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First Detection of Ampicillin Resistant Gene (*bla*TEM) Isolated from *Vibrio* Species in Northern Italy

Tramuta C[1]*, Decastelli L[1], Bianchi DM[1], Boteva C[1], Pitti M[1], Masotti C[2], Serracca L[2] and Battistini R[2]

[1]Sicurezza e Qualità degli Alimenti, Istituto Zooprofilattico Sperimentale Piemonte, Liguria e Valle d'Aosta, Torino, Italy

[2]Levante Ligure, Istituto Zooprofilattico Sperimentale Piemonte, Liguria e Valle d'Aosta, La Spezia, Italy

***Corresponding author:** Clara Tramuta, Sicurezza e Qualità degli Alimenti, Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta, Via Bologna 148, 10154, Turin, Italy

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Abstract

With this study we investigated the resistance and the presence of resistance genes in 22 *Vibrio* spp. strains isolated from *Crassostrea gigas* oysters collected from the Golfo della Spezia (Liguria, Italy). Colonies were identified by MALDI-TOF mass spectrometry and tested for antibiotic susceptibility using a broth microdilution method. Primer pairs for gene amplification of *tet*, *bla*, *qnr*, *sul* and *mcr* were used to screen for resistance determinants. Potentially pathogenic *Vibrio* species were detected: *V. alginolyticus* (68%), *V. harveyi* (18%), and *V. parahaemolyticus* (14%). Multiplex PCR revealed the *bla*TEM gene, which was responsible for ampicillin resistance in 68% of identified strains. High levels of resistance were observed also for ciprofloxacin (91%), ampicillin (73%), and colistin (73%); 45% of the isolates were resistant to three antibiotics. To the best of our knowledge, this is the first study to report *Vibrio* strains encoding the *bla*TEM gene in *Crassostrea gigas* oyster samples from Northern Italy and to find an association between phenotypic and genotypic ampicillin resistance. This finding indicates raw oysters as a possible source of antibiotic-resistant *Vibrio* carrying resistance determinants and a potential for spread of resistance through the food chain.

Keywords: *Vibrio* species; *bla*TEM genes; Antimicrobial resistance; PCR

Background

Oysters are filter organisms that can accumulate microorganisms from the surrounding environment. Consumption of raw shellfish has long been associated with individual cases and sporadic outbreaks of gastroenteritis and other enteric illnesses. Most foodborne diseases are caused by enteropathogenic *Vibrio* strains, which are often implicated in the diffusion, particularly in aquatic and marine environments, of genetic determinants of antibiotic resistance and their transfer to humans through the food chain [1]. The acquisition of antibiotic-resistant bacteria is linked to specific genes that can be easily transferred to other bacteria by transformation, conjugation, and transduction [2].

It has been reported that the spread of resistance genes through plasmid transfer plays an important role in the diffusion of resistance genes in Gram-negative enteric pathogens [3]. For this reason, the European Food Safety Authority (EFSA) focuses on the role of food as a possible source of acquisition of antibiotic-resistant bacteria or genes that determine antibiotic resistance in humans [4].

With this study we wanted to determine antibiotic resistance and antibiotic-resistance genes in *Vibrio* spp. isolated from *Crassostrea gigas* oysters collected in the Golfo della Spezia, a heavily populated area where fish farms employing antibiotics for disease control coexist with bivalve mollusc farming.

Material and Methods

Bacterial isolation and identification

A total of 22 *Vibrio* sp. strains were isolated during the summer of 2021 from oysters of *Crassostrea gigas* species collected from a shellfish farm in Golfo della Spezia (Liguria, Italy). The strains were isolated according to ISO 21872-2:2017 with minor modifications [5]. Briefly, 25 g of pulp and intravalve liquid were homogenized with 225 ml Alkaline Peptone Water (APW) and incubated at room temperature for 18-24 h. After incubation in APW broth, a loop full of enrichment broth was aseptically streaked on sterile surface dried Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar plates and CHROMagar *Vibrio* plates and incubated at room temperature for 18-24 h. Presumptive positive isolates were then streaked onto Columbia blood agar for further identification by MALDI-TOF mass spectrometry (bioMèrieux) using Gram-negative microorganism cards.

PCR assay

The strains were tested with PCR-based biomolecular methods to determine the presence of 23 resistance genes associated with several of the main classes of antibiotics used in human and veterinary medicine. Sets of multiplex PCR endpoints were used for amplification of: *tet* (B), *tet* (C), *tet* (D), *tet* (A), *tet* (E), *tet* (G), *tet* (K), *tet* (L), *tet* (M), *tet* (O), *tet* (S) (tetracycline resistance) [6]; *bla*CTX-M, *bla*TEM, *bla*OXA, *bla*SHV (β-lactam resistance) [7]; *mcr*-1 and *mcr*-2 (colistin resistance) [8]; *qnr*A, *qnr*B, and *qnr*S (quinolone resistance) [9]; *sul*1, *sul*2 and *sul*3 (sulfonamide resistance) [10].

Antibiotic susceptibility test

Antibiotic susceptibility of the *Vibrio* isolates was tested by the two-fold broth microdilution reference method according to ISO 20776-1:2019 [11] using Sensititre EUVSEC plates (Thermo Fisher Scientific) to obtain the Minimum Inhibitory Concentration (MIC). MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight

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Table 1: Antibiotic-resistance genes detected in 22 *Vibrio* species.

ID strain	Species	*tet* genes	*β-lactamase* genes	*qnr* genes	*sul* genes	*mcr genes*
1	*Vibrio harveyi*	-	+ *bla*TEM	-	-	-
2	*Vibrio parahaemolyticus*	-	-	-	-	-
3	*Vibrio harveyi*	-	+ *bla*TEM	-	-	-
4	*Vibrio alginolyticus*	-	+ *bla*TEM	-	-	-
5	*Vibrio alginolyticus*	-	+ *bla*TEM	-	+ *sul*S	-
6	*Vibrio alginolyticus*	-	+ *bla*TEM	-	-	-
7	*Vibrio alginolyticus*	-	+ *bla*TEM	-	-	-
8	*Vibrio parahaemolyticus*	-	+ *bla*TEM	-	-	-
9	*Vibrio alginolyticus*	-	+ *bla*TEM	-	-	-
10	*Vibrio alginolyticus*	-	+ *bla*TEM	-	-	-
11	*Vibrio alginolyticus*	-	+ *bla*TEM	-	-	-
12	*Vibrio alginolyticus*	-	+ *bla*TEM	-	-	-
13	*Vibrio alginolyticus*	-	-	-	-	-
14	*Vibrio alginolyticus*	-	-	-	-	-
15	*Vibrio alginolyticus*	-	+ *bla*TEM	-	-	-
16	*Vibrio alginolyticus*	-	+ *bla*TEM	-	-	-
17	*Vibrio alginolyticus*	-	-	-	-	-
18	*Vibrio harveyi*	-	-	-	-	-
19	*Vibrio alginolyticus*	-	+ *bla*TEM	-	-	-
20	*Vibrio alginolyticus*	-	+ *bla*TEM	-	-	-
21	*Vibrio parahaemolyticus*	-	-	-	-	-
22	*Vibrio harveyi*	-	-	-	-	-

incubation. Briefly, 96 well-plates containing 14 antibiotics (range of concentration in µg/mL) - nalidixic acid (NAL 4-128), ampicillin (AMP 1-64), azithromycin (AZI 2-64), cefotaxime (FOT 0.25-4), ceftazidime (TAZ 0.5-8), ciprofloxacin (CIP 0.03-8), chloramphenicol (CHL 8-128), colistin (COL 1-16), gentamycin (GEN 0.5-32), meropenem (MEM 0.06-16), sulfamethoxazole (SMX 8-1024), tetracycline (TET 2-64), tigecycline (TGC 0.25-8), trimethoprim (TMP 0.25-32) - were seeded with an inoculum of 7.5 x 10^5 CFU/ml (per well) of exponentially growing *Vibrio* cells. The plate was incubated at 35°C and the results were obtained after 18 h incubation by taking the read at the lowest antibiotic concentration at which no turbidity could be detected visually. Resistance breakpoints published by the Clinical and Laboratory Standards Institute [12] and European Committee on Antimicrobial Susceptibility Testing [13] were used.

Results

We identified three different potentially pathogenic *Vibrio* species from a total of 22 strains isolated: 68% (n=15) of strains belonging to *V. alginolyticus*; 18% (n=4) to *V. harveyi*; and 14% *V. parahaemolyticus* (n=3) (Table 1). A set of PCR assays were carried out using specific primers to determine whether there was a relationship between phenotypic resistance of *Vibrio* species and antibiotic resistance genes: 68% of the strains were positive for the *bla*TEM gene responsible for resistance to β-lactams and to ampicillin in particular (Table 2), while *bla*CTX-M, *bla*OXA, and *bla*SHV genes were not detected. No strain presented resistance determinants associated with tetracyclines (*tet* genes), colistin (*mcr* genes), sulfonamides (*sul* genes), and quinolones (*qnr* genes), except one *V. alginolyticus* isolate with the *qnr*S gene (Table 1).

Table 2 presents the results of testing by the broth microdilution method for antibiotic resistance of the *Vibrio* isolates to individual antibiotics. We noted high rates of resistance against ciprofloxacin (91%; MIC range 0.12-0.25 µg/mL), ampicillin (73%; MIC range 32-64 µg/mL), and colistin (73%; MIC range 4-16 µg/mL); ten strains (45%), including 9 *V. alginolyticus* and 1 *V. parahaemolyticus*, were resistant to three antibiotics. Twelve *bla*TEM-positive strains (80%) were phenotypically resistant to ampicillin. All *Vibrio* isolates (100%) were sensitive to nalidixic acid (MIC range 4-8 µg/mL), azithromycin (MIC range 2-8 µg/mL), cefotaxime (MIC ≤ 0.25 µg/mL), ceftazidime (MIC ≤ 0.5 µg/mL), chloramphenicol (MIC ≤ 8 µg/mL), gentamycin (MIC range 0.5-1 µg/mL), meropenem (MIC ≤ 0.03 µg/mL), sulfamethoxazole (MIC ≤ 8 µg/mL), tetracycline (MIC ≤ 2 µg/mL), tigecycline (MIC ≤ 0.25 µg/mL) and trimethoprim (MIC 1-2 µg/mL).

Discussion

The *Vibrio* species in this study (*V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus*) are widely found in marine environments and often implicated in outbreaks of gastrointestinal infections in humans after the consumption of bivalve molluscs and raw or lightly cooked fish products. The molluscs can accumulate numerous species of *Vibrio* resistant to different classes of antibiotics. The percentage of resistance to ampicillin, ciprofloxacin, and colistin recorded for Italy, and for Liguria in particular, are in line with data from previous

Table 2: Antibiotic-resistance profiles obtained with the broth microdilution method.

ID strain	Species	NAL	AMP	AZI	FOT	TAZ	CIP	CHL	COL	GEN	MEM	SMX	TET	TGC	TMP
1	*Vibrio harveyi*	S	S	S	S	S	R	S	S	S	S	S	S	S	S
2	*Vibrio parahaemolyticus*	S	S	S	S	S	R	S	R	S	S	S	S	S	S
3	*Vibrio harveyi*	S	S	S	S	S	R	S	R	S	S	S	S	S	S
4	*Vibrio alginolyticus*	S	R	S	S	S	R	S	R	S	S	S	S	S	S
5	*Vibrio alginolyticus*	S	R	S	S	S	R	S	R	S	S	S	S	S	S
6	*Vibrio alginolyticus*	S	R	S	S	S	R	S	S	S	S	S	S	S	S
7	*Vibrio alginolyticus*	S	R	S	S	S	R	S	R	S	S	S	S	S	S
8	*Vibrio parahaemolyticus*	S	R	S	S	S	R	S	R	S	S	S	S	S	S
9	*Vibrio alginolyticus*	S	R	S	S	S	R	S	S	S	S	S	S	S	S
10	*Vibrio alginolyticus*	S	S	S	S	S	R	S	R	S	S	S	S	S	S
11	*Vibrio alginolyticus*	S	R	S	S	S	R	S	R	S	S	S	S	S	S
12	*Vibrio alginolyticus*	S	R	S	S	S	R	S	R	S	S	S	S	S	S
13	*Vibrio alginolyticus*	S	R	S	S	S	R	S	S	S	S	S	S	S	S
14	*Vibrio alginolyticus*	S	R	S	S	S	R	S	R	S	S	S	S	S	S
15	*Vibrio alginolyticus*	S	R	S	S	S	S	S	R	S	S	S	S	S	S
16	*Vibrio alginolyticus*	S	R	S	S	S	R	S	R	S	S	S	S	S	S
17	*Vibrio alginolyticus*	S	R	S	S	S	S	S	R	S	S	S	S	S	S
18	*Vibrio harveyi*	S	S	S	S	S	R	S	S	S	S	S	S	S	S
19	*Vibrio alginolyticus*	S	R	S	S	S	R	S	R	S	S	S	S	S	S
20	*Vibrio alginolyticus*	S	R	S	S	S	R	S	R	S	S	S	S	S	S
21	*Vibrio parahaemolyticus*	S	R	S	S	S	R	S	S	S	S	S	S	S	S
22	*Vibrio harveyi*	S	S	S	S	S	R	S	R	S	S	S	S	S	S

NAL: Nalidixic Acid; AMP: Ampicillin; AZI: Azithromycin; FOT: Cefotaxime; TAZ: Ceftazidime; CIP: Ciprofloxacin; CHL: Chloramphenicol; COL: Colistin; GEN: Gentamycin; MEM: Meropenem; SMX: Sulfamethoxazole; TET: Tetracycline; TGC: Tigecycline; TMP: Trimethoprim; S: Sensitivity; R: Resistance.

studies conducted on seafood. The probable cause is attributable to anthropic factors, such as industrial, agricultural and domestic wastes, which drive the spread and maintenance of resistance to these antibiotics [14-15]. We observed a strong correlation between phenotypic and genotypic resistance for ampicillin probably because of the acquisition of transferable genetic material between bacterial strains. Indeed, *bla*TEM gene encodes TEM enzymes, which are the predominant plasmid-mediated β-lactamases in Gram-negative bacteria, previously found in ampicillin-resistant *Vibrio* isolates of different origin [16-17]. The MIC ranges we noted showed that most of the *Vibrio* strains were phenotypically resistant to colistin and ciprofloxacin, but we were unable find an association between high levels of resistance and any of the *mcr* and *qnr* genes. Such phenotypic resistance in the absence of colistin-encoding mobile elements may results from chromosomal mutations in the mgrB, phoPQ, and pmrAB genes which confer lipid a modifications [18]. Similarly, mutations in the gyrA and gyrB genes of DNA gyrase and in the parC gene of topoisomerase IV are associated with decreased susceptibility to ciprofloxacin [19]. A plausible explanation for *Vibrio* strains phenotypically resistant to colistin and ciprofloxacin not harboring *mcr* or *qnr* genes is that some other factors besides the assessed *mcr* and *qnr* variants may contribute colistin and ciprofloxacin resistance to the isolates.

Our findings indicate that potentially pathogenic strains (*V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus*) highly resistant to ampicillin, colistin, and ciprofloxacin are present in northern Italy. Since bivalve molluscs are consumed raw or lightly cooked, they may be a source of bacteria and antibiotic-resistant genes. The results from this preliminary study extend our knowledge of the Golfo della Spezia, provide data where none are currently available, and useful information about consumer risk associated with seafood consumption.

Raw oysters can be a source of antibiotic-resistant *Vibrio* carrying resistance determinants with a potential for spread through the food chain.

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