurellosis. In the present study, flumequine showed the lowest M.I.C. value (0.97 µg mL^{-1}) against *V. metschnikovii*, *V. parahaemolyticus* and *V. fluvialis,* suggesting its marked antibiotic effect. However, some problems concern the environmental impact resulting from the use of flumequine. Quinolones are known to remain active in sediments for prolonged periods of time as they do not have biodegradable characteristics (Samuelsen *et al.,* 1992), and for flumequine a half-life up to 60 days in the uppermost layer of

sediments is reported (Hektoen *et al.,* 1995; Migliore *et al.,* 2002). Flumequine, oxolinic acid and oxytetracycline have been indicated as the most relevant chemicals to be monitored in fish farming due to their chronic persistence (Hektoen *et al.,* 1995). Particular attention should be given to possible side-effects of these antibiotics in sediment fish farms and surrounding environments (Lalumera *et al.,* 2004). When assessing fish farming impact, further investigations are needed especially on the mechanisms of antibiotic resistance. Taking into consideration that quinolone resistance can be transmitted only by selection of mutation and not by other genetic mechanisms (Hooper, 2002), a more responsible use of flumequine in aquaculture is recommended. This information may be helpful to farmers to improve rearing practices and reduce the potential impact on the environment.

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