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Antimicrobial resistance and presence of the SXT mobile element in *Vibrio* spp. isolated from aquaculture facilities

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SUMMARY

The aim of this work was to assess the susceptibility of *Vibrio* spp. strains isolated from fish cultures against some usually applied antibiotics and the occurrence of the SXT mobile genetic element among them. Antimicrobial resistance was assessed by the standard disk diffusion technique while the presence of the SXT mobile genetic element was determined by conventional PCR. High levels of resistance to ampicillin (70%), cefoxitin (44%), streptomycin (31%), aztreonam (25%) and sulfamethoxazole (21%) were detected, and a high inter-and-intraspecies diversity in the resistance profile was observed for the majority of the analysed isolates. The SXT mobile genetic element was detected in only 4 isolates belonging to the species *V. diazotrophicus* (1), *V. mediterranei* (2) and *V. vulnificus* (1), which showed a variable antibiotic resistance profile. Horizontal antibiotic resistance gene transfer from the *V. diazotrophicus* SXT-positive strain to a laboratory *E. coli* strain was demonstrated under laboratory conditions. Our results suggest that the *Vibrio* spp. isolated from aquaculture facilities analysed in this study, although not being pathogenic, they constitute a source of antimicrobial resistance genes that could be mobilized to other bacterial populations through mobile genetic elements. However, the low occurrence of the SXT element in these isolates supports the hypothesis that this element is not involved in the development of resistance in the majority of *Vibrio* spp. in the examined aquaculture facilities.

KEY WORDS: Antimicrobial, Aquaculture, Mobile genetic element, *Vibrio*.

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INTRODUCTION

In recent decades, the number of fish farming facilities has increased worldwide, especially for marine fish, and this could have an important environmental impact on the coastal ecosystem. *Vibrio* spp. are autochthonous inhabitants of marine and estuarine environments and are typically isolated from marine organisms. Although some of them are recognised as important human pathogens such as *V. cholerae*, *V. parahaemolyticus*, or *V. vulnificus* (Rippey, 1994), others are responsible for major economic losses in the aquaculture sector, such as *V. anguillarum* and *V. harveyi* that cause vibriosis with high rates of mortality (Moriarty, 1997). The use of antibiotics in aquaculture as prophylactic or therapeutic measures is a major issue since it can alter the bacterial populations inhabiting the surrounding area of the fish farm by promoting the selection of resistant clones (Labella *et al.,* 2013). Hence, the increasing number of antibiotic-resistant bacteria is of great human concern since it can compromise human or animal healthcare management systems (Cabello *et al.,* 2013). In this context, bacteria harbouring antibiotic resistance genes in the environment have been proposed as a reservoir of resistance genes that can be mobilized amongst the bacterial populations through mo-

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bile genetic elements, and therefore contribute to the emergence and spread of new antibiotic resistance phenomena (Taylor *et al.,* 2011). Other antibiotic resistance mechanisms include antibiotic efflux pumps, chromosomal mutations or the exchange of conjugative plasmids or transposons (Kitaoka *et al.,* 2011).

The SXT genetic mobile element (an integrative and conjugative element, ICE) originally described in *V. cholerae* O139 has been reported to mobilise plasmid and genomic DNA from strain to strain. This self-transmissible element has a molecular weight of approximately 62 kb and it has been associated with multidrug resistance such as chloramphenicol, streptomycin, sulfamethoxazol and trimethoprim resistance (Waldor *et al.,* 1996). Since their discovery, different ICEs belonging to the SXT/R391 family have been identified in isolates from *Vibrionaceae* from clinical and environmental settings (Bani *et al.,* 2007; Taviany *et al.,* 2008). SXT/R391-related ICEs share highly conserved module structures that code for the genes necessary for their self-transmissibility (Burrus and Waldor, 2004). However, genetic variability in the accessory genes carried by these genetic mobile elements has also been reported (Song *et al.,* 2013).

Some reports have addressed the study of antibiotic resistance focusing on the most relevant *Vibrio* pathogens in clinical microbiology, but only few reports have addressed antibiotic resistance determinants in the whole environmental *Vibrio* spp. that are in fact a reservoir of resistance genes. The objective of this research was to assess the antibiotic resistance pattern for some *Vibrio* isolates from fish culture facilities to assess the presence of resistant *Vibrio* species and to study the occurrence of the mobile genetic element SXT in these isolates to gain knowledge on the distribution and spread of antibiotic-resistant bacteria in these environments.

MATERIALS AND METHODS

Bacterial strains, media and culture conditions

The *Vibrio* strains used in this study were isolated from sea water and various marine organisms inhabiting aquaculture facilities from different European countries (Austin *et al,* 1997; García-Aljaro *et al.,* 2012). They included 100 isolates classified according to Noguerola and Blanch (2008) and sequencing of the 16S rRNA gene into 26 different species. All the *Vibrio* isolates were grown in modified Tryptic Soy Broth or Agar (TSB or TSA) supplemented with 1.5% (w/v) NaCl (Difco, Barcelona, Spain) and incubated at 30ºC unless otherwise stated.

DNA isolation

Genomic DNA was isolated from bacterial cultures prepared in TSB at 1.5% (w/v) NaCl incubated at 30ºC overnight. One millilitre culture was centrifuged and resuspended in 100 µl double distilled H_2O and boiled for 10 minutes. The samples were then chilled and centrifuged for 5 min at 16,000 *g* and 5 µl of the supernatant was used as template for the PCR studies.

PCR analysis of resistance genes, SXT integrase and sequencing

Primers used in the study to detect different antibiotic resistance genes and SXT integrase are listed in Table 1. The primers and reagents for PCR were purchased from Roche Diagnostics (Barcelona, Spain). The conditions used for the PCR are described elsewhere (García-Aljaro *et al.,* 2004). DNA sequencing was performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems), according to the manufacturer's instructions.

Antibiogram

Antimicrobial susceptibility was determined using standard disk diffusion techniques, according to the recommendations of the Clinical Laboratory Standards Institute (National Committee for Clinical Laboratory Standards, 2004). The method was adjusted for vibrios, as suggested by other authors (Roque *et al.*, 2001; Zanetti *et al.,* 2001). Briefly, cultures were grown overnight at 30ºC in TSA at 1.5% (w/v) NaCl and used to inoculate a saline suspension at 1.5% (w/v) NaCl that was adjusted to 0.5 McFarland. Mueller-Hinton agar plates supplemented with 1.5% (w/v) NaCl were inoculated, antibiotic discs placed on the top and the plates were incubated overnight at 30ºC and the diameters of the inhibition zone measured.

TABLE 1 - *Primers used in this study.*

Primer	*Sequence (5'-3')*	*Target gene*	*Amplicon (bp)*	*Reference*
SXT-F	ATGGCGTTATCAGTTAGCTGGC	SXT integrase	1035	Bhanumathi *et al.,* 2003
SXT-R	GCGAAGATCATGCATAGACC			
SUL2-F	TGTGCGGATGAAGTCAGCTCC	*sul2*, sulfonamides resistance	625	Falbo *et al.,* 1999
SUL2-R	AGGGGGCAGATGTGATCGAC			
DFR18-F	ACTGCCGTTTTCGATAATGTGG	*dfr18*, trimethoprim resistance	389	Hochut *et al.,* 2001
DFR18-R	TGGGTAAGACACTCGTCATGGG			
DFRA1-F	CAAGTTTTACATCTGACAATGAGAACGTAT	*dfrA1*, trimethoprim resistance	278	Falbo *et al.,* 1999
DFRA1-R	ACCCTTTTGCCAGATTTGGTA			
STRB-F	CCGCGATAGCTAGATCGCGTT	*strB*, streptomycin resistance	515	Hochut *et al.,* 2001
STRB-R	CGACTACCAGGCGACCGAAAT			
ln-F	GGCATCCAAGCAGCAAG	*blaP1*, β-lactamase resistance	874	Ceccarelli *et al.,* 2006
blaP1-B	CTGGTTCATTTCAGATAGCG			
FLOR-F	TTATCTCCCTGTCGTTCCAGCG	*floR*, chloramphenicol resistance	526	Iwanaga *et al.,* 2004
FLOR-2	CCTATGAGCACACGGGGAGC			
TETA-F	GTAATTCTGAGCACTGTCGC	*tetA*, tetracyclin resistance	950	Schmidt *et al.,* 2001
TETA-R	CTGCCTGGACAACATTGCTT			

The discs used were Neo-Sensitabs (Rosco, Taastrup, Denmark): amikacin (30 µg), ampicillin (10 µg), aztreonam (30 µg), cefotaxime (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), trimethoprim/sulfamethoxazole (1.25 µg/23.75 µg), streptomycin (10 µg), gentamicin (10 µg), imipenem (10 µg), nalidixic acid (30 µg), nitrofurantoin (300 µg), sulfamethoxazole (240 µg), and tetracycline (30 µg).

CONJUGATION

Conjugation experiments between the SXT integrase-carrying *Vibrio* strains and the laboratory *E. coli* DH5α strain were performed. Briefly, the donor and recipient strains were grown in Luria Bertani broth (LB) until mid-exponential phase at 30ºC and 2 ml of the bacterial cultures were harvested by centrifugation and resuspended in 200 µl of fresh LB. 100 µl of the donnor strain were mixed with 200 µl of the recipient strain and incubated for 10 minutes at room temperature. A drop of 50 µl was placed onto TSA plates and incubated overnight at 30ºC. Selection of transconjugants was performed in Chromocult coliform agar plates (Merck, Darmstadt, Germany) containing 10 mg/l streptomycin or 20 mg/l tetracycline at 37ºC. The presence of the integrase gene as well as the resistance coding genes was confirmed by PCR as stated above.

RESULTS

The different strains were classified as susceptible or resistant (including intermediate and resistant profiles) according to the break points of each antibiotic indicated by the Clinical Laboratory Standards Institute (National Committee for Clinical Laboratory Standards, 2004).

TABLE 2 - *Antibiotic resistance in the different Vibrio spp and related genera.*

	n.	*amc*	*sxt*	*amp*	*cipr*	*clr*	*ctx*	*nal*	*caz*	*imi*	*azt*	*str*	*ni*	*tet*	*cfo*	*gen*	*sul*
Aliivibrio logei	1	1		1											1		
A. salmonicida	1			1													
A. wodanis	1			1									1	1	1		1
Photobacterium damselae	4			3								2					4
P. leiognathi	1	1															
P. ganghwense	1																1
V. alginolyticus	5			5							2	2		1	3		
V. anguillarum	38	34	1	37		1	4	1	1	2	17	15	1	1	33	1	2
V. chagasii	1			1													
V. cholerae	1													1			1
V. diazotrophicus	2	1									1	2		1			1
V. fluvialis	2	2		2													1
V. harveyi	7			7							3	1					3
V. ichthyoenteri	2			1													1
V. lentus	1											1					
V. litoralis	1			1													
V. mediterranei	5			1			2		1		1	2			1		2
V. metschnikovii	5			4								1	4		2		3
V. mimicus	1																
V. mytili	2																
V. natriegens	1																
V. parahaemolyticus	3			3		1				1		2		1	2		
V. proteolyticus	2	2		2								1					
V. rotiferianus	1													1			
V. splendidus	2																
V. vulnificus	9		1		1		1	1	1		1	2		3	1	1	1
No. of resistant strains	100	41	2	70	1	2	7	2	3	3	25	31	6	10	44	2	21

Amc, amikacin; sxt, trimethoprim+sulfamethoxazole; amp, ampicillin; cipr, ciprofloxacin; clr, chloramphenicol; ctx, cefotaxime; nal, nalidixic acid; caz, ceftazidime; imi, imipenem; azt, aztreonam; str, streptomycin; ni, nitrofurantoin; tet, tetracyclines; cfo, cefoxitin; gen, gentamicin; sul, sulfamethoxazole.

Antibiotic resistance was found in all of the isolates with the exception of 8 isolates belonging to *V. anguillarum* (1), *V. ichthyoenteri* (1), *V. mimicus* (1), *V. mytili* (2), *V. natriegens* (1), *V. splendidus* (2) that were sensitive to all the antimicrobials (Table 2). High levels of resistance were observed to ampicillin (70%), cefoxitin (44%), streptomycin (31%), aztreonam (25%) and sulfamethoxazole (21%).

In the case of *V. anguillarum*, the species with the highest number of isolates (38), most isolates (84%) showed resistance to amikacin, ampicillin and cefoxitin and 11 isolates were multiresistant to 5 or more antibiotics. Interestingly, there was high intra- and interspecies variability in the observed antibiotic resistance profile as shown in Table 3, with a total of 15 different antibiotic resistance profiles for the 38 *V. anguillarum* isolates. Apart from *V. anguillarum*, multiresistance to 5 or more antibiotics was also found in 1 isolate of *V. mediterranei* (ampicillin, cefoxatine, ceftazidime, aztreonam, stremtomycin, and cefoxitin), 2 isolates of *V. vulnificus* (trimethoprim-sulfamethoxazol, ciprofloxacin, nalidixic acid, tetracycline and sulfamethoxazole/ amikacin, aztreonam, streptomycin, cefoxitin, and gentamicin), 1 isolate of *V. alginolyticus* (ampicillin, aztreonam, stremtomycin, tetracycline, and cefoxitin) and 1 isolate of *V. metschnikovii* (ampicillin, stremtomycin, nitrofurantoin, cefoxitin, and sulfamethoxazole).

The SXT element was detected in 4 isolates belonging to the species *V. mediterranei* (2 isolates), *V. vulnificus* and *V. diazotrophicus*. Sequencing of the integrase amplicon revealed a maximum nucleotide identity between 97% and 100% with the integrase present in *V. fluvialis*

TABLE 3 - *Diversity of antibiotic resistant profiles in V. anguillarum isolates. R: resistance (including intermediate and full resistance) to the established MIC criteria*

n.	*amp*	*amc*	*cfo*	*azt*	*str*	*ctx*	*imi*	*sul*	*sxt*	*clr*	*nal*	*caz*	*ni*	*tet*	*gen*	*cipr*
1	R	R	R	R	R	R	R	R					R		R	
1	R	R	R	R	R			R	R		R			R		
1	R	R	R	R	R	R										
1	R	R	R	R	R							R				
6	R	R	R	R	R											
4	R	R	R	R												
1	R	R	R				R			R						
3	R	R	R		R											
1	R	R	R			R										
13	R	R	R													
1	R			R	R	R										
1	R	R		R	R											
1	R	R		R												
1	R		R													
1	R															
1																

Amc, amikacin; sxt, trimethoprim+sulfamethoxazole; amp, ampicillin; cipr, ciprofloxacin; clr, chloramphenicol; ctx, cefotaxime; nal, nalidixic acid; caz, ceftazidime; imi, imipenem; azt, aztreonam; str, streptomycin; ni, nitrofurantoin; tet, tetracyclines; cfo, cefoxitin; gen, gentamicin; sul, sulfamethoxazole.

(GenBank accession number JQ180502), *Photobacterium damselae* (AJ870986) and *Proteus mirabilis* (AJ634266), for *V. mediterranei*, *V. vulnificus* and *V. diazotrophicus* isolates, respectively. However, the translated sequence was identical in all four isolates, being 100% identical to *Proteus mirabilis* (CAG24070), *P. damselae* (CAI35912 and CAQ34932) SXT integrase gene. The sequences of the 4 integrase genes have been deposited in GenBank under accession numbers KF307591, KF307592, KF307593 and KF307594. These isolates were resistant to sulfamethoxazole with the exception of *V. vulnificus*, which only showed resistance to cefotaxime. *V. diazotrophicus* was the only isolate in which genes coding for tetracycline, chloramphenicol, streptomycin and sulfamethoxazole were detected by PCR.

Conjugation experiments were carried out for the three *Vibrio* strains carrying appropiate selective markers including 2 strains of *V. mediterranei* and 1 strain of *V. diazotrophicus*. Only conjugation with the *V. diazotrophicus* strain produced transconjugants which were confirmed by PCR to carry the genes coding for the SXT-integrase and the genes coding for resistance to streptomycin and sulfamethoxazole.

DISCUSSION

Studies on the antibiotic resistance of *Vibrio* species from different ecosystems have yielded different results. For instance, a report on *V. harveyi* isolated from diseased *Peneus monodon* showed that the majority of the strains were resistant to erythromycin, kanamycin, penicillin, and streptomycin (Baticados *et al.,* 1990), while another study reported a high resistance to ampicillin, streptomycin and ciprofloxacin (Vaseeharan *et al.,* 2005). Oxytetracycline and florphenicol (derived from chloramphenicol) are two commonly used antibiotics in aquaculture. Resistance to oxytetracycline and chloramphenicol has been found to be plasmid mediated allowing horizontal gene transfer (Kim *et al.,* 1993; Towner, 1995). However, in our study only 10% and 2% of our isolates were resistant to these antibiotics, respectively. Moreover, in the case of the *V. anguillarum* isolates studied, only one isolate was resistant to each antibiotic which is considerably lower compared to the results of Nash *et al.* (Nash *et al.,* 1992) that showed that 29% of *V. anguillarum* isolates were resistant to oxytetracycline. However, Blanch *et al.* observed that *Vibrio* isolates from sea water and exhibition aquaria showed very low resistance to antibiotics suggesting that different management practices can keep the resistance level low in the population (Blanch *et al.,* 2009). Therefore, the lower resistance observed in this study supports a more appropriate fish-health management practice. Similarly, a recent study also found significant differences in the occurrence of antibiotic-resistant *Vibrio* isolates in the Adriatic coast and aquaculture centres depending on the fish farm origin (Labella *et al.,* 2013). This study also showed an increase in the incidence of multiresistant bacteria in aquaculture centres compared to the resistance occurring in natural marine environments associated with the impact of aquaculture activities. The relatively low level of resistance to tetracycline (17%) observed by Labella *et al.* (2013) is in accordance with our results. A high percentage of the *Vibrio* isolates analysed in our study were resistant to ampicillin, being mostly related to *V. anguillarum*, *V, harveyi* and *V. alginolyticus*. This is in agreement with previous results in which isolates belonging to these species have previously been reported to present constitutive resistance to ampicillin (Noguerola and Blanch, 2008). Similarly, Laganà and co-workers investigated the antibiotic resistance in isolates from *Photobacterium damsela* ssp. *piscicida*, *Vibrio fluvialis*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio metschnikovii* isolated from Italian fish farms, which were mainly resistant to β-lactams (Laganà *et al.,* 2011).

The SXT element was detected in only 4 of the 100 isolated *Vibrio* strains showing a variable antimicrobial resistance profile, which differed from the original resistance gene cassette described by Waldor *et al.* (1996). However, this variable profile is in agreement with other previous reports. For instance, some environmental strains of *V. cholerae* were sensitive to the common antibiotics carried by the SXT element, but these strains failed to amplify the *int* gene coding for the integrase (Miyazato *et al.,* 2004). Another recent report demonstrated the SXT integrase to be highly prevalent in *Vib-*

rio spp. isolated from wastewaters, including *V. vulnificus*, *V. parahaemolyticus*, *V. metschnikovii* and *V. fluvialis*, although in agreement with our study the antibiotic gene profile was variable among the isolates (Okoh and Igbinosa, 2011; Igbinosa *et al.,* 2011; Iwanaga *et al.,* 2004). Similarly, many strains of *V. cholerae* seem to have acquired SXT elements worldwide, although genetic variability has been reported for this element (Kitaoka *et al.,* 2011; Song *et al.,* 2013). For instance, in the case of a Laos *V. cholerae* isolate the SXT had lost resistance to trimethoprim and gained genes encoding a putative exonuclease and helicase (Iwanaga *et al.,* 2004). Our study found a lower occurrence of the SXT element in the studied *Vibrio* isolates but also a low level of antibiotic resistance against the antibiotic resistance genes most frequently carried by this element. This suggests that the management practices in the fish farms were not promoting the transmission of antibiotic resistance genes through the SXT element, although horizontal antibiotic gene transfer from *V. diazotrophicus* to *E. coli* was demonstrated in this study by conjugation under laboratory conditions.

In conclusion, although the majority of *Vibrio* spp. analysed in our study are not pathogenic they constitute a source of antimicrobial resistance genes that could be mobilized to other bacterial populations through mobile genetic elements such as the SXT element detected in our study. However, the low occurrence of the SXT element suggests other mobile genetic elements may be implied in the development of resistance in the majority of the *Vibrio* spp. in these environments. Further investigations are needed to disclose the mechanisms of gene transfer in the environmental isolates.

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