Persistence of antibiotic resistant *Vibrio* spp. in shellfish hatchery environment.

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Abstract

Characterization of antibiotic resistant vibrios isolated from shellfish aquaculture is necessary to elucidate the potential transfer of resistance and to establish effective strategies against vibriosis. With this aim, we analyzed a collection of bacterial isolates obtained from 15 failed hatchery larval cultures that, for the most part, had been treated experimentally with chloramphenicol to prevent vibriosis. Isolates were obtained during a 2–year study from experimental cultures of five different clam species. Among a total of 121 *Vibrio* isolates studied, 28 were found to be chloramphenicol resistant, suggesting that the shellfish hatchery had been using a sublethal concentration of the antibiotic. Interestingly, chloramphenicol resistant vibrios showed also resistance to tetracycline and amoxicillin (Group A; n=19), or to streptomycin (Group B; n=9). Chloramphenicol resistant vibrios were subjected to a PCR amplification and DNA sequencing of the chloramphenicol acetyltransferase genes (*cat*), and the same approach was followed to study the tetracycline resistance markers (*tet*). 16S rRNA gene sequencing revealed that chloramphenicol resistant vibrios pertained mostly to the Splendidus clade. Conjugation assays demonstrated that various R–plasmids which harbored the *cat* II/*tet*(D) genes and *cat* III gene in Groups A and B respectively, were transferred to *E. coli* and bivalve pathogenic vibrios. Most interestingly, transconjugants exhibited the antibiotic resistance patterns of the donors, despite having been selected only on the basis of chloramphenicol resistance. This is the first report carried out in a bivalve hatchery elucidating the persistence of resistant vibrios, the mechanisms of antibiotic resistance and the transfer of different R–plasmids.

Keywords: *Vibrio* spp., antibiotic resistance genes, R–plasmids, bivalve cultures, shellfish hatchery.

Introduction

The fast growth of the shellfish culture industry has increased the need for intensive farming practices to maximize production. The bivalve spat from hatchery is currently the only sustainable alternative. Recurrent outbreaks of disease caused by some *Vibrio* species are the main bottleneck in larval production, leading to mortality rates of more than 90% in 24–48 h and causing considerable economic losses to hatcheries [1]. Consequently, antimicrobial agents have routinely been applied to water to treat and prevent disease during the first stages of development.

In Europe, chloramphenicol (CHL) has been banned for use in food animals, including aquaculture, because it has been associated with aplastic anemia and it is difficult to establish a safe level of human exposure [2]. However, this antibiotic is commonly used in experimental shellfish hatcheries during the development and optimization of the culture protocols due to its wide antibacterial spectra. Indeed, some authors have reported that its utilization in bivalve hatcheries during larvae and juvenile stages produces higher survival rates and have supported its use as an effective control of *Vibrio* populations [3–6]. Moreover, assuming that these facilities have an effluent treatment system to prevent environmental contamination, the antibiotic residue of this brief use may not be harmful to consumers or the environment because the bivalve hatcheries provide 2–mm seeds which are later grown in the sea for at least 1–2 years before reaching commercial size [3]. The greatest risk associated with extensive antimicrobial use in aquaculture is the development of resistant bacteria, including vibrios, in the aquatic environment that can disseminate the antimicrobial resistance genes to other bacteria, i.e. animal or human pathogens, by horizontal gene transfer [7–11].

Despite the influence of vibrios in larval cultures, no specific studies have been carried out to date in bivalve hatcheries regarding the persistence of antibiotic–resistant vibrios and the characterization of their antibiotic resistance mechanisms. The aim of the present study was to characterize the molecular determinants responsible for antibiotic resistant phenotypes in vibrios isolated from unsuccessful larval cultures in an experimental shellfish hatchery, with particular attention being paid to CHL and tetracycline (TET). We also hypothesize on the persistence of resistant vibrios and provide clues on the plasmid–mediated horizontal gene transfer of resistance genes.

Materials and methods

Microbiological Samples

The Centro de Investigacións Mariñas (CIMA) of Ribadeo (Galicia, NW Spain) is a governmental research center in shellfish aquaculture. A variety of culture protocols have been developed for several clam species in this bivalve hatchery to increase production as well as extend the number of species cultured in Spain. Occasionally, hatchery cultures fail during development and optimization of bivalve cultures due to recurrent outbreaks of vibriosis. Hence, when necessary, protocols include the preventive use of chloramphenicol (CHL) at 2.5 mg/L, following Helm et al. [5], with every change of seawater during larval development until settlement.

A collection of 271 bacterial isolates were obtained from failed larval cultures, as described in Table 1. The larvae in these clam cultures had high mortality rates and did not reach the settlement. Most larval cultures had been preventively treated with CHL (batches II–IV and VII–XV) and some had not (batches I, V and VI) (Table 1). Isolates were obtained from experimental cultures of the following clam species: pod razor shell (*Ensis siliqua*), grooved razor shell (*Solen marginatus*), wedge clam (*Donax trunculus*), Manila clam (*Ruditapes philippinarum*), and carpet shell clam (*R. decussatus*).

For microbiological samplings, larvae and seawater were sampled and immediately processed *in situ* as previously described by Prado et al. [12]. The culture media used were Marine Agar (MA; Difco, USA) for marine heterotrophic bacteria, and Thiosulphate–Citrate–Bile–Sucrose (TCBS; Oxoid, UK) for vibrios. Larvae were streaked directly on the plates with an inoculating loop (10 µL) to detect the presence/absence of bacteria and to obtain semi–quantitative estimates of the bacterial numbers in the different samples. Seawater corresponding to larval culture tanks was diluted in sterile seawater and 100 µL of the appropriate decimal dilutions was spread on the plates to determine the bacterial counts expressed in colony forming units per milliliter (CFU/mL). TCBS and MA plates were incubated at room temperature (20±2ºC) to reproduce the environmental conditions of the larval cultures in shellfish hatchery, in which the seawater tank is kept at 19–22ºC, depending on the bivalve species reared. TCBS and MA plates were incubated for 48 h or 7–10 days, respectively. Predominant types of colonies were isolated, purified and maintained frozen at –80ºC in Marine Broth (MB; Difco, USA) supplemented with 20% glycerol (v/v). All isolates were analyzed for their basic phenotypic properties using a set of tests described by Prado et al. [12] and

only those that shared the main phenotypic features of the genus *Vibrio* [13] were selected for further studies.

Characterization of CHL–resistant *Vibrio*

CHL sensitivity of the presumptive vibrios was evaluated by the agar disc diffusion method as described by CLSI [14] using 30 µg discs (Oxoid, UK) on Müeller–Hinton Agar supplemented with 1% NaCl (w/v) (MHA–1; Oxoid, UK). According to the incubation conditions (24/48 h at 20±2ºC) proposed by CLSI [14] for bacteria isolated from aquatic animals, the zones of inhibition around the discs were measured and compared against recognized zone size ranges established by the manufacturer (Oxoid, UK). The susceptibility of the CHL–resistant isolates to commercial discs (Oxoid, UK) impregnated with tetracycline (TET, 30 µg), trimethoprim–sulphamethoxazole (SXT, 25 µg), amoxicillin (AMX, 25 µg), florfenicol (FFC, 30 µg), streptomycin (STR, 30 µg), oxolinic acid (OA, 2 µg) and kanamycin (KAN, 50 µg) was also evaluated.

Only the DNA of the CHL–resistant vibrios was extracted with Instagene Matrix (Bio–Rad, USA) and their 16S rRNA genes were amplified and sequenced to confirm their identities using specific bacterial primers (27F, 926F, 1100R and 1510R) [15] to obtain an almost complete sequence (approx. 1400 bp). Bioinformatics analyses were performed as described by Prado et al. [1].

Molecular Characterization of Chloramphenicol and Tetracycline Resistance Genes

In order to prove that CHL resistance was mediated by chloramphenicol acetyltransferase genes (*cat*), we tested for the presence of these genes in the resistant vibrios using the multiplex PCR developed by Yoo et al. [16]. Moreover, CHL–TET–resistant vibrios were examined for the most frequently reported *tet* genes in aquaculture environments which encode the efflux proteins, *tet*(A–E) and *tet*(G) (17), and the ribosomal protection proteins Tet(M) and Tet(S) [18]. PCR primer sets used for the detection of *cat* and *tet* genes are listed in the supplementary data Table S1. PCR products were analyzed by electrophoresis in 2% agarose gel and sequenced.

Conjugal Transfer Assays

All CHL–resistant vibrios were used as donors in a plate mating assay to assess the transfer of R–plasmids to the kanamycin–resistant *E. coli* K^R CAG 18420 strain (mating assay 1) following Balado et al. [19]. Transconjugants grew on selective Tryptone Soy Agar supplemented with 1% NaCl (w/v) (TSA–1; Pronadisa–Lab Conda, Spain) and 20 mg/L of CHL (Sigma, USA) plus 50 mg/L of KAN (Sigma, USA) and the frequency of conjugation was calculated dividing the number of transconjugants per milliliter by the number of recipients.

Moreover, representative isolates were conjugated with two larval pathogens: *Vibrio tubiashii* subsp. *europaensis* PP–638^T (mating assay 2) and *V. ostreicida* PP–203^T (mating assay 3). MA, MB and TCBS were the culture media used in the mating assays between *Vibrio* spp., since donors and recipients were selected based on their growth appearance on TCBS. Therefore, representative CHL–resistant donors that grow as green colonies on TCBS were mated with *V. tubiashii* subsp. *europaensis* PP–638^T whose colonies are yellow. In contrast, *V. ostreicida* PP–203^T was used as recipient since its colonies are green on TCBS. In the vibrios mating assay, the pellet from the donor overnight bacterial culture was suspended in 100 µL of MB and mixed with the recipient in a microfuge tube. This small volume was dropped onto a fresh MA plate and incubated for 5 h at 25ºC. The bacterial spot was suspended in 1 mL of MB and 100 µL of serial decimal dilutions was spread on TCBS plates supplemented with 10 mg/L of CHL in which donors and transconjugants were differentiated by the color of their colonies.

All mating assays were run in duplicate and at least ten transconjugants were picked out and tested for antibiotic resistance by disc diffusion method and the specific PCR detailed previously to confirm the presence of specific resistance genes in the transconjugants.

Isolation of R–plasmids

Plasmids were extracted from the selected transconjugants using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, USA). Plasmid extractions were evaluated by electrophoresis in 0.6% (w/v) agarose (NZYTech, Portugal) gels using en electric potential of 70V for 1 h. Moreover, plasmid DNA was used as template to confirm the presence of resistance genes using the specific PCR detailed previously. Plasmids were digested with the restriction enzyme *Hind*III (New England Biolabs, USA) and the band patterns were visualized by electrophoresis under the conditions mentioned above.

Nucleotide Sequence Accession Numbers

Sequences corresponding to 16S rRNA, *cat* and *tet* genes obtained in this study have been deposited in the DDBJ/EMBL/GenBank database under accession numbers HF937133 to HF937207 (see supplementary data, Table S2).

Results

Analysis of Bacterial Load in Larval Cultures and Identification of CHL–resistant *Vibrio*

A bacterial collection of 271 isolates from larval batches with high mortality rates was obtained along a two–year period of study (Table 1). Among them, a total of 121 isolates shared the main phenotypic features of the genus *Vibrio* [13]: Gram–negative rods, facultative anaerobic, grew on TCBS, were able to reduce nitrates to nitrites, were positive for catalase, oxidase and indole production and grew at 4–25ºC and 3–6% NaCl (data not shown). *Vibrio* populations were detected in all larval samples by semi–quantitative analysis, and in the CHL–treated batches 92/219 isolates showed these characteristics (Table 1). Considerably more isolates were obtained from the larval samples (81/164) than from the seawater in the larval culture tanks (11/55). In addition, only 29/52 isolates were identified phenotypically as vibrios in the larval cultures that did not receive antibiotic treatment (27/43 from larvae and 2/9 from seawater samples). In most cases the preventive treatment did not reduce the range of vibrios associated to the larvae (Table 1). Hence, results clearly suggest that the antibiotic concentration (2.5 mg/L) was not enough to totally eliminate the vibrios associated to bivalve larvae. In addition, vibrios were uncommon in quantitative analysis of inlet seawater samples used by the hatchery ($<10^1$ cfu/mL or even not detected), regardless of whether the tank had received antibiotic treatment (Table 1).

A total of 28 vibrios (Table 1) were resistant (diameter ≤14 mm) to CHL (30 μg). As expected, most of the CHL–resistant vibrios were isolated from antibiotic treated larval cultures (n=24) but 4 isolates were obtained from cultures without antibiotic treatment (Table 1). Moreover, 25 CHL–resistant vibrios were found in larvae samples and only three isolates (Vm–26, Vd–3159 and Vd–3160) in seawater.

195 Two bacterial groups (A and B) were defined on the basis of their antibiotic resistance phenotypes (Table
196 2). Group A was constituted by 19 vibrios resistant to CHL, TET and AMX, whereas Group B included 9
197 isolates resistant to CHL and STR but not to the other chemotherapeutics assayed.
198 We confirmed the identity of CHL–resistant isolates (n=28) by 16S rRNA gene sequencing. Analysis of
199 16S rRNA gene sequence similarities confirmed that all CHL–resistant isolates belonged to the genus
200 *Vibrio*, namely to Splendidus (n=24) and Harveyi (n=4) clades (Table 2). With respect to the Splendidus
201 clade (see supplementary data, Fig. S1), most of the isolates were identified in both groups as *V. splendidus*–
202 related isolates, which includes the species *V. hemicentroti* (n=5) and the larval pathogen *V. splendidus*
203 (n=17), since these species are not distinguishable by 16S rRNA gene sequence. The remaining isolates
204 were close to *V. kanaloae* (n=2).
205 With respect to the Harveyi clade (n=4) (see supplementary data, Fig. S2), the isolate Vp–307 was
206 identified as *V. neocaledonicus*, whereas the isolates Vs–63, Vs–229 and Vs–292 showed 16S rRNA gene
207 sequence similarities lower than the threshold value (98.70 %) proposed by Stackebrandt and Ebers (20) to
208 define new taxa.
209
210 Characterization of *cat* and *tet* Genes
211
212 All CHL–resistant vibrios were selected for further studies and the genetic determinants responsible for
213 CHL resistance were analyzed. The *cat* II gene was found in all isolates in Group A, whereas *cat* III gene
214 was specific to Group B (see supplementary data, Fig. S3a). The detection of the *tet*(D) gene (see
215 supplementary data, Fig. S3b) in all Group A isolates explained TET resistance and the concurrence with
216 *cat* II gene was the most common concurrence in the batches analyzed (Table 2). Group A isolates were
217 isolated in samples during the first days of larval culture (0, 4, 6 and 7 days) (II, V, IX, X, XI) (Table 1). In
218 some batches (V and XI), CHL–resistant vibrios persisted throughout the larval culture, seeing as they were
219 detected at the beginning and at the end. Only in the *V. splendidus*–related isolates we detected both *cat*
220 II/*tet*(D) genes and *cat* III gene (Table 2), although never simultaneously in the same bacterial isolate.
221 Interestingly, *cat* genes were not detected in the vibrios with intermediate CHL susceptibility (15–17 mm)
222 (data not shown).
223 Interestingly, the phylogenetic analysis revealed that the resistance genes identified in the present study
224 share high similarities with *cat* and *tet* sequences described in plasmids isolated from fish and human

pathogens and also from environmental bacteria (Fig. 1; see also supplementary data, Fig. S4). Some of these isolates, such as *Klebsiella pneumoniae* blaNDM–1, *K. pneumoniae* NK245, *Salmonella* sp. TC67, *Vibrio cholerae* ICDC–1447 or *Enterobacter cloacae* pEC–IMPQ, simultaneously harbored the *cat* II and *tet*(D) genes in their plasmids (Fig. 1). Interestingly, the resistance genes contained in plasmid p9014 of the fish pathogen *Photobacterium damselae* subsp. *piscicida* were the closest relatives of the Group A sequences identified in the present study. Partial sequences of *cat* II, *cat* III and *tet*(D) genes obtained by sequencing of PCR products had 100% similarity to the best–match known *cat* and *tet* genes retrieved from GenBank database.

Conjugation Experiments

The mating assays demonstrated that all CHL–resistant vibrios were able to transfer the CHL resistance to *E. coli* (Table 2). The frequency of transfer in Group A ranged from 10^{-2} to 10^{-4} transconjugants per recipient, whereas in Group B this frequency was approximately 10^{-4}, and slightly higher in the *V. splendidus*–related isolates than in *V. jasicida*. Moreover, we found that all transconjugants from Group A also exhibited resistance to TET and AMX, and similarly all Group B transconjugants were resistant to STR in addition to CHL, even though they had been selected only on the basis of their resistance to CHL (Table 2). PCR tests demonstrated positive amplification of *cat* II and *tet*(D) genes in all transconjugants in Group A and *cat* III gene in Group B. Moreover, PCR using the R–plasmids as templates confirmed that the resistant determinants were on those plasmids. Together, these results clearly suggest the existence of a genetic linkage of the transferred resistance markers within a single mobile genetic element in the donor strain.

In order to gain information on the potential transmissibility of these resistances to bacterial species that inhabit the marine environment, representative CHL–resistant vibrios were selected as putative donors to be used in mating assays 2 and 3. Isolates Vd–3154 and Vs–58, identified as *V. splendidus*–related isolates, were chosen as donors in assay 2 to mate with *V. tubiashii* subsp. *europaensis* PP–638^T, whereas isolate Vs–63 (*V. jasicida*) was used in mating assay 3 using *V. ostreicida* PP–203^T as recipient. In both mating assays we found that the donors were able to transfer the resistance to larval pathogens. The antibiotic resistance profile of the recipients conditioned the phenotype of the transconjugants, although they kept the original resistance of the donors in all cases (see supplementary data, Table S3).

Definitive evidence for the role of R–plasmids in the transfer of the studied resistance genes was gained by analysis of plasmid restriction profiles (Fig. 2). In Group A (see supplementary data, Fig. 2a) two distinct R–plasmid patterns (A1 and A2) were differentiated. Patterns of Group A isolates Vt–268, Vp–348, Vp–307, Vm–26, Vm–402 and Vd–608 could not be obtained, likely due to the very low copy number of their R–plasmids. In Group B isolates, we were able to discern two types of R–plasmids (see supplementary data, Fig. 2b) which were associated to *V. jasicida* (plasmid B1) and to *V. splendidus*–related isolates (plasmid B2). Analyses of the *E. coli* transconjugants, including plasmid purification (see Fig. S5 in the supplemental material), antibiotic resistance profiles by disc diffusion method and PCR, and further restriction patterns, provided strong evidences that Group A and Group B isolates harbored R–plasmids encoding CHL–TET–AMX and CHL–STR resistance genes, respectively.

Discussion

The understanding of the mechanisms implicated in the antimicrobial resistance of vibrios is necessary to evaluate their role in shellfish aquaculture and to establish an effective strategy against vibriosis. Vibrios are highly abundant in aquatic environments and they have long been recognized as reservoirs and vehicles of antibiotic resistance by horizontal genetic material exchange [21]. Monitoring over these bacterial populations should be carried out to avoid potential dissemination of resistance genes to other bacteria and environments. Some reports have demonstrated the successful utilization of CHL to prevent vibriosis and to improve the survival rates of larvae and juvenile, using 2–8 mg/L [3, 5] or 6 and 10 mg/L [4, 6]. In the hatchery studied, workers preventively used chloramphenicol at 2.5 mg/L during the optimization and development protocols of clam cultures when high larval mortalities were detected. However, our results suggested a sublethal effect of the antibiotic at this concentration due to the high number of CHL susceptible vibrios isolated in the batches. Hence, the antibiotic concentration used in the experimental shellfish hatchery should be re–evaluated in further studies. Interestingly, Andersson and Hughes [22] reported the microbiological effects of subinhibitory concentrations of antibiotics at three different levels: as selectors of resistance (by enriching for pre–existing resistant bacteria and by selecting for *de novo* resistance); as generators of genetic and phenotypic variability (by increasing the rate of adaptive evolution, including resistance development); and as signaling molecules (influencing various physiological activities, including

285 virulence, biofilm formation and gene expression). The horizontal gene transfer and recombination of the
286 antimicrobial genetic determinants promoted by subinhibitory, and even residual, concentrations between
287 different bacterial species have been reported by some authors [23–27]. Successful alternatives to
288 antibiotics use in larval batches have been reported [28, 29].

289 Our results showed the good microbiological quality of the inlet seawater used in the shellfish hatchery,
290 regardless of CHL treatment. Nevertheless, vibrios, including CHL–resistant isolates, were commonly
291 detected in larvae even in samples corresponding to the first days of larval cultures. Persistence of these
292 populations could be explained by the fact that bivalves represent an important ecological niche for vibrios
293 which are regular components of their microbiota [30]. Vibrios are an important component of marine
294 biofilms which may contribute to the attachment of bacteria to marine organisms [31]. In biofilms these
295 bacteria can use trapped and absorbed nutrients, and establish beneficial transactions with other members
296 of the biofilm such as the acquisition of resistance genes [21, 32].

297 Interestingly, the present study demonstrated the persistence of CHL–resistant vibrios also in untreated
298 batches. Some reports supported that the persistence of antimicrobial resistance in aquatic systems in
299 absence of selective pressure can be promoted and maintained by the stability of resistance genes, by
300 subinhibitory and residual concentrations of antibiotics in the environment (sediments, water…), by other
301 factors such as high organic matter content or by a natural resistance against antimicrobial agents that
302 bacteria use to protect themselves, thus expanding the marine antibacterial resistome [27, 33–36].

303 Most of the CHL–resistant vibrios analyzed belonged to the Splendidus clade, which include the
304 dominant *Vibrio* species in coastal marine sediments, seawater and bivalves [32]. Dang et al. [21] reported
305 that this clade constituted the most common multidrug resistant bacteria in sea cucumber and sea urchin
306 cultures and resistant *V. splendidus*–related isolates were the predominant species. Moreover, the regular
307 detection of resistant isolates related to the larval pathogen *V. splendidus* [36] could explain the failure of
308 larval batches. This fact revealed the undesirable effect of the subinhibitory CHL concentration as selector
309 of resistance to contribute to the prevalence of resistant *V. splendidus*–like isolates during the course of the
310 larval cultures. Further studies should determine the possible pathogenicity of these isolates and the isolates
311 related to the Harveyi clade, which also includes aquaculture pathogens [38].

312 Detection of R–plasmids identified the horizontal transfer mechanism involved. Acquisition and
313 maintenance of different R–plasmids confirmed the versatility of the *V. splendidus*–related isolates.
314 Interestingly, the use of CHL promoted the co–selection of resistance to other antibiotics, despite the fact

315 that it was the only antibiotic registered in the history of the shellfish hatchery. The concurrence of these
316 resistant genes in the same R–plasmid should be taken into account due to the mechanisms of co–resistance
317 and cross–resistance [39]. Comparative analyses of the resistance genes revealed that similar *cat* II, *cat* III
318 and *tet*(D) sequences were detected in R–plasmids isolated from fish and human pathogens in different
319 environments. Concurrence of similar *cat* II and *tet*(D) genes in the same R–plasmid was found in the fish
320 pathogen *Photobacterium damselae* subsp. *piscicida* and in human pathogens such as *Klebsiella*
321 *pneumoniae, Salmonella* sp., *V. cholerae* or *Enterobacter cloacae* [40–45]. The different environments and
322 geographical origins of the hosts support the worldwide dissemination of these genes. The high abundance
323 of the Splendidus clade in the aquatic environment and their versatility to develop and keep antibiotic
324 resistances should be taken into account, since they were detected in larval samples taken during the first
325 days of culture or even in batches without antibiotics. The transfer of R–plasmids to other bacteria,
326 including bivalve and human pathogens, constitutes a potential risk to the aquatic environment.
327 Shellfish hatcheries constitute a potential source of antibiotic resistant bacteria. Hence, bivalve larvae
328 could serve as delivery vehicles of resistant bacteria, including pathogenic vibrios, in different geographical
329 locations and aquatic environments due to aquaculture exports. Moreover, the use of antibiotics in shellfish
330 hatcheries without an effluent treatment system is highly unadvisable, since these hatcheries constitute a
331 potential source of antibiotic residues and resistant bacteria to the surroundings. The exposure to these risks
332 for aquatic environment should be taken into account.
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334 **Conflict of interest**
335 The authors declare that they have no conflict of interest.
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338 **References**
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Figure legends

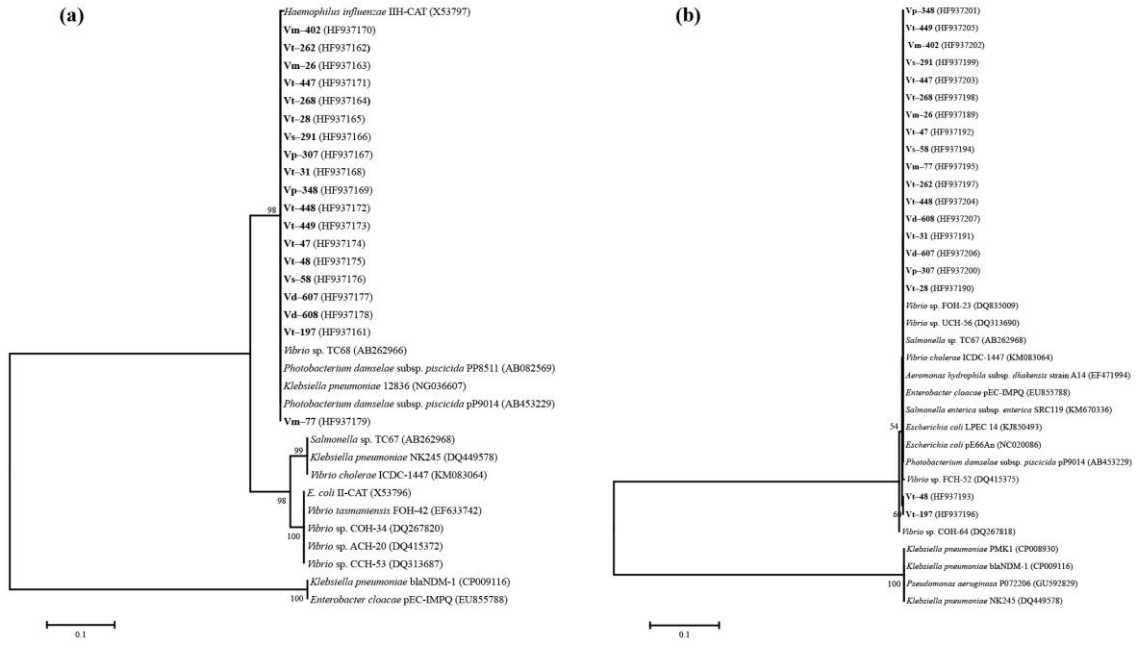

Fig. 1 Phylogenetic tree based on partial *cat* II (a) and *tet*(D) (b) gene sequences of the isolates included in the Group A. constructed using the NJ algorithm. GenBank sequence accession numbers are given in parentheses. The stability of the groupings was estimated by bootstrap percentages from 1000 replicates. Bar. 0.1 substitutions per nucleotide position.

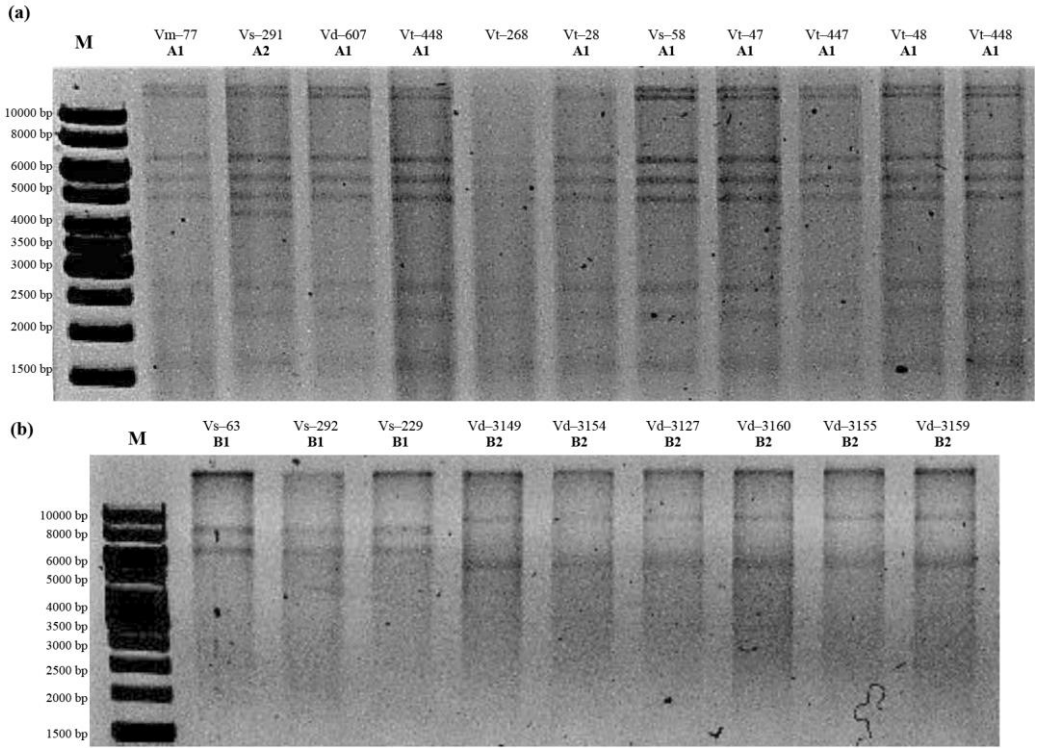

Fig. 2 Restriction enzyme patterns (A1, A2, B1 and B2) of the R–plasmids digested with *Hind*III corresponding to chloramphenicol resistant vibrios of the Groups A (a) and B (b).

M: GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, USA).

Table 1 *Vibrio* populations detected in the larval batches treated with chloramphenicol (batches II–IV and VII–XV) and in absence of antibiotic (batches I, V and VI) corresponding to the clam species *Ensis siliqua* (I–III), *Donax trunculus* (IV–VIII), *Ruditapes philippinarum* (IX–X), *Solen marginatus* (XI) and *R. decussatus* (XII–XV).

High mortalities rates were detected in all batches at the end of larval cultures.

Clam	Batch	Days of larval culture	Larvae[a]	Seawater (CFU/mL)[b]	CHL–resistant *Vibrio*	Clam	Batch	Days of larval culture	Larvae[a]	Seawater (CFU/mL)[b]	CHL–resistant *Vibrio*
E. siliqua	I	4	++	$<10^1$		*R. philippinarum*	X	2	+	$<10^1$	
		11	++++	$<10^1$	Vs–229			7	+	$5.0x10^1$	Vp–307
		18	+	$2.0x10^1$				14	+	$4.0x10^1$	
	II	1	++	$2.1x10^2$		*S. marginatus*	XI	3	+	$2.2x10^2$	
		4	+++	$<10^1$	Vs–58			6	+	$2.4x10^2$	Vm–26
		11	++	$1.0x10^2$	Vs–63						Vm–77
		18	+	$<10^1$	Vs–292			13	++	$<10^1$	
	III[d]	16	+	nd[c]	Vs–291			20	++	$1.6x10^2$	
D. trunculus	IV[d]	20	+	nd[c]	Vt–197			27	+	nd[c]	Vm–402
	V	0	++	$<10^1$	Vt–262			21	+	$<10^1$	
		4	++	$<10^1$		*R. decussatus*	XII	2	+	$<10^1$	
		11	+	$9.0x10^1$				5	++	$<10^1$	
		18	++	$1.6x10^2$	Vt–268			7	++	$<10^1$	
	VI	4	+	$2.0x10^1$				14	++	$<10^1$	
		11	++	$4.0x10^1$				19	+	$<10^1$	
		18	++	$2.1x10^2$	Vt–28			26	++	$<10^1$	Vd–607
					Vt–31						Vd–608
	VII	4	+	$<10^1$			XIII	0	+	nd[c]	
		11	+	$2.0x10^1$							
		18	+	$<10^1$				2	++	nd[c]	
		25	+	$<10^1$	Vt–47			4	+	$<10^1$	
		29	+	nd[c]	Vt–447			12	+	$<10^1$	
	VIII	4	+	$<10^1$				19	+++	$2.0x10^1$	Vd–3149
		11	++	$1.4x10^2$							Vd–3154
		18	++	$7.0x10^1$							Vd–3159
		25	++	$<10^1$	Vt–48		XIV	0	+	nd[c]	
		29	++	nd[c]	Vt–448			2	+++	nd[c]	
		29	++	nd[c]	Vt–449			4	++	$9.0x10^1$	
R. philippinarum	IX	1	+	$<10^1$				12	+	$<10^1$	
		6	+	$<10^1$	Vp–348			19	+++	$7.0x10^1$	Vd–3155
		13	+	$7.0x10^1$							Vd–3160
		20	+	$<10^1$			XV	0	+	nd[c]	
		29	+	$<10^1$				5	+	$<10^1$	
								12	++	$2.0x10^1$	Vd–3127

[a] Presence of vibrios in larvae on TCBS plates and expressed in semi–quantitative estimates: + (1–29 CFU) ++ (30–49 CFU) +++ (50–100 CFU) ++++ (>100 CFU).

[b] Vibrios load counted on TBCS plates corresponding to the seawater used in the larval tanks diluted decimally and expressed in CFU/mL..

[c] Sample was not taken during this sampling.

[d] Batches III and IV were only sampled punctually, when the first signs of disease were detected.

Table 2 Characterization of the CHL–resistant vibrios.

Donors and transconjugants exhibited the same antibiotic resistance patterns and resistance genes.

Group	Antibiotic resistance pattern	Resistance genes	Clam	Batch	Isolate	Closest sequence match (%)	Frecuency of conjugation
A:	CHL, TET, AMX	*cat* II / *tet*(D)	*E. siliqua*	II	Vs–58	*V. hemicentroti* (100)[a]	$1.1x10^{-2}$
				III	Vs–291	*V. splendidus* (99.8)[a]	$4.9x10^{-3}$
			D. trunculus	IV	Vt–197	*V. hemicentroti* (99.9)[a]	$2.4x10^{-3}$
				V	Vt–262	*V. hemicentroti* (100)[a]	$7.1x10^{-4}$
					Vt–268	*V. splendidus* (99.8)[a]	$7.0x10^{-5}$
				VI	Vt–28	*V.kanaloae* (99.9)[a]	$3.5x10^{-4}$
					Vt–31	*V.kanaloae* (99.9)[a]	$6.0x10^{-5}$
				VII	Vt–47	*V. splendidus* (99.8)[a]	$1.4x10^{-3}$
					Vt–447	*V. splendidus* (99.8)[a]	$1.9x10^{-3}$
				VIII	Vt–48	*V. splendidus* (99.8)[a]	$2.0x10^{-5}$
					Vt–448	*V. splendidus* (99.8)[a]	$3.9x10^{-3}$
					Vt–449	*V. splendidus* (99.8)[a]	$6.4x10^{-3}$
			R. philippinarum	IX	Vp–348	*V. hemicentroti* (99.9)[a]	$5.3x10^{-3}$
				X	Vp–307	*V. neocaledonicus* (99.8)[b]	$2.2x10^{-3}$
			S. marginatus	XI	Vm–77	*V. splendidus* (99.7)[a]	$3.5x10^{-4}$
					Vm–26	*V. hemicentroti* (99.9)[a]	$1.4x10^{-3}$
					Vm–402	*V. splendidus* (99.7)[a]	$4.5x10^{-4}$
			R. decussatus	XII	Vd–607	*V. splendidus* (99.8)[a]	$2.9x10^{-3}$
					Vd–608	*V. splendidus* (99.8)[a]	$2.7x10^{-3}$
B:	CHL, STR	*cat* III	*E. siliqua*	I	Vs–229	*V. jasicida* (98.2)[b]	$2.0x10^{-5}$
				II	Vs–63	*V. jasicida* (98.2)[b]	$1.0x10^{-5}$
					Vs–292	*V. jasicida* (98.2)[b]	$2.0x10^{-5}$
			R. decussatus	XIII	Vd–3159	*V. splendidus* (99.5)[a]	$1.3x10^{-4}$
					Vd–3149	*V. splendidus* (99.5)[a]	$1.6x10^{-4}$
					Vd–3154	*V. splendidus* (99.8)[a]	$7.0x10^{-5}$
				XIV	Vd–3155	*V. splendidus* (99.8)[a]	$1.5x10^{-4}$
					Vd–3160	*V. splendidus* (99.5)[a]	$2.2x10^{-4}$
				XV	Vd–3127	*V. splendidus* (99.8)[a]	$2.7x10^{-4}$

Resistant to: CHL, chloramphenicol (30 µg); TET, tetracycline (30 µg); AMX, amoxicillin (25 µg); STR, streptomycin (30 µg).

[a,b] Isolates belonged to Splendidus (a) or Harveyi (b) clade (see also Fig. S1 and S2).