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ORIGINAL ARTICLE

Prevalence, antibiotic resistance, virulence and plasmid profiles of *Vibrio parahaemolyticus* from a tropical estuary and adjoining traditional prawn farm along the southwest coast of India

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Abstract *Vibrio parahaemolyticus*, autochthonous to estuarine, marine, and coastal environments throughout the world, is a leading cause of foodborne gastroenteritis in Asia as well as in other countries. Considering their role in disease outbreaks, water and sediment samples from two different environments along the southwest coast of India, such as the Cochin estuary, which is a tourist hot spot and a traditional prawn farm, were examined for the detection of *V. parahaemolyticus.* A total of 120 presumptive vibrios were further screened using a chromogenic medium. The isolates producing the specific colour for *V. parahaemolyticus* on the chromogenic medium were confirmed by molecular methods. Using a PCR assay targeting the species-specific *tlh* gene, 75 isolates were confirmed as *V. parahaemolyticus.* The incidence of *V. parahaemolyticus* was found to be 71.6 % in the Cochin estuary and 53.3 % in the shrimp farm. The virulence genes *tdh* and *trh* were detected in two isolates from the Cochin estuary by multiplex PCR. Using 16S rRNA sequence analysis, our isolate exhibited 100 % similarity to the *V. parahaemolyticus* O3:K6 pandemic clone. The isolates were evaluated for their susceptibility towards 16 different antibiotics. All the isolates exhibited multiple antibiotic resistance (MAR). The MAR index of the isolates from the Cochin estuary ranged from 0.312 to 0.75 and that from the shrimp farm ranged from 0.1875 to 0.5. The plasmid profiles of the isolates were also checked. About 50.6 % of the isolates harboured plasmids and ten different profiles were observed. No correlation was observed between the antibiotic resistance patterns and plasmid profiles.

Keywords *Vibrio parahaemolyticus* · *tdh* · *trh* · Pandemic clone · Multiple antibiotic resistance · Plasmid profile · Southwest coast

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Introduction

Vibrios are Gram-negative halophilic bacteria, found naturally in shallow coastal waters to the deepest parts of the ocean, and are highly abundant in aquatic environments, including estuaries, marine coastal waters and sediments, and aquaculture settings worldwide. Vibrios comprise more than 74 species (Thompson et al. 2004), and most of the *Vibrio* sp. are known to be pathogenic to both humans and animals. *V. parahaemolyticus* is regarded as the primary source of increase in vibriosis incidence (Newton et al. 2012), and highly pathogenic serotypes of the species are emerging on a global scale. The pandemic serovar O3:K6 that emerged in India in 1996 has since been found to account for many cases of *V. parahaemolyticus* outbreaks worldwide. *V. parahaemolyticus* is a leading cause of bacterial gastroenteritis in Asia as well as in other countries (Chiou et al. 2000; Daniels et al. 2000; Wong et al. 2000; McLaughlin et al. 2005; Shigeaki et al. 2012), and is attracting increasing interest worldwide where raw or undercooked seafood is often consumed (Chao et al. 2009). It causes wound infections in those exposed to contaminated water (Miyoshi et al. 2008). It also causes septicemia, particularly in immunocompromised people (Daniels et al. 2000). Hence, the prevalence

and distribution of Vibrios in aquatic environments is of great public health importance.

During the past few decades, there has been a rapid emergence of antimicrobial resistance in many bacterial genera, due to the excessive use of antibiotics in humans, agriculture, and aquaculture systems (Cabello 2006). Release of sewage also results in the entry of a large number of drug resistant bacteria from various sources into the environment. Resistant genes are further transferred from non-pathogens to pathogens through horizontal gene transfer via conjugation, transduction and transformation. This could lead to the transfer of drug resistance features to extremely autochthonous microflora such as vibrios. Thus, the search for genetic elements like plasmids, transposons, and integrons associated with antibiotic resistance in microorganisms also becomes important. Multiple drug resistance among *Vibrio* sp. in estuarine-marine environments may have future implications for those who consume seafood contaminated with these pathogenic vibrios, and also for the recreational and commercial users of these environments (Shaw et al. 2014).

Vembanad Lake is connected to the Arabian Sea through the Cochin estuary. This is the largest brackish, tropical wetland ecosystem along the southwest coast of India, and is of extraordinary importance for its hydrological function, biodiversity and rich fishery resources. It is also one of the famous hot spots for tourism. The backwaters of Kerala support as much biological productivity and diversity as can be found tropical rain forests. These waters are responsible for the rich fishery potential of Kerala. The backwaters also act as nursery grounds for commercially important prawns and fishes. The fields around the backwaters are suitable for aquaculture. These areas support traditional, seasonal and perennial prawn fishery. The fishery products from these areas are exported worldwide.

Considering all these factors, the present study aimed to investigate the incidence of *V. parahaemolyticus* at various stations of the Cochin estuary (influenced by urban, industrial, human and hospital waste water) and the adjoining traditional shrimp farm at Edavanakkadu. The study area is one of the most famous tourist hot spots, and shrimps grown here are exported to various countries. The study also checked for multiple drug resistance and plasmid profiling among the isolates.

Materials and methodology

Sample collection, processing and enrichment

Considering the size of the estuaries, water and sediment samples were collected from ten stations in the Cochin estuary (9°40′- 10°12′ N, 76°10′- 76°30′ E). Four traditional shrimp farms and the feeder canal to these ponds located along Cochin backwaters at Edavanakkadu were also selected for the study. The farms in this area are well connected to the Cochin estuary through the feeder canal system, which brings water to this area from the Cochin backwaters. The water samples were collected using a Niskin water sampler, and sediment samples were collected using the Van-Veen grab. All samples were processed within 2 h of collection. Aseptic procedures were strictly followed during collection, transportation and analysis of the samples. Five hundred millilitres of water samples from each station were filtered using 0.45 μm bacteriological filters, and each filter was transferred to 100 ml alkaline peptone water for pre-enrichment and incubated at 37 °C for 18–24 h. Sediment samples were analysed after making tenfold dilutions in isotonic saline. One millilitre of the diluted soil sample was transferred to 99 ml of alkaline peptone water and enriched by incubation at 37 °C for 18–24 h.

Isolation and identification of *Vibrio* sp.

A loopful of enrichment broth was aseptically streaked onto a sterile surface of dried thiosulphate citrate bile salt sucrose (TCBS; Himedia, India) agar plates, and incubated at 37 °C for 24 h. About three to four typical sucrose non-fermenting colonies having a green or bluish-green colour were picked from each TCBS plate. Preliminary screening of isolates was done on the basis of Gram staining, oxidase test, catalase test and reactions on triple sugar iron agar. The presumptive isolates were then streaked onto a chromogenic medium for further confirmation (Hichrome Vibrio agar, Himedia). The isolates producing bluish-green colonies on Hichrome Vibrio agar were picked and maintained on nutrient agar slants supplemented with 3 % NaCl for further molecular identification.

Extraction of genomic DNA

Genomic DNA of these isolates was extracted by the boiling method (Devi et al. 2009). The colonies were inoculated in Luria Bertani (LB) broth supplemented with NaCl (2 % w/v), and incubated at 37 °C with shaking (120 rpm) for 16–18 h. The broth cultures were centrifuged (10,000 rpm, 4 °C, 1 min) to obtain a pellet, which was then washed with normal saline (0.8 % NaCl w/v) and re-suspended with DNA-free sterile distilled water (0.5 ml). The resulting suspension was heated at 98±2 °C for 15–20 min in a water bath to lyse the cells, and release the DNA. The lysate was centrifuged to remove the cell debris (10,000 rpm, 4 °C, 5 min), and the supernatant was stored (−20 °C) until further use.

Detection of species specific *tlh* gene by PCR

The detection of the *tlh* gene was performed using the primers TLHF (5′-AAAGCGGATTATGCAGAAGCACTG-3′) and

TLHR (5′-GCTACTTTCTAGCATTTTCTCTGC-3′). PCR amplification was optimized in a total reaction volume of 25 μl consisting of sterile Milli Q water (15.5 μl), 10× PCR buffer (2 μl), primer (1 μl each), dNTP mix (1 μl, 200 mM), template (4 μl), and Taq DNA polymerase (0.5 μl). The PCR conditions included an initial denaturation of 94 °C for 3 min, followed by 30 cycles of denaturation (94 °C for 1 min), primer annealing (58 °C for 1 min), and primer extension (72 °C for 1 min), followed by a final extension (72 °C for 5 min).

Detection of virulence genes by multiplex PCR

The detection of the genes *tdh* (thermostable direct hemolysin) and *trh* (thermostable direct hemolysin-related hemolysin) was done using the primer pairs TDHL (5′-GTAAAGGTCT CTGACTTTTGGAC-3′) and TDHR (5′- TGGAATAGAA CCTTCATCTTCACC-3′) for *tdh* and TRHL (5′-TTGGCT TCGATATTTTCAGTATCT-3′) and TRHR (5′-CATAACAA ACATATGCCCATTTCCG-3′) for *trh*. PCR amplification was performed in a total reaction volume of 25 μl consisting of sterile Milli Q water (13.5 μl), 10× PCR buffer (2 μl), primer (1 μl each), dNTP mix (1 μl, 200 mM), template (4 μl), and Taq DNA polymerase (0.5 μl). The PCR conditions were the same as for the *tlh* gene.

Gel documentation and image analysis

The PCR amplified products were separated by electrophoresis on an agarose (1.5 % w/v) gel in 1× TBE Buffer (Himedia, India) containing 0.5 μg/ml of ethidium bromide. The amplicon sizes were compared with a 100 bp DNA ladder. The gels were then visualized under a UV transilluminator and recorded as tiff files using the Gel Documentation System, (GelDoc EZ imager, Bio-Rad).

16S rRNA amplification, sequencing and accession number

To confirm the identification of *V. parahaemolyticus*, the isolate PM1S2 was subjected to 16S rRNA gene amplification using the universal primer set (27F: 5′-AGA GTT TGA TCC TGG CTC AG-3′, 149R: 5′-GGT TAC CTT GTT ACG ACT T-3′). The reaction conditions included an initial denaturation at 95° for 2 min, followed by 30 cycles of denaturation at 95° for 2 min, annealing at 58 °C for 1 min, and extension at 72 °C for 2 min. A final extension at 72 °C for 10 min was also included.

The PCR product was sent for sequencing at Scigenom, Kochi. The sequence obtained was submitted to the basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST) at NCBI (National Center for Biotechnology Information) to determine the percentage similarity with already identified 16S rRNA sequences in the GenBank database. The sequence was deposited in the Genbank and an accession number was allotted to it.

Antibiotic sensitivity test

The antibiotic sensitivity of the 75 *V. parahaemolyticus* isolates, i.e., 43 isolates from the Cochin estuary and 32 isolates from the shrimp farm, were analysed and compared using the disc diffusion method (Bauer et al. 1966). Antibiotic impregnated discs (Himedia, India) of 8-mm diameter were used for the test. Discs containing the following antibacterial agents were placed on the plates swabbed with enriched bacterial culture and incubated overnight: amoxycillin (Amx-10 μg), ampicillin (Amp-10 μg), amikacin (Ak-10 μg), carbenicillin (Cb-100 μg), sulphamethoxazole (Sm-300 μg), oxytetracycline (O-30 μg), chloramphenicol (C-30 μg), nalidixic acid (Na-30 μg), norfloxacin (Nx-10 μg), nitrofurantoin (Nit- 100 μg), enrofloxacin (Ex-5 μg), erythromycin (E-15 μg), streptomycin (S-10 μg), netillin (Net-10 μg), trimethoprim (Tr-5 μg), and furazolidone (Fr-50 μg). The antibiotics belonged to nine different classes according to their chemical structure: aminoglycosides (amikacin, streptomycin, gentamicin, netilmicin), macrolides (erythromycin), beta-lactams (amoxycillin, ampicillin, carbenicillin), quinolones (nalidixic acid), fluoroquinolones (norfloxacin, enrofloxacin), tetracyclines (tetracycline, oxytetracycline), nitrofurans (nitrofurantoin, furazolidone), sulphadrugs (sulphamethoxazole) and folate pathway inhibitors (trimethoprim).

After incubation, the diameter of the zone of inhibition was measured and the results were interpreted based on recommendations of Clinical Laboratory Standards Institute (CLSI 2011). Based on resistance to more than three antibiotics, the isolates were grouped as multiple antibiotic resistant isolates.

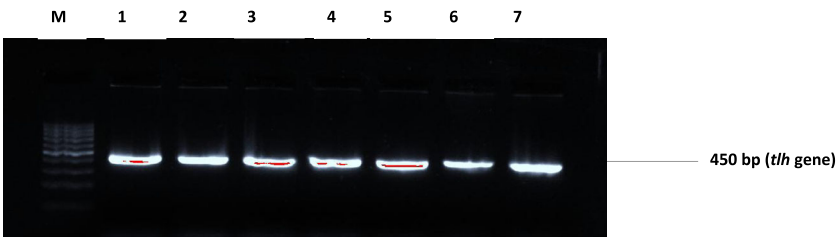

Fig. 1 PCR amplified *V. parahaemolyticus*-specific *tlh* gene. *Lane M*: 1 kb molecular weight ladder; *lanes 1–7*: *V. parahaemolyticus* isolates

Table 1 Antibiotic resistance patterns and plasmid profiles of *V. parahaemolyticus* isolates from Cochin estuary

Isolated strain	MAR index	Antibiotic resistance pattern	Plasmid profile
IW1	0.56	Amx, Amp, Cb, Sm, Ex, E, Nit, Fr, S	P1[a]: 33 kb
1W2	0.4375	Amx, Amp, Nit, Ex, , Tr, O, Fr	ND
1W3	0.5	Amx, Amp, Cb, Nit, E, Ex, Fr, Sm,	P2: 33 kb; 15 kb
1W5	0.5	Amx, Amp, Cb, Nit, O, Ex, Tr, Fr	P3: 33 kb; 1.5 kb; 1 kb
1W6	0.5	Amx, Amp, Cb, Nit, Ex, Tr, O, Fr	ND
1W7	0.3125	Amx, Amp, Nit, Ex, Fr	ND
1S1	0.4375	Amx, Amp, Sm, Ex, Tr, Nit, Fr,	P3: 33 kb; 1.5 kb; 1 kb
1S3	0.5	Amx, Amp, Ak, Cb, Sm, Ex, Nit, Fr	P1: 33 kb
2S1	0.56	Amx, Amp, Cb, Nit, C, Ex, Tr, Fr, Sm	ND
2S5	0.375	Amx, Amp, Cb, C, Fr, Sm	P2: 33 kb; 15 kb
3S3	0.4375	Amx, Amp, Cb, Sm, Nit, Ex, Fr	ND
3S4	0.625	Amx, Amp, Ak, Cb, Sm, Ex, Nit, Fr, S, Gen	ND
3W6	0.5	Amx, Amp, Cb, Sm, Ex, Nit, Fr, S	P1: 33 kb
3W7	0.625	Amx, Amp, Ak, Cb, Sm, Ex, E, Nit, Fr, S	ND
6S2	0.5	Amx, Amp, Ak, Cb, Sm, Ex, Nit, Fr	ND
6S3	0.4375	Amx, Amp, Cb, Sm, Nit, Ex, Fr	P3: 33 kb; 1.5 kb; 1 kb
6S4	0.4375	Amx, Amp, Cb, Sm, Nit, Ex, Fr	P1: 33 kb
7W5	0.5	Amx, Amp, Cb, Sm, Nit, Ex, Tr, Fr	ND
8W3	0.68	Amx, Amp, Cb, Sm, Ex, O, E, Tr, Nit, Fr, Gen	P1: 33 kb
8S6	0.56	Amx, Amp, Cb, Nit, E, Ex, Fr, Sm, S	ND
9S4	0.6	Amx, Amp, Cb, Sm, Nit, Ex, E, Tr, O, Fr	ND
9S5	0.5	Amx, Amp, Ak, Cb, Sm, Ex, Nit, Fr	ND
10W1	0.375	Amx, Amp, Sm, Nit, Ex, Fr	P1: 33 kb
10W2	0.5	Amx, Amp, Cb, Sm, Nit, Ex, Tr, Fr	P4: 33 kb; 8.9 kb
10W4	0.5	Amx, Amp, Cb, Sm, Nit, Ex, Tr, Fr	P1: 33 kb
10S4	0.43	Amx, Amp, Cb, Sm, Nit, Ex, Fr	ND
M1W1	0.56	Amx, Amp, Cb, Nit, Sm, E, Ex, Fr, S	P1: 33 kb
M1W4	0.56	Amx, Amp, Cb, Nit, Sm, Ex, O, Fr, S	ND
M6W4	0.375	Amx, Cb, Nit, Ex, Fr, Sm	P1: 33 kb
M7S2	0.312	Amx, Nit, Ex, Fr, Sm	P1: 33 kb
M7S3	0.56	Amx, Amp, Cb, Nit, E, Ex, Fr, Sm, S	P5: 33 kb; 4 kb
M8W2	0.5	Amx, Amp, Cb, Sm, Nit, Ex, E, Fr	P1: 33 kb
M10S2	0.75	Amx, Amp, Na, Cb, Nit, O, E, Ex, Tr, Fr, Sm, S,	ND
M10S4	0.75	Amx, Amp, Na, Cb, Nit, O, E, Ex, Tr, Fr, Sm, S	ND
PM1W3	0.625	Amx, Amp, Ak, Cb, Sm, Ex, Tr, Nit, Fr, S	2 kb
PM1S1	0.6	Amx, Amp, Ak, Cb, Nit, E, Ex, Fr, Sm, S	ND
PM1S2	0.43	Amx, Amp, Cb, Nit, E, Ex, Fr	ND
PM1S5	0.5	Amx, Amp, Cb, Sm, Nit, Ex, E, Fr	P6: 33 kb; 2 kb; 1 kb; 0.5 kb
PM3W5	0.375	Amx, Amp, Cb, Sm, Nit, E, Fr	P7: 33 kb; 7 kb; 8 kb; 9 kb
PM3S2	0.56	Amx, Amp, Cb, Nit, O, E, Ex, Fr, Sm	P2: 33 kb; 15 kb
PM6W6	0.56	Amx, Amp, Cb, Nit, O, E, Ex, Fr, Sm	P2: 33 kb; 15 kb
PM6W7	0.687	Amx, Amp, Ak, Cb, Nit, E, Ex, Fr, Sm, S	ND
PM8W1	0.6	Amx, Cb, Nit, O, E, Ex, Tr, Fr, Sm, S	P8: 33 kb; 5 kb; 2 kb; 1.5 kb

ND none detected

[a] P1, P2, P3, P4, P5, P6, P7, P8—Profile 1 to profile 8

Multiple antibiotic resistance (MAR) indexing of the isolates was determined by calculating the ratio between the number of antibiotics to which an isolate is resistant and the total number of antibiotics to which the isolate was exposed (Krumperman 1983).

Plasmid profiling

The multiple drug resistant strains were screened for the presence of plasmids. Bacterial strains were grown in 10 ml of Luria Bertani broth (Hi media, India) containing 1 % sodium chloride and ampicillin, and were incubated overnight at 37 °C in a shaker incubator (200 rpm) (Scigenics Biotech, India) for 16–18 h. About 1.5 ml of this culture was used for plasmid extraction by the alkaline lysis method (BirnBoim and Doly 1979). Briefly, 1.5 ml of the culture was transferred into a microfuge tube and centrifuged at 6, 000 rpm for 5 min. Supernatant was removed and the pellet was resuspended in 100 μl distilled water followed by 100 μl lysis buffer (10 % SDS, 0.5 M EDTA, 10 N NAOH). The tubes were kept in a boiling water bath for 10 min, then 50 μl of 1 mM $MgCl_2$ was added to them, in the hot condition itself. Tubes were kept on ice for 2 min, and then centrifuged at 12,000 rpm for 2 min. Then 3 mM potassium acetate was added and kept on ice for 2 min and then centrifuged at 12,000 rpm for 2 min. The supernatant was transferred to a new tube and 600 μl of isopropanol was added and the tube was kept on ice for 10 min. Tubes were then centrifuged at

10,000 rpm for 10 min. The supernatant was discarded and the pellet was rinsed in ice-cold 70 % ethanol at 5, 000 rpm for 5 min and air-dried for about 10 min to allow the ethanol to evaporate. The pellet was resuspended in 30 μl distilled water and kept at 4 °C overnight for dissolving.

Electrophoresis was carried out on a 0.8 % agarose gel (Agarose, Himedia, Mumbai, India) 1 % (w/v) in 1× TBE Buffer (Himedia, India) containing 0.5 μg/ml of ethidium bromide. Electrophoretic separation was carried out at 75 V for 1 h and a molecular weight marker (Supercoiled DNA ladder, Himedia, India) was included. The gels were then visualized under a UV transilluminator and recorded as tiff files using the Gel Documentation System, (GelDoc EZ imager, Bio-Rad).

Results

Characterisation of the bacterial isolates and detection of virulence genes

A total of 120 *V. parahaemolyticus*-presumptive, bluish-green colonies (60 each from the Cochin estuary and shrimp farm) were isolated on the TCBS agar. Of the 120, 75 isolates, i.e., 43 isolates from the Cochin estuary and 32 isolates from the shrimp farm, produced bluish-green–coloured colonies (specific for *V. parahaemolyticus*) on Hichrome Vibrio agar. All 75 isolates were found to be *V. parahaemolyticus* by PCR assay targeting the species-specific *tlh* (450 bp) gene (Fig. 1). The incidence of *V. parahaemolyticus* was found to be 71.6 % in the Cochin estuary and 53.3 % in the shrimp farms. Multiplex PCR assay revealed two *V. parahaemolyticus* isolates from the Cochin estuary that possessed the virulent genes *tdh* and *trh*, with amplicon sizes of 270 and 500 bp, respectively (data not given).

Confirmation by 16S rRNA sequencing analysis

When the sequence similarity search was done using the BLAST tool, our sequence was found to be 100 % identical with the 16S rRNA sequences of the pandemic clone *V. parahaemolyticus* O3:K6 in GenBank (RIMD 2210633). The sequence was submitted to GenBank and was assigned the accession number KM406325.

Antibiotic sensitivity of Cochin estuary isolates

All 43 isolates from the Cochin estuary were found to be multiple drug resistant. The MAR index of the isolates ranged from 0.312 to 0.75. The MAR index and antibiotic resistance pattern are shown in the Table 1. The most repeated resistance pattern was Amp, Amx, Cb, Sm, Nit, Ex, Fr. The percentage resistance of the isolates is shown in Fig. 2. All of the isolates (100 %) were resistant to amoxycillin (beta lactam) and furazolidone (nitrofuran). A high percentage of resistance was also exhibited towards ampicillin and carbenicillin (beta lactams), nitrofurantoin (nitrofuran), sulphamethoxazole (sulphonamide) and enrofloxacin (fluoroquinolone). A medium-to-low level resistance was shown towards erythromycin (macrolide), streptomycin and amikacin (aminoglycoside), trimethoprim (folate pathway inhibitors), oxytetracycline (tetracycline), gentamicin (aminoglycoside) and nalidixic acid (quinolone). All of the strains were sensitive to netillin (aminoglycoside) and norfloxacin (fluoroquinolone).

Antibiotic sensitivity of shrimp farm isolates

All the isolates from the shrimp farm were also multiple drug resistant. The MAR index of the isolates ranged from 0.1875 to 0.5. The MAR index and antibiotic resistance patterns are

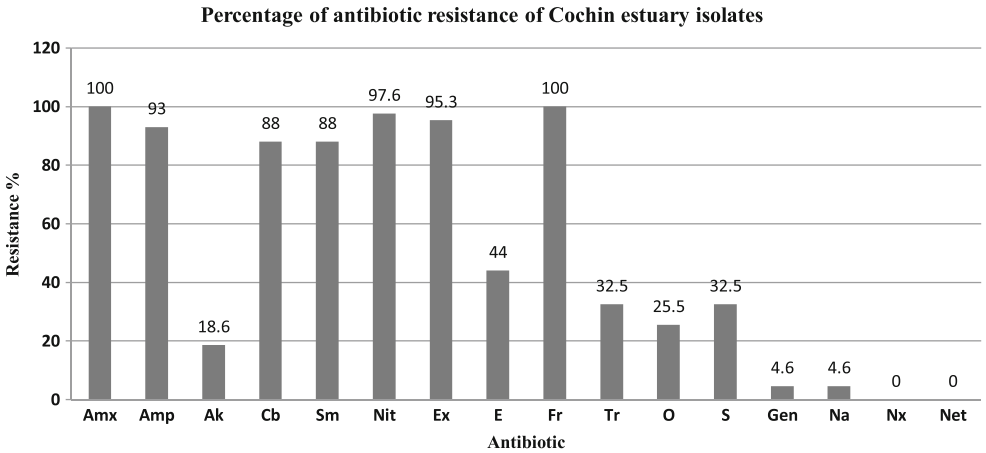

Fig. 2 Antibiotic resistance of *V. parahaemolyticus* strains from Cochin estuary. Antibiotics tested are as follows: *Amx*—amoxycillin (10 μg), *Amp*—ampicillin (10 μg), *Ak*—amikacin (10 μg), *Cb*—carbenicillin (100 μg), *Sm*—sulphamethoxazole (100 μg), *Nit*—nitrofurantoin (100 μg), *Ex*—enrofloxacin (5 μg), *E*—erythromycin (15 μg), *Fr*—furazolidone (50 μg), *Tr*—trimethoprim (5 μg), *O*—oxytetracycline (30 μg), *S*—streptomycin (10 μg), *Gen*—gentamicin (10 μg), *Na*—nalidixic acid (30 μg), *Nx*—norfloxacin (10 μg), *Net*—netillin (10 μg)

shown in Table 2. The most repeated resistance pattern was Amp, Amx, Cb. The percentage resistance of the isolates is shown in Fig. 3. All ofthe isolates (100 %) exhibited resistance towards amoxycillin (beta lactam). A high percentage of resistance was also observed towards ampicillin (beta lactam) and sulphamethoxazole (sulphonamide) and erythromycin (macrolide). A low level of resistance was observed towards nitrofurantoin (nitrofuran), enrofloxacin (fluoroquinolone), furazolidone (nitrofuran), trimethoprim (folate pathway inhibitors), oxytetracycline (tetracycline), carbenicillin (beta lactam), nalidixic acid (quinolone) and norfloxacin (fluoroquinolone). All isolates from the shrimp farm demonstrated sensitivity towards streptomycin, gentamicin and netillin (aminoglycoside).

Table 2 Antibiotic resistance patterns and plasmid profiles of *V. parahaemolyticus* isolates from a traditional shrimp pond

Isolated strain	MAR index	Antibiotic resistance pattern	Plasmid profile
MWV2	0.25	Amx, Amp, Cb, Tr	P1: 33 kb
MWV11	0.5	Amx, Amp, Ak, Cb, E, Ex, Fr, Sm	ND*
V2	0.312	Amx, Amp, Cb, E, Fr	ND
ASV18	0.312	Amx, Amp, Cb, Tr, Sm	ND
AST5	0.312	Amx, Amp, Cb, E, Sm	P4: 33 kb; 8.9 kb
WV151	0.375	Amx, Amp, Na, Cb, Ex, Tr	P1: 33 kb
SV178	0.43	Amx, Amp, Cb, E, Tr, Fr, Sm	P9: 33 kb; 1 kb
DWA18	0.5	Amx, Nx, Cb, O, E, Tr, Fr, Sm	P1: 33 kb
JSA23	0.312	Amx, Amp, Ak, E, Tr	P1: 33 kb
ASA17	0.25	Amx, Amp, E, Tr	P10: 33 kb; 0.75 kb
AWT5	0.43	Amx, Amp, Nit, E, Ex, Fr, Sm	P1: 33 kb
AST23	0.5	Amx, Amp, Ak, Nit, E, Ex, Fr, Sm	ND
ASA8	0.375	Amx, Amp, Nit, E, Ex, Fr	ND
JSA18	0.312	Amx, Amp, Na, E, Sm	ND
SSV24	0.25	Amx, Amp, Na, E	ND
MAYSA4	0.1875	Amx, Amp, Cb	ND
JULYWA15	0.25	Amx, Amp, Na, Sm	ND
JULYWA12	0.25	Amp, Amx, E, Sm	ND
WA197	0.1875	Amx, Amp, Cb	P1:33 kb
ASA5	0.1875	Amx, Amp, Cb	ND
ASA1	0.1875	Amx, Amp, Cb	ND
FSA19	0.3125	Amx, Ak, Tr, Fr, Nit	ND
AUGWT23	0.4375	Amx, Amp, O, E, Na, Sm, Tr	ND
FWA16	0.25	Amx, Amp, E, Sm	P1:33 kb
MAYWA10	0.375	Amx, Amp, E, Sm, Nit, Fr	P1:33 kb
MAYSV14	0.1875	Amx, Amp, E	ND
MAYSV7	0.375	Amx, Amp, Ak, Ex, Sm, Fr	P1: 33 kb
MSV8	0.1875	Amx, Amp, Sm	P1: 33 kb
MSV11	0.1875	Amx, Amp, Cb	ND
JSA1	0.1875	Amx, Amp, Sm	ND
AUGST33	0.1875	Amx, Amp, Sm	P1:33 kb

ND none detected

Plasmid profiling of the isolates

Plasmid profiling of the 75 multiple drug resistant isolates was performed. Plasmids were found in 50.6 % (38/75) of the isolates and ten different plasmid profiles (P1–P10) were observed (Tables 1 and 2). Comparing between the two environments, 55.8 % of the isolates from the Cochin estuary revealed the presence of one to four plasmids of a size ranging from 0.5 to 33 kb (Table 1), and 43.75 % of the isolates from the shrimp farm revealed the presence of one to two plasmids of a size ranging from 0.75 to 33 kb (Table 2). Eleven isolates from the Cochin estuary harboured one 33-kb plasmid each, seven isolates had two plasmids, three isolates harboured three plasmids, and three isolates revealed the presence of four plasmids. Eleven isolates from the shrimp farm also harboured one plasmid each, and three isolates had two plasmids. The plasmid profile 1(P1: 33 kb) was the most common profile among the isolates from both of the environments. We had strains with the same resistance patterns, but some presented different plasmid profiles and some were devoid of plasmid. Hence, no correlation was observed between the presence of plasmids and antibiotic resistance among the isolates. Plasmid curing experiments have to be done in the future to confirm the correlation.

Discussion

Vibrio parahaemolyticus has been recognized as one of the most important foodborne pathogens and the leading causal agent of human acute gastroenteritis, following the consumption of raw, undercooked or mishandled seafood and marine products (Su and Liu 2007; Pal and Das 2010; Velazquez-Roman et al. 2012). The present study investigated the presence of *V. parahaemolyticus* in sediment and water samples from various stations of the Cochin estuary and the adjoining traditional shrimp farm. A high incidence of the species was found in both the environments. The Cochin estuary (71.6 %) showed a much higher incidence of *V. parahaemolyticus* when compared to the shrimp farm (53.3 %). Incidence of *V. parahaemolyticus* in India has almost doubled in the last 5 years (Chowdhury et al. 2000). By 16S r RNA sequencing, we observed that our isolate had 100 % similarity to the pandemic clone *V. parahaemolyticus* O3:K6. Strains belonging to pandemic O3:K6 have been previously isolated from environmental samples in several countries, including Bangladesh (Islam et al. 2004), Japan (Hara-Kudo et al. 2003), India (Deepanjali et al. 2005), and Italy (Caburlotto et al. 2010). They may prove endemic if favorable conditions occur in

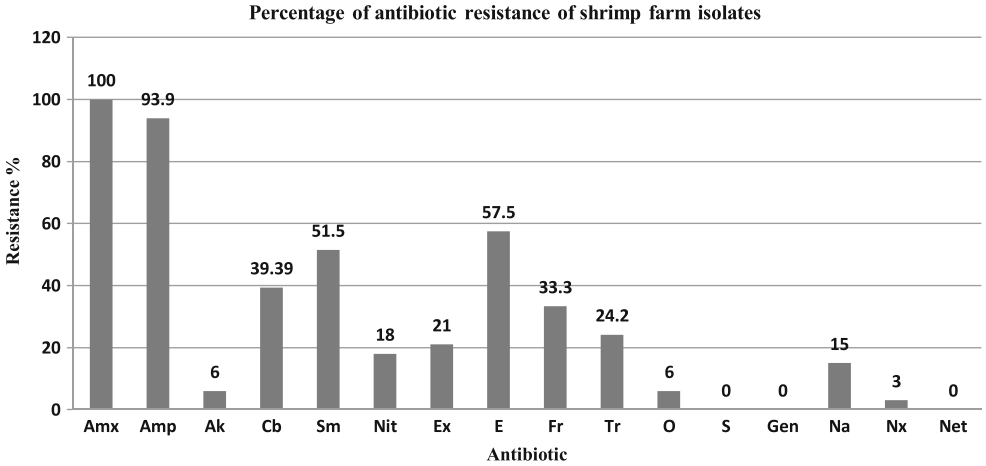

Fig 3 Antibiotic resistance of *V. parahaemolyticus* strains from traditional shrimp farm. Antibiotics tested are as follows: *Amx*—amoxycillin (10 μg), *Amp*—ampicillin (10 μg), *Ak*—amikacin (10 μg), *Cb*—carbenicillin (100 μg), *Sm*—sulphamethoxazole (100 μg), *Nit*—nitrofurantoin (100 μg), *Ex*—enrofloxacin (5 μg), *E*—erythromycin (15 μg), *Fr*—furazolidone (50 μg), *Tr*—trimethoprim (5 μg), *O*—oxytetracycline (30 μg), *S*—streptomycin (10 μg), *Gen*—gentamicin (10 μg), *Na*—nalidixic acid (30 μg), *Nx*—norfloxacin (10 μg), *Net*—netillin (10 μg)

the environment. In our study, the virulence genes *tdh* and *trh* could be detected in two strains (2.8 %) that were isolated from the Cochin estuary. This is in agreement with previous reports stating that most of the environmental strains are not pathogenic to humans, and only 1–2 % of the environmental strains have been shown to be positive for these genes (Hervio-Heath et al. 2002; Robert-Pillot et al. 2004). On the contrary, a previous study revealed a much higher presence of *tdh* (8.4 %) and *trh* (25.3 %) genes in *V. parahaemolyticus* isolated from seafoods along the southwest coast of India (Devi et al. 2009).

Vibrio is generally considered to be highly susceptible to most clinically used antimicrobials (Oliver 2006). However, in the past few decades, antimicrobial resistance has emerged and evolved in many bacterial genera due to the excessive use of antimicrobials in human, agriculture, and aquaculture systems (Mazel and Davies 1999; Cabello 2006). This emerging issue has gained great concern due to an increased resistance of pathogenic *V. parahaemolyticus* towards clinically used antibiotics. Recently, a higher frequency of drug-resistant *Vibrio* has been reported (Okoh and Igbinosa 2010; Hua and Apun 2013). Our findings reveal that all the *V. parahaemolyticus* isolates from the Cochin estuary and shrimp farm have acquired multiple drug resistance. However, there was a difference in the pattern of antibiotic resistance from the two environments. This could be due the difference in the types and amounts of antibiotic residues the isolates were exposed to. As pointed out by Hsu et al. (1992), differences in the percentage of bacterial resistance to various antibiotics may reflect the history of antibiotic application, and hence there is a possibility of using bacterial drug resistance as an indicator of antibiotic application. A high percentage of β lactam resistance was exhibited by our isolates. Resistance towards β-lactam antibiotics has been previously reported in *V. parahaemolyticus* and other vibrios from different sources (Molina-Aja et al. 2002; Manjusha et al. 2005; Devi et al. 2009). A previous study reported sensitivity of *V. parahaemolyticus* from the southwest coast towards nitrofurantoin and trimethoprim (Devi et al. 2009). Similarly, in another study from Tunisia, more than 70 % of isolates of Vibrio showed susceptibility to trimethoprim-sulfamethoxazole (Lajnef et al. 2012). In contrast in the present study, Cochin estuary isolates demonstrated very high resistance towards the nitrofuran drugs (Nit—97.6 % and Fr—100 %) and sulphamethoxazole (88 %), and low level resistance towards trimethoprim (32.5 %). The shrimp farm isolates exhibited resistance towards the nitrofuran drugs (18 % and 33.3 %), trimethoprim (24.2 %), and sulphamethoxazole (51.5 %). Resistance towards enrofloxacin was shown by 95.3 % isolates from the Cochin estuary, while only 21 % of shrimp farm isolates had acquired resistance towards it. All isolates from the shrimp farm were sensitive towards gentamicin and streptomycin, whereas Cochin estuary isolates exhibited low level resistance towards both the drugs. Treatment recommendations for *Vibrio* infections include tetracyclines, flouroquinolones, aminoglycosides and folate pathway inhibitors (Daniels and Shafaie 2000; CDC 2013). In the present study, most of the *V. parahaemolyticus* isolates from the Cochin estuary have attained resistance towards life-saving drugs like enrofloxacin, nitrofurantoin, trimethoprim, sulphamethoxazole, streptomycin, amikacin and nalidixic acid. Shrimp farm isolates also exhibited resistance towards enrofloxacin, nitrofurantoin, trimethoprim, sulphamethoxazole, nalidixic acid. These strains act as potential reservoirs of drug resistant genes in the environment. Resistant genes are further transferred from non-pathogens to pathogens through horizontal gene transfer via conjugation, transduction

and transformation. Based on our data, treatment of Vibrio infections may benefit from the use of aminoglycoside, netillin, which was the only aminoglycoside that was 100 % effective against vibrios recovered in this study.

All the isolates from the Cochin estuary showed a MAR index higher than 0.2, and it ranged up to 0.75. About 72 % of isolates from the shrimp farm also had a MAR index that was greater than 0.2. MAR indices higher than 0.2 are often considered to have originated from higher-risk sources (Krumperman 1983) of contamination, such as those from hospital sewage, commercial poultry farm waste, etc., that somehow find their way to the open sea via illegal dumping of waste or transferral by infected humans. The study area is one of the most famous tourist hot spots, and shrimps grown in this area are exported to various countries, making the findings of our study all the more important. Therefore, continued monitoring of both the prevalence of and the antimicrobial susceptibility profile of *V. parahaemolyticus* is important to better ensure seafood and public health safety from our study area.

The plasmid is known to be one of the most important mediators facilitating the fast spread of antibiotic resistance among bacteria (Dale and Park 2004). In the present study, we could detect the presence of plasmids among 50.6 % of our isolates. Ten different plasmid profiles were obtained. The plasmid of size 33 kb (profile1) was the common profile observed among the isolates from both environments; this is similar to the finding by Zhang et al. (2006), stating the presence of > 30 kb plasmids in environmental *Vibrio* isolates. Bacterial antibiotic resistance patterns are known to sometimes be associated with the presence of large plasmids, as well as the abilities of plasmids in conjugation. Transferable R plasmids are usually as big as 30 kb, and the indispensable components of a conjugative plasmid make it big in size compared to other plasmids (Guiney and Landa 1989). In our study, when we compared the antibiotic resistance patterns and the plasmid profiles, we could not find any correlation. Even among the strains with same resistance pattern, the plasmid profiles were different and some strains even lacked plasmids, which was similar to findings by Lajnef et al. 2012. So, in some strains resistance may be plasmid coded, and in some it may be chromosomally borne. Further experiments have to be done to confirm the origin of antibiotic resistance among the isolates. Plasmid profiles have been previously studied in *Vibrio* species such as *V. parahaemolyticus* (Devi et al. 2009), *V. ordalii* (Tiainen et al. 1995), *V. vulnificus* (Radu et al. 1998) and *V. salmonicida* (Sorum et al. 1990), and most extensively in *V. anguillarum* (Pedersen et al. 1996; Pedersen 1999), where a high diversity of profiles have been observed. A high incidence of plasmids in *Vibrio* sp. of both polluted and pristine environments may be ecologically important to the survival of these bacteria in the environment (Zhang et al. 2006). In the present study, we were not able to find any correlation between antibiotic resistance and presence of plasmids in the isolates.

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Conflict of interest The authors declare they have no conflict of interest.

References

Bauer AW, Kirby WMM, Sheris JC, Turck M (1966) Antibiotics susceptibility testing by standardized single disk method. Am J Clin Pathol 45:493–496

BirnBoim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7(6): 1513–1523

Cabello FC (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ Microbiol 8:1137–1144

Caburlotto G, Gennari M, Ghidini V, Tafi M, Lleo MM (2010) Serological and molecular characterization of *Vibrio parahaemolyticus* marine strains carrying pandemic genetic markers. ISME J 4:1071–1074

Centers for Disease Control and Prevention (2013) *Vibrio vulnificus*: General information. In: National Center for Emerging and Zoonotic Infectious Diseases DoF, Waterborne, and Environmental Disease (ed)

Chao G, Jiao X, Zhou X, Yang Z, Huang J, Zhou L, Qian X (2009) Distribution, prevalence, molecular typing, and virulence of *Vibrio parahaemolyticus* isolated from different sources in coastal province Jiangsu, China. Food Control 20:907–912

Chiou CS, Hsu SY, Chiu SI, Wang TK, Chao CS (2000) *Vibrio parahaemolyticus* serovar 03:K6 as a cause of unusually high incidence of food-borne disease outbreaks in Taiwan from 1996 to 1999. J Clin Microbiol 38:4621–4625

Chowdhury NR, Chakraborty S, Ramamurthy T, Nishibuchi M, Yamasaki S, Takeda Y, Nair GB (2000) Molecular evidence of clonal *Vibrio parahaemolyticus* pandemic strains. Emerg Infect Dis 6: 631–636

CLSI-Clinical Laboratory Standards Institute (2011) Performance Standards for Antimicrobial Disc Susceptibility Tests, Vol: 3(1)

Dale JW, Park S (2004) Molecular Genetics of Bacteria, 4th edn. Wiley, Chichester

Daniels NA, Shafaie A (2000) A review of pathogenic *Vibrio* infections for clinicians. Infect Med 17:665–685

Daniels NA, Mackinnon L, Bishop R, Altekruse S, Ray B, Hammond RM, Thompson S, Wilson S, Bean NH, Griffin PM, Slutsker L (2000) *Vibrio parahaemolyticus* infections in the United States, 1973–1998. J Infect Dis 181(5):1661–1666

Deepanjali A, Kumar HS, Karunasagar I, Karunasagar I (2005) Seasonal variation in abundance of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters along the south west coast of India. Appl Environ Microbiol 71:3575–3580

Devi R, Surendran P, Chakraborty K (2009) Antibiotic resistance and plasmid profiling of *Vibrio parahaemolyticus* isolated from shrimp farms along the southwest coast of India. World J Microbiol Biotechnol 25:2005–2012

Guiney DG, Landa E (1989) Conjugative transfer of Inc plasmids. In: Thomas CM (ed) Promiscuous Plasmids of Gram-Negative Bacteria. Academic, London, pp 27–56

Hara-Kudo Y, Sugiyama K, Nishibuchi M, Chowdhury A, Yatsuyanagi J, Ohtomo Y, Saito A, Nagano H, Nishina T, Nakagawa H, Konuma H, Miyahara M, Kumagai S (2003) Prevalence of pandemic thermostable direct hemolysin-producing *Vibrio parahaemolyticus* O3:K6 in seafood and the coastal environment in Japan. Appl Environ Microbiol 69:3883–3891

Hervio-Heath D, Colwell RR, Derrien A, Robert-Pillot A, Fournier JM, Pommepuy M (2002) Occurrence of pathogenic vibrios in coastal areas of France. J Appl Microbiol 92:1123–1135

Hsu CH, Hwang SC, Liu JK (1992) Succession of bacterial drug resistance as an indicator of antibiotic application in aquaculture. J Fish Soc Taiwan 19:55–64

Hua LM, Apun K (2013) Antimicrobial susceptibilities of *Vibrio parahaemolyticus* isolates from tiger shrimps (*Penaeus monodon*) aquaculture in Kuching, Sarawak. Res J Microbiol 8:55–62

Islam MS, Tasmin R, Khan SI, Bakht HBM, Mahmood ZH, Rahman MZ, Bhuiyan NA, Nishibuchi M, Nair GB, Sack RB, Huq A, Colwell RR, Sack DA (2004) Pandemic strains of O3:K6 *Vibrio parahaemolyticus* in the aquatic environment of Bangladesh. Can J Microbiol 50:827–834. doi:10.1139/w04-072

Krumperman PH (1983) Multiple antibiotic resistance indexing of *Escherischia coli* to identify high-risk sources of fecal contamination of food. Appl Environ Microbiol 46:165–170

Lajnef R, Snoussi M, Jesus Lopez Romalde JL, Nozha C, Hassen A (2012) Comparative study on the antibiotic susceptibility and plasmid profiles of *Vibrio alginolyticus* strains isolated from four Tunisian marine biotopes. World J Microbiol Biotechnol 28: 3345–3363

Manjusha S, Sarita GB, Elyas KK, Chandrasekaran M (2005) Multiple antibiotic resistances of *Vibrio* isolates from coastal and brackish water areas. Am J Biochem Biotechnol 1:201–206

Mazel D, Davies J (1999) Antibiotic resistance in microbes. Cell Mol Life Sci 56:742–754

McLaughlin JB, DePaola A, Bopp CA, Martinek KA, Napolilli NP, Allison CG, Murray SL, Thompson EC, Bird MM, Middaugh JP (2005) Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. N Engl J Med 353:1463–1470

Miyoshi S, Nitanda Y, Fujii K, Kawahara K, Li T, Maehara Y, Takeda Y, Shinoda S (2008) Differential gene expression and extracellular secretion of the collagenolytic enzymes by the pathogen *Vibrio parahaemolyticus*. FEMS Microbiol Lett 283:176–181

Molina-Aja A, Garcia-Gasca A, Abreu-Grobois A, Bolan-Mejia C, Roque A, Gomez-Gill B (2002) Plasmid profiling and antibiotic resistance of *Vibrio* strains isolated from cultured penaeid shrimp. FEMS Microbiol Lett 213:7–12

Newton A, Kendall M, Vugia DJ, Henao OL, Mahon BE (2012) Increasing rates of vibriosis in the United States, 1996–2010: review of surveillance data from 2 systems. Clin Infect Dis Off Publ Infect Dis Soc Am 54(Suppl 5):391–395

Okoh AI, Igbinosa EO (2010) Antibiotic susceptibility profiles of some *Vibrio* strains isolated from wastewater final effluents in a rural community of the Eastern Cape Province of South Africa. BMC Microbiol 10:143

Oliver JD (2006) *Vibrio vulnificus.* The biology of vibrios. ASM Press, Washington, DC

Pal D, Das N (2010) Isolation, identification and molecular characterization of *Vibrio parahaemolyticus* from fish samples in Kolkata. Eur Rev Med Pharmacol Sci 14:545–549

Pedersen K (1999) The fish pathogen *Vibrio anguillarum*. Doctoral Thesis. The Royal Veterinary and Agricultural University, Denmark

Pedersen K, Tiainen T, Larsen JL (1996) Plasmid profiles, restriction fragment length polymorphisms and O-serotypes among *Vibrio anguillarum* isolates. Epidemiol Infect 117:471–478

Radu S, Elhadi N, Hassan Z, Rusul G, Lihan S, Fifadara N, Yuherman R, Purwati E (1998) Characterization of *Vibrio vulnificus* isolated from cockles (Anadaragranosa): antimicrobial resistance, plasmid profiles and random amplification of polymorphic DNA analysis. FEMS Microbiol Lett 165:139–143

Robert-Pillot A, Guenole A, Lesne J, Delesmont R, Fournier JM, Quilici ML (2004) Occurrence of the *tdh* and *trh* genes in *Vibrio parahaemolyticus* isolates from waters and raw shellfish collected in two French coastal areas and from seafood imported into France. Int J Food Microbiol 91:319–325

Shaw KS, Rosenberg Goldstein RE, He X, Jacobs JM, Crump BC, Sapkota AR (2014) Antimicrobial susceptibility of *Vibrio vulnificus* and *Vibrio parahaemolyticus* recovered from recreational and commercial areas of Chesapeake Bay and Maryland Coastal Bays. PLoS ONE 9(2):e89616. doi:10.1371/journal.pone.0089616

Shigeaki M, Natsumi O, Toshio K, Takeshi H, Tetsuya I (2012) A cytotoxic type III secretion effector of *Vibrio parahaemolyticus* targets vacuolar H+ -ATPase subunit c and ruptures host cell lysosomes. PLoS Pathogens

Sorum H, Hvaal AB, Heum M, Daae FL, Wiik R (1990) Plasmid profiling of *Vibrio salmonicida* for epidemiological studies of cold-water vibriosis in Atlantic salmon (*Salmosalar*) and cod (*Gadusmorhua*). Appl Environ Microbiol 56:1033–1037

Su Y-C, Liu C (2007) *Vibrio parahaemolyticus*: a concern of seafood safety. Food Microbiol 24:549–558

Thompson F, Lida T, Swings J (2004) Biodiversity of Vibrios. Microbiol Mol Biol Rev 68:403–422

Tiainen T, Pedersen K, Larsen JL (1995) Ribotyping and plasmid profiling of *Vibrio anguillarum* serovar O2 and *Vibrio ordalii*. J Appl Bacteriol 79:384–392

Velazquez-Roman J, Leon-Sicairos N, Flores-Villasenor H, Villafana-Rauda S, Canizalez-Roman A (2012) Association of pandemic *Vibrio parahaemolyticus* O3:K6 present in the coastal environment of Northwest Mexico with cases of recurrent diarrhea between 2004 and 2010. Appl Environ Microbiol 78:1794–1803. doi:10.1128/AEM.06953-11

Wong HC, Liu SH, Wang TK, Lee CL, Chiou CS, Liu DP, Nishibuchi M, Lee BK (2000) Characteristics of *Vibrio parahaemolyticus* O3:K6 from Asia. Appl Environ Microbiol 66:3981–3986

Zhang R, Wang Y, Gu JD (2006) Identification of environmental plasmid-bearing *Vibrio* species isolated from polluted and pristine marine reserves of Hong Kong, and resistance to antibiotics and mercury. Antonie Van Leeuwenhoek 89:307–315