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Antibiotic resistance of Aeromonas ssp. strains isolated from Sparus aurata reared in Italian mariculture farms

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Abstract

Selective pressure in the aquatic environment of intensive fish farms leads to acquired antibiotic resistance. This study used the broth microdilution method to measure minimum inhibitory concentrations (MICs) of 15 antibiotics against 104 Aeromonas spp. strains randomly selected among bacteria isolated from Sparus aurata reared in six Italian mariculture farms. The antimicrobial agents chosen were representative of those primarily used in aquaculture and human therapy and included 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). The most prevalent species selected from positive samples was Aeromonas media (15 strains). The bacterial strains showed high resistance to SZ, AMX, AM, E, CF, S and TMP antibiotics. Conversely, TE and CL showed MIC90 values lower than breakpoints for susceptibility and many isolates were susceptible to OXA, GM, FF, FM, K and OT antibiotics. Almost all Aeromonas spp. strains showed multiple antibiotic resistance. Epidemiological cut-off values (ECVs) for Aeromonas spp. were based on the MIC distributions obtained. The results showed a high frequency of Aeromonas spp. contamination in Sparus aurata reared on the Italian coast and an elevated biodiversity in isolated bacterial strains. Aeromonas isolates comprise potentially pathogenic species for humans, often resistant to several antibiotics and able to transfer the genes responsible for antibiotic resistance to microorganisms pathogenic for humans throughout the food chain. The few ECV studies available on many antibiotics against Aeromonas spp. strains isolated from the aquaculture environment highlight the need for further research in this area, while regular monitoring programmes should be stepped up to check for antibiotic resistance.

1. Introduction

World fish consumption has been growing in the last thirty years reaching 20 kg per capita in 2014. For the first time, the aquaculture production of fish for human consumption has overtaken the supply of wild-caught fish and is expected to rise to 62% by 2030 (FAO, 2016). Gilthead sea bream (Sparus aurata) is a very suitable species for mariculture in the Mediterranean basin and has become one of Europe's main fish species in aquaculture. Greece, Turkey and Spain are the main producers worldwide while Italy is the third main producer in the EU (EC, 2017). Large-scale aquaculture is characterized by the intensive and semi-intensive production systems with high stocking density, which leads to poor hygiene conditions and the emergence of infectious diseases (Diana et al., 2013). The genus Aeromonas comprises a group of bacteria with a ubiquitous distribution in natural habitats, including the aquatic environment (Janda and Abbott, 2010) where species such as A. hydrophila, A. caviae, A. salmonicida and A. veronii biovar sobria cause disease in marine fish (Radu et al., 2003). Aeromonas spp. are also important human opportunistic pathogens able to cause intestinal, blood, skin and soft tissue and trauma-related infections, particularly in young children and the elderly

(Janda and Abbott, 2010; Real et al., 1994). Aeromonas species have been frequently isolated from fish and other foods (Callister and Agger, 1987; Gobat and Jemmi, 1993). These bacteria are responsible for food spoilage and may serve as vectors for disease transmission to humans (Tsai and Chen, 1996). Infection can also occur after contactwith contaminated water or fish (Janda and Abbott, 2010). Aeromonas pathogenicity is linked to the production of a number of extracellular hydrolytic enzymes such as lipases and proteases, which aid in bacterial invasion and the establishment of infection (Galindo et al., 2006). Among an array of other virulence factors, the biological activities of cytotoxic enterotoxin (Act) include haemolysis, cytotoxicity, enterotoxicity and lethality (Chopra et al., 1991). The worldwide expansion of intensive fish farming has increased the use of antibiotics to treat bacterial infections (Díaz-Cruz et al., 2003). In aquaculture, antimicrobials are generally added to the feed or directly to the water to prevent the spread of infectious fish disease (Defoirdt et al., 2011) and in some circumstances to promote fish growth illegally (Serrano, 2005). Regulations governing the use of antibiotics in aquaculture differ widely with little to no enforcement in many of the world's major aquaculture-producing countries (Pruden et al., 2013). The extensive use of antibiotics in aquaculture has in turn resulted in the emergence of antibiotic resistance in both foodborne and opportunistic human pathogens (Marshall and Levy, 2011). The resistance of Aeromonas species to diverse groups of antibiotics is a major concern for human health (Figueira et al., 2011) as resistant bacteria can spread from the aquatic environment to humans via the food chain or direct contact (Taylor et al., 2011). In addition, resistance genes can be transferred by mobile genetic elements such as plasmids, phages and transposons (Levy and Marshall, 2004). Janda and Abbott (2010) reviewed the general susceptibility profiles of Aeromonads to various antimicrobial classes, showing resistance to sulfamethoxazole, cephalosporins, penicillins (amoxicillin, ampicillin, ampicillin-sulbactam, ticarcillin, oxacillin and penicillin) and macrolides (clarithromycin). Aeromonas species resistant to penicillins and first generation cephalosporins are associated with the production of chromosomally encoded beta-lactamases (Janda and Abbott, 2010). Other important resistance determinants to beta-lactam antimicrobials and tetracyclines are blagenes and tet genes respectively encoded in mobile genetic elements (Agersø et al., 2007; Wu et al., 2011) or integrons, responsible for resistance to tetracyclines, aminoglycosides, chloramphenicol and trimethoprim (Chang et al., 2007; Kadlec et al., 2011). Indeed, acquired antibiotic resistance among fish pathogens could determine serious therapeutic problems in humans following the use of molecules whose class and structure are similar or, in some cases, identical to those used in mariculture (Cabello, 2006). Despite recent efforts by international agencies such as the European Centre for Disease Prevention and Control and the National Antimicrobial Resistance Monitoring System (EFSA, 2014), the role of antibiotic usage in aquaculture in the development and dissemination of antibiotic resistance genes is still poorly understood. The potential risk of transferring such resistance from the aquaculture environment to humans is underestimated (Cabello et al., 2013) so the effectiveness of antibiotics used in fish farming should be carefully monitored. Little information is available on the susceptibility of Aeromonas spp. isolated from mariculture to antibiotics used in both fish farming and human therapy. Antimicrobial susceptibility is generally tested by measuring the drug's minimum inhibitory concentration (MIC). MIC breakpoints are the MICs at which an organism should be considered susceptible, intermediate or resistant. Breakpoint values are published by organizations such as the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the American Clinical Laboratory Standard Institute (CLSI), based on pharmacokinetic/pharmacodynamics data and clinical studies. MIC50 and MIC90 indicate the lowest concentrations of the antimicrobial agent inhibiting visible growth of 50% and 90% of the bacterial population respectively. However, few interpretation criteria for Aeromonas spp. have been published to date. The only available breakpoints proposed by the CLSI are from clinical isolates adapted from Enterobacteriaceae, while no criteria have been established by EUCAST. Epidemiological cut-off values (ECVs) must be set to discriminate wild-

type strains (with no acquired resistance mechanism to the tested antibiotic) from non-wild-type strains (with one or more acquired resistance mechanisms) (Kahlmeter et al., 2003). These cut-off values are the upper limit of the MIC distribution of fully susceptible strains. The purpose of the present study was to estimate the MICs of Aeromonas spp. strains isolated from Sparus aurata against 15 antimicrobial agents, and to determine the ECVs for Aeromonas spp.

2. Materials and methods

2.1. Fish sampling

The study was conducted on gilthead sea bream (Sparus aurata) collected from six offshore mariculture farms in three Italian regions (Sardinia, Sicily and Tuscany). All fish farms were characterized by intensive rearing systems in sea cages. Water salinity was ca. 33% and the temperature ranged between 16 °C and 22 °C. Twenty commercial size (~250 g) Sparus aurata specimens were randomly collected at each farm during two different visits conducted four months apart. After collection, fish were slaughtered by immersion in fusing ice, placed in expanded polystyrene boxes and covered with a plastic film then transported to the laboratory under refrigeration and processed within 3 h after collection.

2.2. Microbiological analysis

Samples of skin, gills, muscle and intestinal content were aseptically collected from each specimen for microbiological analysis. The initial suspension and decimal dilution for microbiological examination were prepared according to ISO 6887–1:1999. Each matrix was tested for Aeromonas species (presence/absence) inoculating 0.1 mL of homogenized PBS (pH 7.4) on plates of Aeromonas Medium Base (Ryan's medium) (Oxoid, Basingstoke, UK) supplemented with ampicillin selective supplement at 5 mg/L. The agar plates were incubated at +30 °C for 48 h. Colonies with typical growth characteristics, opaque dark green with darker centres, were picked and subcultured on brain heart infusion (BHI) agar plates (BHI, Oxoid, Basingstoke, UK). After incubation, isolates were tested as follows: morphology in Gram staining, cytochrome oxidase, amylase and trealose fermentation. After presumptive genus identification, strains were stored at −80 °C for subsequent genetic confirmation and species identification.

2.3. Bacterial identification

Genus identification of isolates was confirmed by PCR (Khan et al., 2009). To avoid over-representation of clones, 16S ribosomal DNA sequencing was conducted on a selection of strains to identify bacterial species. A hierarchical method was used to select up to three strains from each of the following nested criteria: region of collection (three levels), fish farm (two levels), fish specimens (40 levels) and fish matrix (4 levels). For species identification, colonies with morphological and biochemical features of Aeromonas spp. were grown overnight at 37 °C in tryptone soya broth (Oxoid). DNA was extracted using the following protocol: 1 mL of broth culture (108 CFU/mL) was centrifuged at 12,000g for 5 min, then the pellet was resuspended in 1 mL of phosphate- buffered saline, boiled for 5 min, and centrifuged again (Bottero et al., 2004). The supernatant was stored at −20 °C until use. The DNA was quantified using a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific). All extracted DNA were subjected to sequencing analysis with the MicroSeq 500 16S rDNA bacterial sequencing kit (Thermo Fisher Scientific). 16S rDNA amplicons were purified by Exo-Sap treatment according to the manufacturer's recommendations (USB Europe, Staufen, Germany). Forward and reverse sequencing reactions were performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 (Thermo Fisher Scientific). The extended products were purified with DyeEx 2.0 Spin kit (Qiagen, Valencia, CA, USA) and resolved by capillary electrophoresis using an ABI 310 Genetic Analyzer (Thermo Fisher Scientific). 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.

2.4. Antibiotic susceptibility

Antibiotic susceptibility was determined for Aeromonas strains at the genus level. MICs of 15 antibiotics were measured by the broth microdilution method (CLSI, 2011). The antimicrobial agents chosen among those mainly used in aquaculture and human therapy were: 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). To obtain stock solutions, the antibiotic powders (Sigma Aldrich, MI, Italy) were weighed and dissolved in the following solvents (Sigma Aldrich): phosphate buffer, pH 8.0, 0.1 mol/L (AM), phosphate buffer, pH 6.0, 0.1 mol/L (AMX and CF), ethanol 95% (CL and E), methanol 96% (FF), aqueous alkaline solution NaOH 0.1M+ethanol 2:1 (FM), aqueous alkaline solution NaOH 0.1 M, pH 10 (OXA), methanol- water 2:1 (OT and TE), aqueous acidic solution HCl 10% (TMP), and deionized water (GM, K, S and SZ). Once dissolved, each stock solution (2560 μg/mL) was dispensed in 1.5 mL aliquots into polypropylene vials and frozen at −80 °C until use. Each microtitre plate was prepared with 12 serial twofold dilutions of each antibiotic stock solution (Work Station - Micro Star, Hamilton, Bonaduz GR, Switzerland) with deionized water (phosphate buffer, pH 6.0, 0.1 mol/L, only for AMP and AMX antibiotics). The antibiotic concentrations obtained ranged between 0.06 μg/mL and 128 μg/mL (0.12–256 μg/mL for SZ antibiotic). Strains were subcultured twice in BHI plates before preparation of the inoculum. After overnight incubation at 37 °C, two or more colonies were picked from BHI plates and dissolved in salt solution (0.85% w/v) to obtain 0.5 McFarland turbidity, measured using a portable photometric reader (Densimat, bioMérieux, Lyon, France). Each bacterial suspension was further diluted (1:100) in cation-adjusted Mueller Hinton broth (CAMHB, Oxoid, Basingstoke, UK) supplemented with NaCl (1%) to obtain an inoculum concentration of ca.106 cfu/mL. Fifty μL of the final suspension were transferred into microtitre wells (one strain for each row of the microplate) containing 50 μL of each antimicrobial agent. The density of the final inoculum in each well was ca. 5×105 cfu/mL. The reference strain E. coli ATCC 25922 was used as quality control. Each microplate was subsequently incubated under aerobic conditions for 20 h at 35 °C. The MIC of each antibiotic was compared with breakpoint values to determine resistance (CLSI, 2005, 2007, 2011, 2016; NCCLS, 1998, 1999, 2002). The MIC range and mode, MIC50 and MIC90 of each antimicrobial agent were also determined. Multiple antibiotic resistance (MAR) among Aeromonas spp. strains was evaluated applying the MAR index defined as a/b, where “a” was the number of antimicrobials the isolate was resistant to and “b” was the number of antibiotics against which the isolate was tested. According to Krumperman (1983), a MAR index below 0.2 is interpreted as strains originating from animals in which antibiotics are seldom or never used, while a MAR index above 0.2 is interpreted as strains originating from an elevated selective pressure environment where antibiotics are frequently used.

2.5. Epidemiological cut-off values

The distribution of MIC values served to determine the ECVs. The statistical determination of ECV values for each antimicrobial agent at genus level was conducted according to Turnidge et al. (2006) using the freely available ECOFFinder Microsoft Excel spreadsheet calculator (https://clsi.org/education/microbiology/ecoffinder/). The spreadsheet is designed to estimate the ECVs based on the observed MIC of the tested bacterial population, i.e. it estimates the MIC value best describing where wild type distribution ends.

3. Results

3.1. Isolation and identification of Aeromonas spp.

Aeromonas spp. were observed in 98 skin samples (30.6%), 154 gills (48.2%) and 40 gut contents (12.5%) whereas the bacteria were never detected in muscle. One hundred and four Aeromonas spp. strains isolated from Sparus aurata were speciated by 16S ribosomal DNA sequencing. Of the total

strains, 59 originated from Tuscany, 40 from Sicily and five from Sardinia. The Aeromonas strains were isolated from skin (n. 48), gut content (n. 20) and gills (n. 36). Sequencing identified 23 different Aeromonas species or species-complex. The most frequently recovered species were Aeromonas media (15 strains, 14.4%), eromonas salmonicida/bestiarium/hidrophila/caviae species-complex (12 strains, 11.5%), A. molluscorum (11 strains, 10.6%) and A. bivalvum (10 strains, 9.6%). Table 1 reports a complete list of Aeromonas species and species-complex identified and the relative number of strains. Fig. 1 shows their distribution by region of origin.

3.2. Antimicrobial susceptibility

For some of the selected antimicrobial agents, the reference strain used as quality control for the MIC determination assay indicated the Aeromonas strains in compliance with CLSI recommendations (CLSI, 2005). Over 90% of the speciated Aeromonas strains showed susceptibility to CL, FF and GM antibiotics. Table 1 lists the resistance profile for each Aeromonas species. All tested Aeromonas strains showed resistance to two or more antibiotics. One strain of A. bivalvium and one strain of the A. punctata/hydrophila/enteropelogenes species-complex were resistant to 11 antibiotics while one A. molluscorum strain was resistant to 12 antibiotics. Table 2 reports the MAR index indicating the multiple antibiotic resistance of Aeromonas spp. strains by region of origin. Table 3 shows the MIC50, MIC90, mode and range and cut-off of MICs for each tested antibiotic, and the number of sensitive, intermediate and resistant strains with reference to the CLSI breakpoints. The most frequent combination of antibiotic resistance profiles was AM, AMX, CF, E, S, SZ and TMP. For CF and SZ, the MIC50 and MIC90 values were above the tested range (128 and 256 μg/mL respectively).

3.3. Determination of wild-type strains

The ECVs were computed for 12 out of 15 antimicrobial agents. In addition to CF and SZ, no values were computed for TMP due to a high number of isolates with MIC values greater than the upper limit of the tested dilutions (128 μg/mL). The wild-type strains ranged between 59.6% and 96.2% of the tested strains. Thirty-one strains (29.8%) were wild-type for all antibiotics with a computable ECV. One strain (A. bivalvium) resulted wildtype exclusively for E, one strain for GM and K (A. punctata/hydrophila/enteropelogenes species-complex) and one strain (A. molluscorum) for GM, K and S. The remaining 70 strains were wild-type for five up to 11 different antibiotics, yielding 31 different combinations of antibiotic wild-type profiles. More than 80% of wild-type strains were resistant to eight antibiotics (CL, E, FF, GM, K, OT, S, TE). Table 3 reports the complete results on the ECVs and percentage of wild-type strains.

4. Discussion

The worldwide growth of aquaculture has seen the development of intensive fish farming. This in turn has been associated with an extensive use of antibiotics to treat or prevent bacterial infections.
Regulations governing the antimicrobial agents authorized in fish farming differ from country to country. The selective pressure exerted by intensive fish farming has resulted in the emergence of antibiotic resistant food-borne pathogens, opportunistic pathogens and human commensal flora of food animals (Sorum, 2006; Teuber, 2001; Witte, 2000). The potential transfer of antibiotic resistance from the aquatic environment to humans through direct contact or via the food chain is a serious concern for human health (Marshall and Levy, 2011). Antibiotic resistance monitoring fails to collect extensive information of the classes of antimicrobials used in aquaculture and the efficacy of antibiotics (Cabello et al., 2013). The present study provided useful information on the resistance of Aeromonas spp. isolated from gilthead sea bream (Sparus aurata) reared in Italian fish farms. Aeromonas spp. were widely distributed in skin, gills and intestinal content of Sparus aurata whereas they were never detected in muscle. Aeromonas spp. can potentially cause human illness by direct contact or through the ingestion of contaminated fish (Janda and Abbott, 2010). Clinical breakpoints are useful to assess the efficacy of antibiotics during treatments, while the determination of ECVs will establish the emergence of antibiotic resistance mechanisms within a bacterial population. Based

on these values, the present study documented high resistance rates for β-lactams, erythromycin, sulfadiazine and trimethoprim. The MIC90 of ampicillin, amoxicillin and cephalothin > 128 μg/mL) were higher than the reference breakpoints for resistance and the number of resistant strains ranged between 40.4% and 86.5%. The literature reports resistance rates for these antibiotics as high as 100% (Hatha et al., 2005; Snoussi et al., 2011). The MIC50 for ampicillin and amoxicillin was 16 μg/mL, an intermediate value between
the reference breakpoints for susceptibility and resistance, while the MIC50 for cephalothin was greater than the reference value for resistance(> 128 μg/mL). Amoxicillin and ampicillin are susceptible to β-lactamase and to rapid onset antibiotic resistance especially when the antibiotic is repeatedly used in a short time period, typical of intensive fish farming systems. Three different types of β-lactamase have been observed in Aeromonas spp. (Walsh et al., 1997), but little information
is available on the ECVsT for Aeromonas spp. and limited to few antibiotics, hampering a comparison with the MIC distribution observed in our microbial population. Despite the high resistance rates observed for β-lactam antibiotics, based on the ECVs computed for amoxicillin and ampicillin, an elevated percentage of strains could be considered wildtype. Among the quinolone antibiotics, various countries have authorized oxolinic acid (a first generation quinolone) for therapeutic use in aquaculture, while flumequine is the only one of the five fluoroquinolone antibiotics listed in Reg. EC 37/2010 authorized for fish farming. These antibiotics are used in mariculture for the treatment of furunculosis caused by Aeromonas salmonicida (Giraud et al., 2004). Oxolinic acid and flumequine showed resistance in 32.7% and 22.1% of the tested Aeromonas spp. strains respectively. The antibiotic resistance of Aeromonas spp. in the present study is in agreement with previous investigations conducted in mariculture farms where resistance was between 25% and 50% (Inglis et al., 1991; Snoussi et al., 2011; Cattoir et al., 2008). The ECV computed in the present study was 0.25 μg/mL for both oxolinic acid and flumequine while the literature reported values of 0.031 μg/mL and 0.06 μg/mL respectively (Baron et al., 2017; Smith and Kronvall, 2015). However, these results are not comparable as the values obtained in our study coincided with the lowest dilution tested. Due to their broad-spectrum activity, low toxicity and cost, tetracyclines are the most commonly used antibiotics in both human and veterinary medicine. In mariculture, oxytetracycline is authorized for therapeutic immersion in Europe, while elsewhere (USA and Asian countries) it is also administered with medicated foods. The widespread use of tetracycline has resulted in the dissemination of resistance to many marine bacteria (Furushita et al., 2003) with the number of resistant strains ranging from 7.7% (TE) to 11.5% (OT). These results are comparable with previous studies where Aeromonas spp. strains showed sensitivity to tetracycline and oxytetracycline (Awan et al., 2009). Aeromonas spp. strains showed high in vitro sensitivity against both oxytetracycline (80.8%) and tetracycline (85.6%) in the tetracycline class with MIC50 values below the reference breakpoint of susceptibility (≤1 μg/mL) and MIC90 of 4 μg/mL and 16 μg/mL for tetracycline and oxytetracycline, respectively. The MIC50 and MIC90 observed in our study were within the range reported in previous investigations conducted on Aeromonads isolated from freshwater fish (Baron et al., 2017; Čížek et al., 2010). The ECV of Aeromonas spp. was 2 μg/mL for both tetracycline and oxytetracycline, values greater than those reported by Baron et al. (2017). Among the Macrolides, erythromycin is the bacteriostatic drug of choice against Gram-positive bacteria. Although it is not approved for aquaculture use in most European countries, the EU has established maximum residue limits (MRLs) (Reg. EC 37/2011). In the present study, erythromycin showed little effectiveness against Aeromonas spp. The MIC90 of erythromycin was higher than the reference breakpoints (8 μg/mL) with 84.6% of the tested Aeromonas strains showing resistance. This high resistance rate and the MIC50, MIC90 and ECV for erythromycin are in agreement with other studies (Mejdi et al., 2010; Baron et al., 2017). Trimethoprim is mainly used in fish culture and often combined with sulfadiazine in commercial preparations. Because of the potential carcinogenic effect of both antibacterial agents, the EU set MRLs in fish muscle. The present study tested the two antimicrobials independently. Low efficacy was obtained for sulfonamides with resistance rates of 92.3% and 69.2% of strains for sulfadiazine and trimethoprim, respectively. For both sulfadiazine and trimethoprim the MIC50 and MIC90 were

above the reference breakpoints, so the ECVs could not be stimated. These values could not be compared with other studies on Aeromonas spp. as these antibacterials are generally used in combination. Aminoglycoside antibiotics showed intermediate MIC90 values for gentamicin (8 μg/mL) and kanamycin (32 μg/mL), whereas they were above the breakpoint for streptomycin (64 μg/mL) to which 39.4% of strains were resistant. The ECVs for gentamicin and streptomycin were greater than those observed by Baron et al. (2017), while the MIC50 for streptomycin was comparable with data obtained by Goñi-Urriza et al. (2000). In the present study, chloramphenicol and florfenicol MIC90 were lower than the reference breakpoints. These results were expected for chloramphenicol as it has been banned from use in animal food production since 1994 (EC 1430/94) due to its serious side effects on human health (irreversible aplastic anaemia). While florfenicol is registeredfor use in aquaculture only in some European countries, resistance to the fenicol category ranged between 2.9% and 3.8% of the tested strains. The MIC50 and ECVs for these two antimicrobials were in agreement with values reported by Baron et al. (2017). Overall, Aeromonas spp. showed elevated multiple resistance to the antibiotics tested. Most of the strains (82.7%) showed a MAR index between 0.3 and 0.5 (corresponding to resistance to four to eight different antibiotics) while six strains were resistant to nine to 11 antibiotics. One strain, A. punctata/hydrophila/enteropelogenes, was resistant to 11 out of 15 antibiotics tested, indicating that the isolates were exposed to high-risk sources of contamination with broad use of antibiotics, as in intensive fish farming. This result is in agreement with previous studies indicating the high antibiotic resistance of Aeromonas spp. (Dumontet et al., 2000; Nguyen et al., 2014). The antibiotics most frequently associated with multiple resistance were amoxicillin, ampicillin, cephalothin, erythromycin, streptomycin, sulfadiazine and trimethoprim.

5. Conclusions

The present study confirms that selective pressure in the aquatic environment of intensive fish farms leads to acquired antibiotic resistance by Aeromonas spp. in gilthead sea bream reared in Italy.
Compared to clinical breakpoints, measuring epidemiological cut-off values allows a better distinction between wild-type strains and strains which have acquired drug resistance due to selective pressure. The multiple antibiotic resistance of almost all strains raises serious concerns due to the possible transfer via food of antibiotic-resistant bacteria to humans or the acquisition of antibiotic resistance by human pathogens. In the light of these findings, regular monitoring programmes should be stepped up to check for antibiotic resistance in the aquaculture production of fish for human consumption.

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Table 1
Resistance of *Aeromonas* species and species-complex to 15 antimicrobial agents.

Species	N (%)	OXA	AMX	AM	CF	CL	E	FM	FF	GM	K	OT	S	SZ	TE	TMP
A. media	15 (14.4%)	8	8	6	13	1	13	5	–	1	3	1	6	13	1	12
A. enteropelogenes	1 (0.9%)	–	1	1	1	–	1	–	–	–	–	–	–	1	–	1
A. bivalvium	10 (9.6%)	2	6	6	10	–	10	1	–	1	1	2	6	10	1	8
A. media/veronii	4 (3.8%)	2	4	4	4	–	4	2	1	–	–	–	–	4	–	4
A. salmonicida/bestiarium/hidrophila/caviae	12 (11.5%)	1	7	8	12	1	11	–	1	–	–	1	7	12	1	9
A. punctata/hydrophila/enteropelogenes	1 (0.9%)	1	1	1	1	1	1	1	–	–	–	1	1	1	–	1
A. salmonicida/bestiarium	8 (7.7%)	–	4	5	8	–	8	–	–	–	–	1	6	8	1	3
A. popoffii	3 (2.9%)	2	1	2	3	–	3	–	–	–	–	–	1	2	–	2
A. molluscorum	11 (10.6%)	7	1	1	4	1	3	6	1	1	2	5	4	11	3	6
A. encheleia	2 (1.9%)	1	1	1	2	–	2	–	–	–	–	–	2	2	–	2
A. hydrophila/salmonicida/bestiarum	4 (3.8%)	1	–	–	4	–	3	–	–	–	–	–	–	4	–	4
A. punctata	6 (5.8%)	1	3	1	6	–	6	1	–	–	–	–	1	4	–	6
A. bivalvium/popoffii	3 (2.9%)	–	–	–	2	–	3	–	–	–	–	–	–	3	–	1
A. salmonicida/bestiarium/popoffii	2 (1.9%)	1	–	–	1	–	2	1	–	–	–	–	–	2	–	–
A. media/hydro	2 (1.9%)	1	2	2	2	–	2	1	–	–	–	–	–	2	–	2
A. allosacarophila	1(0.9%)	–	–	–	1	–	1	–	–	–	–	–	–	1	–	1
A. tasmaniensis/hydro/punctata	2(1.9%)	1	2	–	2	–	2	1	–	–	–	1	1	2	1	2
A. media/punctata	5 (4.8%)	4	3	2	3	–	5	3	–	1	2	–	2	3	–	4
A. encheleia/molluscorum	3 (2.9%)	–	2	2	3	–	3	–	–	–	–	–	1	2	–	1
A. salmonicida/sobria/popoffii	4 (3.8%)	–	–	–	4	–	3	–	–	–	–	–	1	4	–	2
A. salmonicida	3 (2.9%)	–	–	–	3	–	1	–	–	–	–	–	–	3	–	1
A. salmonicida/sobria	1 (0.9%)	–	–	–	1	–	1	–	–	–	–	–	1	1	–	–
A. molluscorum/eucrenophila	1 (0.9%)	1	–	–	–	–	–	1	–	–	–	–	1	1	–	–
Total	104 (100%)	34	46	42	90	4	88	23	3	4	8	12	41	96	8	72

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

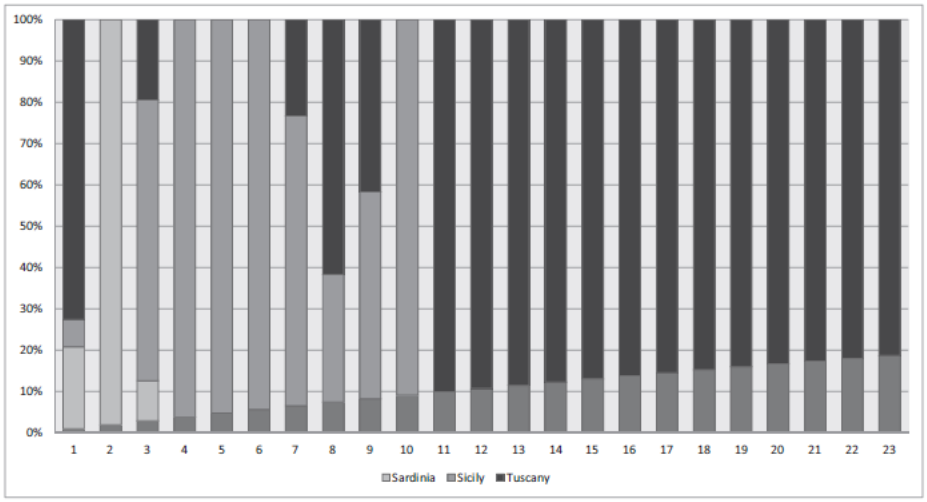

Fig. 1. Percentages of *Aeromonas* species - species complex isolated from 6 mariculture farms in 3 Italian regions.
1 = *A. media*; 2 = *A. enteropelogenes*; 3 = *A. bivalvium*; 4 = *A. media/veronii*; 5 = *A. salmonicida/bestiarium/hidrophila/caviae*; 6 = *A. punctata/hydrophila/enteropelogenes*; 7 = *A. salmonicida/bestiarium*; 8 = *A. popoffii*; 9 = *A. molluscorum*; 10 = *A. encheleia*; 11 = *A. hydrophila/salmonicida/bestiarum*; 12 = *A. punctata*; 13 = *A. bivalvium/popoffii*; 14 = *A. salmonicida/bestiarium/popoffii*; 15 = *A. media/hydro*; 16 = *A. allosacarophila*; 17 = *A. tasmaniensis/hydro/punctata*; 18 = *A. media/punctata*; 19 = *A. encheleia/molluscorum*; 20 = *A. salmonicida/sobria/popoffii*; 21 = *A. salmonicida*; 22 = *A. salmonicida/sobria*; 23 = *A. molluscorum/eucrenophila*.

Table 2
Multiple antibiotic resistance (MAR) index of *Aeromonas* spp. strains isolated from gilthead sea bream reared in 3 Italian regions.

MAR index	Region			Total
	Sardinia	Sicily	Tuscany	
0.1	–	$n = 1$	$n = 2$	$n = 3$
0.2	–	–	$n = 13$	$n = 13$
0.3	–	$n = 14$	$n = 31$	$n = 45$
0.4	$n = 3$	$n = 7$	$n = 4$	$n = 14$
0.5	$n = 1$	$n = 14$	$n = 7$	$n = 22$
0.6	–	$n = 1$	$n = 1$	$n = 2$
0.7	$n = 1$	$n = 2$	$n = 1$	$n = 4$
0.8	–	$n = 1$	–	$n = 1$
0.9	–	–	–	–

Table 3
MIC (μg/mL) and antimicrobial susceptibility of *Aeromonas* spp. strains isolated from *Sparus aurata*. * = M45-P (CLSI, 2005); a = M100-S26 (CLSI, 2016); b = M42/49 (CLSI, 2011); c = M31A2 (NCCLS, 2002); d = M31A (NCCLS, 1998).

Antibiotic	Breakpoints	MIC_{50}	MIC_{90}	Moda	Range	S (%)	I (%)	R (%)	ECV	WT strains (%)
OXA	≤0.12–≥1[b]	0.06	16	0.06	0.06–≥128	65 (62.5)	5 (4.8)	34 (32.7)	0.25	69 (66.3)
AMX	≤8–≥32*	16	≥128	8	0.06–≥128	44 (42.3)	14 (13.5)	46 (44.2)	32	66 (63.5)
AM	≤8–≥32*	16	≥128	16–≥128	0.06–≥128	42 (40.4)	20 (19.2)	42 (40.4)	64	77 (74.0)
CF	≤8–≥32*	≥128	≥128	≥128	0.06–≥128	14 (13.5)	–	90 (86.5)	ND	ND
CL	≤8–≥32*	0.5	4	0.5	0.25-64	98 (94.8)	2 (1.9)	4 (3.8)	2	91 (87.5)
E	≤0.5–≥8[c]	16	64	8	0.06–≥128	9 (8.7)	7 (6.7)	88 (84.6)	32	93 (89.4)
FM	≤2–≥4[c]	0.12	16	0.06	0.06–≥128	81 (77.9)	–	23 (22.1)	0.25	62 (59.6)
FF	≤4–≥8[b]	1	4	0.5	0.06-64	101 (97.1)	–	3 (2.9)	2	93 (89.4)
GM	≤4–≥16*	2	8	2	0.12–32	92 (88.5)	8 (7.7)	4 (3.8)	8	100 (96.2)
K	≤16–≥64[a]	8	32	16–32	0.5–≥128	89 (85.6)	7 (6.7)	8 (7.7)	32	96 (92.3)
OT	≤1–≥8[b]	0.5	16	0.5	0.12–≥128	84 (80.8)	8 (7.7)	12 (11.5)	2	84 (80.8)
S	≤6–≥25[d]	16	64	16	1–≥128	9 (8.7)	54 (51.9)	41 (39.4.)	64	90 (86.5)
SZ	≤38–≥76*	≥256	≥256	≥256	0.12–≥256	8 (7.7)	–	96 (92.3)	ND	ND
TE	≤1–≥8[b]	0.5	4	0.5	0.12–≥128	89 (85.6)	7 (6.7)	8 (7.7)	2	85 (81.8)
TMP	≤8–≥16*	32	64	64	0.12–≥128	32 (30.8)	–	72 (69.2)	ND	ND

MIC (Minimum Inhibitory Concentrations), S (susceptible strains), I (intermediate strains), R (resistant strains), ECV (epidemiological cut-off value) (μg/mL), WT strains (wild-type strains), oxolinic acid (OXA), amoxicillin (AMX), ampicillin (AM), cephalothin (CF), cloramphenicol (CL), erythromycin (E), flumequine (FM), florfenicol (FF), gentamicin (GM), kanamycin (K), oxytetracycline (OT), streptomycin (S), sulfadiazine (SZ), tetracycline (TE) and trimethoprim (TMP). N.D. = not determined.