What drives the virulence and antibiotic resistance changes of *Vibrio harveyi* in South China Sea?

Yiqin Deng (yiqindd@126.com)
South China Sea Fisheries Research Institute https://orcid.org/0000-0001-8933-5365
Liwen Xu
South China Sea Fisheries Research Institute
Haoxiang Chen
South China Sea Fisheries Research Institute
Songlin Liu
South China Sea Institute of Oceanology Chinese Academy of Sciences
Qian Wang
South China Sea Fisheries Research Institute
Zhixun Guo
South China Sea Fisheries Research Institute
Juan Feng
South China Sea Fisheries Research Institute

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Abstract

Background

Vibrio harveyi is an important pathogen responsible for severe economic losses in the aquaculture industry worldwide. Bacterial pathogenicity and antibiotic resistance which are of concern for environmental safety and public health exhibit significant spatial distribution patterns. In this study, we determined the prevalence, antibiotic resistance profile, and antibiotic resistance and virulence factor genes of *V. harveyi* isolated from diseased marine fishes in south coastal China (incl. Hainan, Guangdong and Fujian Province), which can help understand the driving environmental factors of bacterial virulence and antibiotic resistance changes.

Results

A total of 107 *V. harveyi* strains were identified by multilocus sequence analysis of 16S rRNA- toxR Vh - rctB (incl. 2 from Fujian, 52 from Hainan, and 53 from Guangdong). Nine typical virulence genes highly presented among isolates with higher average number in Hainan (7.39±0.24) than in Guangdong (6.91±0.28). Five atypical virulence genes were detected in some isolates. Specially, flaC and vvh were detected in more than 60% of the isolates. Its average number was significantly higher in Hainan (2.30±0.20) than in Guangdong (1.70±0.10). *V. harveyi* isolates were generally resistant (>50%) to vancomycin, amoxicillin, and midecamycin, showed moderate resistant (10%~50%) to tobramycin, gentamicin, furazolidone, rifampicin, tetracycline, and trimethoprim-sulfamethoxazole, but were nearly susceptible (<10%) to other antibiotics tested. Aac(6')-Ib , tetW , and sul2 were detected in more than 10% isolates. The mobile genetic elements (MGEs) of int and intI1 were presented in 14.95% and 24.30% isolates, respectively. Both the average number of antibiotic resistance and antibiotic resistance genes were higher in Hainan (5.25±0.27 and 1.11±0.15) than in Guangdong (3.87±0.21 and 0.75±0.10).

Conclusion

This study demonstrated that multiple antibiotic resistances *V. harveyi* was highly prevalent with being the potential pathogen of marine fishes in South China Sea which requires particular attention. Moreover, both virulence genes and drug resistance were presence more / stronger in Hainan than in Guangdong which suggests that warming temperature and antibiotics pollutants probably enhance antibiotic resistance and bacterial infection. In the future, laboratory chamber experiment are recommend to study the response mechanisms of bacterial virulence and drug resistance to environmental factors, especially temperature and drug pollutants.

Background

Vibriosis has become one of the most serious bacterial diseases endangering sustainable aquaculture development [1, 2]. In recent years, losses due to vibriosis among various species of marine fishes have been reported worldwide [3–5]. With the rapid developments in aquaculture, it was found that Vibrio

harveyi is the dominant species that causes serious infection and mortality of marine fishes in southern China, being recognized as a major constraint on production [6, 7]. Despite its role as a serious pathogen of marine animals, the pathogenicity mechanisms are imprecisely understood. Different strains vary in their ability to cause diseases. They infect the hosts by likely mechanisms involving the ability to attach and form biofilms [8], capacity to bind iron [9], quorum sensing [10], various extracellular products [11] including haemolysin, protease, and phospholipase, lipopolysaccharide [12], and interaction with bacteriophage [13] and bacteriocin-like substances [14].

Antibiotics are widly used to prevent or treat bacterial diseases in aquaculture [15]. The regulatory framework of the use of antibiotics in aquaculture is limited, differs greatly among countries, and little or no enforcement is present in many of the major producers of aquacultural products [16]. The intensing and incrasing use of antibiotics caused a large number of antibiotic residues in the environment [17]. This will lead to the selection of antimicrobial-resistant bacteria (AMRB) and promote the evolution of antibiotic resistance mechanisms [16], such as reduce intracellular drug concentration, alterate or protect target site and direct inactivate antibiotics, which will result in multidrug resistance (MDR) [18–20]. For example, Nakayama et al. [21] found that a gradual increase in oxytetracycline concentration and frequent subculture could develop the V. harveyi drug resistance to oxytetracycline.

The virulence and antibiotic resistant genes are the key to the fundamental cause of pathogenicity and drug resistance of V. harveyi. Thus, it would be important to determine the occurrence of the putative virulence genes and antibiotic resistance genes among the isolates of V. harveyi, which would facilitate better association of these with their pathogenic and drug-resistance mechanisms. For example, the presence of vhh has been shown to correlate with pathogenicity of V. harveyi [22]. StrB, tetS and ermB genes were found in V. harveyi isolates coding for streptomycin, tetracycline and erythromycin resistance by Raissy et al. [23]. Both virulence and antibiotic resistance are spatial different which probably be resulted from the different environment conditions, including temperature, nutrient loading and pollutants. Unreasonable use of antibiotics, high nutritional load by overfeeding, pollution by biocides and heavy metals, high temperature by global warming can adjust enzyme activity, affect plasmid replication, change phage activity, and damage immune systems with regulating bacterial resistance and pathogenicity [24–26]. For example, Zhang et al. [27] reported that bacterial virulence genes from wastewater treatment plants of different cities were separated based on latitude (negative correlation), pH (negative correlation) and temperature (positive correlation) which also showed a strong and positive correlation with antibiotic resistance genes [27]. Tendencia and de la Peña [28] identified that MDR of V. harveyi was highest in ponds currently using oxolinic acid, followed by those that have previously used antimicrobials and the least was those that have not used any antimicrobials. Specially, those factors enhance horizontal gene transfer (HGT), an efficient mechanism for introducing new virulence and antibiotic resistance into the genome of bacteria [29].

The marine fish farming has been developed over a period of more than 50 years in China. Specially, it is rapid developed in the South China [30]. Diseases control and strict use of antibiotics are essential for healthy and sustainable aquaculture development. At present, the systematic investigation of

pathogenicity and drug resistance patterns of V. harveyi strains isolated from marine fish farming is otherwise rare. In this study, we isolated and identified potential pathogens of V. harveyi from diseased marine fishes in the breeding area of South China, and analyzed their virulence and drug resistance pattern with the following three objectives: (1) to obtain the frequency of resistance to common antimicrobial agents and to assess the distribution of corresponding resistance genes in V. harveyi isolates; (2) to assess the distribution of the major typical and atypical virulence genes in these isolates; (3) to compare the virulence and antibiotic resistance among different cities and estimate the potential factors that drive the virulence and antibiotic resistance changes of V. harveyi in South China Sea. These are helpful for evaluating its pathogenic potential and guiding effective antibiotic treatment in marine fish culture.

Results

Prevalence and phenotypic characteristics of V. harveyi

A total of 107 V. harveyi strains were identified as phylogenetic tree showed that they clustered together with V. harveyi reference strains while separated from other reference strains (Fig. 1). Among them, 2, 9, 39, 2, 2, 17, 15, and 21 strains were isolated from DongShan (Fujian province), YanDui (Hainan province), XinCun (Hainan province), WanNing (Hainan province), ShanYa (Hainan province), Zhusanjiao (Guangdong Province), Yuexi (Guangdong Province), and Yuedong (Guangdong Province), respectively.

Distribution of virulence factor genes among V. harveyi isolates

The frequencies of the virulence factor genes were reported in Table 1 and Additional file 1: Table S1. Each strain contained 7.15 ± 0.19 typical virulence genes. $ToxR_{vh}$ was presented in all of the tested isolates. The positive rates of other eight typical V. harveyi virulence genes (CqsS, luxN, luxP, aphA, vhh, hflK, luxR, and chiA) were 38.32%, 54.21%, 71.96%, 85.98%, 90.65%, 84.11%, 94.39%, and 95.33%, respectively. Notably, 90.65% isolates possessed at least one atypical virulence gene and each strain contained 1.99 ± 0.10 atypical virulence genes. The atypical virulence genes of flaC and vvh were detected with high frequencies of 83.18% and 63.55%, respectively. 30 (28.04%), 17 (15.89%), and 9 (8.41%) isolates contained the atypical virulence genes of $toxR_{vc}$, hlyA, and tdh, respectively, while no isolate contained the atypical virulence gene trh. One-way ANOVA analysis showed that the average number of typical virulence genes and atypical virulence genes were marginal significantly ($p = 0.111$) higher and significantly higher ($p = 0.002$) in Hainan than in GuangDong, respectively.

Resistance of V. harveyi isolates to antibiotics

Resistance / susceptibility to different antimicrobial agents by V. harveyi was shown in Table 2 and Additional file 1: Table S1. Each strain showed resistance to 4.57 ± 0.18 antibiotics with at least one and at most ten. They were generally resistant to vancomycin (97.22%), amoxicillin (74.3%), midecamycin (79.41%). They showed moderate resistance to tobramycin (43.57%), gentamicin (19.89%), furazolidone (31.78%), rifampicin (30.08%), tetracycline (15.12%), and trimethoprim-sulfamethoxazole (16.47%). They

were however susceptible to other antimicrobials, including erythromycin (4.11%), doxycycline (7.75%), norfloxacin (2.49%), ciprofloxacin (2.49%), chloramphenicol (6.65%), and florfenicol (0.00%). One-way ANOVA analysis indicated that the antibiotic resistance significantly higher in Hainan than in GuangDong ($P = 0.0001$).

Table 1
Distribution of virulence factor genes

Typical virulence genes	**% (n)**	**Atypical virulence genes**	**% (n)**
cqsS	38.32 (41)	$toxR_{Vc}$	28.04 (30)
luxN	54.21 (58)	hlyA	15.89 (17)
luxP	71.96 (77)	flaC	83.18 (89)
ahpA	85.98 (92)	tdh	8.41 (9)
vhh	90.65 (97)	trh	0 (0)
hflk	84.11 (90)	vvh	63.55 (68)
luxR	94.39 (101)	AtyAVG (n)[b]	1.99 ± 0.10
chiA	95.33 (102)	AtyAVG (n)/HN	2.30 ± 0.20
TyAVG (n)[a]	7.15 ± 0.19	AtyAVG (n)/GD	1.70 ± 0.10
TyAVG (n)/HN	7.39 ± 0.24	P A_{tyAVG} (GD vs HN)	0.002
TyAVG (n)/GD	6.91 ± 0.28		
P $_{TyAVG}$ (GD vs HN)	0.111		
a: average number of typical virulence genes in each strain; b: average number of atypical virulence genes in each strain.			

Table 2
Resistance to antibiotics by V. harveyi isolates

Antibiotics	% (n)	Antibiotics	% (n)
Vancomycin	97.22 (104)	Trimethoprim-sulfamethoxazole	16.47 (17)
Amoxicillin	74.3 (80)	Norfloxacin	2.49 (3)
Midecamycin	79.41 (85)	Ciprofloxacin	2.49 (3)
Erythromycin	4.11 (4)	Chloramphenicol	6.65 (3)
Tobramycin	43.75 (47)	Florfenicol	0 (0)
Gentamicin	19.89 (21)	TAR (n)[a]	4.57 ± 0.18
Furazolidone	31.78(34)	TAR (n)/HN	5.25 ± 0.27
Rifampicin	30.8 (33)	TAR (n)/GD	3.87 ± 0.21
Tetracycline	15.12 (16)	P_{TAR} (GD vs HN)	0.0001
Doxycycline	7.75 (8)		
a: average number of antibiotic resistance.			

Distribution of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs), and the correlation of ARGs to MGEs and the resistant phenotype

The presence of the ARGs and MGEs were reported in Table 3 and Additional file 1: Table S1. The frequencies of aac(6')-Ib, tetW, sul2, int, and intI1 were more than 10%. No tetO, tetS, tetQ, tetX, sul 3, qnrAm, and qepA were detected. The frequencies of other genes were between 0%-10%. One-way ANOVA analysis indicated that the presence of antibiotic resistance genes was marginal higher in Hainan than in GuangDong (P = 0.075).

The variations and correlations among virulence genes, ARGs, MGEs and the resistant phenotype

The abundance of ARGs was positively correlated with the abundance of virulence genes and antibiotic resistance (pearson correlation coefficient = 0.207 and 0.195, respectively). In addition, positive correlation was detected between the gene content of macrolides (ermB and ermC) and erythromycin resistance (pearson correlation coefficient = 0.221). Db-RDA showed that the presence of virulence genes was related to the resistance to gentamicin, norfloxacin, trimethoprim-sulfamethoxazole, and vancomycin (Fig. 2, Table 4). The presence of int positively related to the presence of ermB and aac(6')-Ib (pearson correlation coefficient = 0.246 and 0.359, respectively). No positive relationship was detected between MGEs (int and intI1) and different virulence genes.

Table 3
Distribution of ARGs and MGEs in V. harveyi isolates

ARGs	% (n)	ARGs	% (n)
blaTEM	5.61 (6)	sul 3	0 (0)
blaSHV	8.41 (9)	qnrSm	4.67 (5)
ermC	0.93 (1)	qnrAm	0 (0)
ermB	2.8 (3)	qnrBm	9.35 (10)
aac(6')-Ib	14.02 (15)	qepA	0 (0)
tetO	0 (0)	oqxA	4.67 (5)
tetS	0 (0)	oqxB	2.8 (3)
tetW	20.56 (22)	ARG (n)[a]	0.93 ± 0.09
tetQ	0 (0)	ARG (n)/HN	1.11 ± 0.15
tetB	4.67 (5)	ARG (n)/GD	0.75 ± 0.10
tetX	0 (0)	P_{AVG} (GD vs HN)	0.075
sul 1	1.87 (2)	int	14.95 (16)
sul 2	12.15 (13)	intI1	24.3 (26)
a: average number of antibiotic resistance genes.			

Table 4
The relationships between dbRDA coordinate axes and variables

Variable	dbRDA1	dbRDA2
Erythromycin	-0.176	0.225
Gentamicin	0.508	0.105
Rifampicin	-0.266	-0.175
Norfloxacin	0.286	-0.463
Ciprofloxacin	-0.19	-0.028
Chloramphenicol	0.342	0.039
Florfenicol	0.241	-0.003
Tetracycline	-0.156	0.246
Trimethoprim-sulfamethoxazole	-0.132	-0.646
Amoxicillin	-0.099	-0.403
Vancomycin	-0.4	0.085
Tobramycin	0.336	0.035
Midecamycin	-0.137	0.144
Doxycycline	0.064	0.148

Discussion

V. harveyi is the main pathogen of marine fish culture in southern China, causing diseases responsible for severe economic losses in the aquaculture industry. V. harveyi-like (V. harveyi, V. campbellii, V. rotiferianus and V. owensii) isolates and even other Vibrio species are almost indistinguishable by 16S rRNA gene sequencing, frequently leading to species misidentification [31, 32]. In recent years, multilocus sequence analysis (MLSA), such as 16S rRNA-recA-pyrH-rpoD-gyrB-rctB for the identification of genus Vibrio strains [33], is emerged as a powerful tool for identification of closely related bacteria. In this study, we firstly confirmed the genus level of the isolates by 16S rRNA gene sequencing. Then 107 strains have been identified as V. harveyi strains by using two protein-coding loci ($toxR_{Vh}$ and rctB) to produce multilocus genotypes.

Here, the high presence (> 70%) of vhh, hflK, luxR, chiA, and $toxR_{Vh}$ in V. harveyi is similar to the V. harveyi strains which are isolated from Bermuda reef fish and north-east coastal China diseased hybrid grouper [5, 34]. In addition, high presence of luxP and aphA, and moderate presence (35%~70%) of cqsS and luxN

were detected. The presence rates of atypical virulence genes, including $toxR_{VC}$, hlyA, and flaC were similar to the results of Ruwandeepika et al. [35] while higher than the results of Zhu et al. [5]. Moreover, here we identified the presence of atypical virulence genes tdh and vvh, especially vhh which was detected in 63.55% of the isolates, however, have not been identified in the previously studies [5, 35]. LuxN, LuxP, and CqsS are the three receptors of V. harveyi three quorum-sensing (QS) systems, which act in parallel and feed a common signal transduction cascade with LuxR as the main regulator [36, 37]. Haemolysins are potent toxins playing a major role in the virulence of vibrios including V. parahaemolyticus (tdh, trh, and tlh), V. cholerae (hlyA), V. vulnificus (vvh) and V. harveyi (vhh), having haemolytic, cytolytic activities [38]. ToxR is a transmembrane transcription regulator and plays an important role in the regulation of virulence gene expression which has been shown to control the expression of more than 17 virulence genes in V. cholerae [39]. Flagella are responsible for the adhesion during pathogen infection [40]. Motile isolates are usually more virulent than nonmotile mutants [40]. V. anguillarum flaC gene shares 88% similarity with flaD gene that is involved in the virulence of this organism [41]. Many extracellular bacterial proteases such as acid phosphatase (aphA), seine protease (hflK) and chitinase (chiA) are suggested to play important roles in virulence [42–44]. Overall, the high presence of typical virulce genes suggests that QS systems, haemolysin and extracellular bacterial proteases are relative conserved virulence factors in V. harveyi. In addition, the presence of those atypical virulence genes within the genome of some isolates suggesting the HGT of virulence, which probably contirbutes to the isolates virulence variation and be essential for broadening their host rang. However, no positive relationship was detected between int or intI1 and any atypical virulence genes suggesting their transfer by other MGEs.

Our study revealed high degree (> 50%) of resistance to vancomycin and amoxicillin, moderate degree (10% ~ 50%) of resistance to tetracycline, and low degree (< 10%) of resistance to norfloxacin, ciprofloxacin, and chloramphenicol which are consistent with the study of V. harveyi strains from shrimp aquaculture environment in South Indian [45, 46]. However, the low degree of resistance to erythromycin, and moderate degree of resistance to tobramycin, and gentamicin are different from the study in South Indian while similar with the study in Persian Gulf present researches [23, 45, 46]. In addition, lower reiststance to ciprofloxacin, doxycycline, and norfloxacin have been found in our study than in Persian Gulf [23]. Correspondingly, we detected the presence of several groups of antibiotic resistance genes, and only the gene content of macrolides and erythromycin resistance was correlated. Bacteria become resistant to antibiotics via direct inactivation of antibiotics, by a reduction in the intracellular concentration of drug, and by target site alteration or protection [47]. Therefore, some resistance genes are probably not expressed in the isolates though they are present on the chromosomes. Some strains should have developed other resistance mechanisms, such as change and active the antibiotics efflux, and modify the antibiotic targets, but not encode inactivating enzymes by antibiotic resistance genes directly [48]. Vancomycin and amoxicillin are both reported can be intrinsicly resistant, thus exhibitting high degree resistance [49, 50]. Though high concentration of norfloxacin and ciprofloxacin has been detected in the farming area [51], low degree of resistance was shown, suggesting a probably use of (fluoro)quinolones antibiotics in marine fish framing with inhibiting bacteria diseases. Notably, integrons

were found in some isolates and were positively related to the presence of ermB and aac(6')-Ib probably suggesting the HGT of drug resistance especially the resistance to macrolides and aminoglycosides in V. harveyi which has also been reported in V. cholerae [52].

Incrasing use of antibiotics and enhanced global warming lead to serious of drug resistance and infection in aquaculture [53–55]. Continue and over use of antibiotics result into antibiotic residues, which can enhance durg reistance by promoting the enrichment of AMRB and the evolution of antibiotic resistance mechanisms, including the HGT of ARGs [16, 56]. Because of the warming temperature and the advantages of adjacent to South China Sea, Hainan has a longer annual breeding time (almost 12 months / year) than Guangdong (around 9 months / year) and it is one main marine fish hatchery. Temperature is one of main factors that affect HGT, by affecting biofilm formation, membrane permeability, plasmid replication, and the activity of immune systems, phage and HGT-related enzyme etc. For example, Ehlers et al. [57] reported that the conjugation efficiency of Pseudomonas sp. increased 10,000 times after the temperature increased form 15 ℃ to 28 ℃ by promoting the biofilm formation of Pseudomonas sp. Warming temperature also enhances bacterial drug resistance and virulence by inducing the expression of antibiotic resistance genes and virulence genes directly [58]. Therefore, we can roughly speculate that higher temperature and longer annual breeding time (means longer time usage of antibiotics) might lead to stronger drug resistance and bacterial virulence in Hainan than in Guangdong, though we do not have direct evidence. Further, we recommend that laboratory chamber experiment should be conducted to study the response mechanisms of bacterial virulence and drug resistance to environmental factors, especially temperature and drug pollutants.

In addition, it has been reported that bacterial virulence is related to drug resistance [59]. The antimicrobial resistance genes and virulence determinants can be physical linkaged with locating on the same MGE and be horizontal transferred together [59, 60]. In addition, MDR determinants, such as Mtr from Neisseria gonorrhoeae [61] and SapA [62] from Salmonella typhimurium extrude both defensins and antibiotics. Moreover, the overproduction of MexAB-OprM efflux pump lead to multidrug resistance, and affects QS with involving into bacterial virulence in Pseudomonas aeruginosa [63]. Here, the pearson correlation test showed that abundance of virulence genes was positively correlated with the abundance of ARGs which was consistent with the results of Zhang et al. [27]. Moreover, the db-RDA indicated that the virulence of V. harveyi related to the resistance to gentamicin, norfloxacin, trimethoprim-sulfamethoxazole, and vancomycin. However, the detail mechanism should be further studied, including the physical linkaged between virulence genes and antibiotice resistence genes, the regualtion of MDR determinants on bacterial virulence, and the regulation effect of efflux pump on MDR and virulence, etc.

Conclusions

This study represents the first attempt to investigate the pattern of drug resistance, and virulence genes with understanding the driving factors of virulence and antibiotic resistence changes of V. harveyi in South China Sea. Our results showed that QS system, haemolysin, and extracellular bacterial proteases were relative conserved virulence factors in V. harveyi. Abtypical virulence genes were present in V. harveyi

with promoting bacterial virulence and broadening their host rang. All the isolates were resistant to at least one antibiotic and most of them were multidrug resistance. V. harveyi develped various resistance mechnismes besides encoding inactivating enzymes as little correlation has been detected between resistance gene content and drug resistance. Higher temperature and longer time usage of antibiotics might enhance the drug resistance and bacterial virulence in Hainan than in Guangdong. These results are helpful for evaluating the isolates pathogenicity and guiding the effective use of antibiotics in marine fish farming. It also highlights the necessity for more detailed investigations for improving consumer protection and public health safety. However, further research should be focus on: (1) the influence mechanisms of warming temperature and antibiotics pollutants on virulence and antibiotic resistance; (2) the correlation mechanisms between virulence and antibiotic resistance.

Methods

Sampling, isolation and identification of V. harveyi

During the epidemiological investigation of marine fish, thousands suspected pathogenic bacteria strains have been isolated from diseased marine fishes (incl. Trachinotus ovatus, Blotchy rock cod, Sparus macrocephlus, Hypoplectrus indigo, Lates niloticus, Lutjanus erythopterus, Plectropomus leopardus, and Rachycentron canadum). For isolation, the homogenate of lesion tissues (incl. spleen, tail, eye, liver, kidney, mouth, head, body surface, and intestine) have been screened with marine agar 2216E plate. Then the dominant clones have been selected for identification with the gene of 16 s rRNA. Subsequently, Vibrio spp. strains have been chosen for V. harveyi identification by MLSA of $toxR_{Vh}$ and rctB [32, 64]. The phylogenetic tree was constructed from the concatenated sequences of $toxR_{Vh}$-rctB genes using Kimura 2-parameter model with the neighbor-joining method, bootstrapped 1,000 times via MEGA6.0 software. 29 Vibrio spp. strains including five V. harveyi strains sequences were downlated from National Coalition Building Institute (NCBI) database as the references. Sampling was done in South China (incl. Hainan or HN, Guangdong or GD, and Fujian or FJ) from 2011 and 2014. The primer pair sequences used for this study were shown in Additional file 1: Table S1.

Antibiotics Susceptibility Testing

Antibiotics susceptibility of V. harveyi isolates were test by disk diffusion method on Mueller-Hinton agar using commercial antibiotic disks (Oxoid) according to Clinical and Laboratory Standards Institute (CLSI) guidelines [65]. Escherichia coli ATCC 35218 were used as quality control strain in each run. 15 antibiotics including furazolidone 300 μg/disk, erythromycin 150 μg/disk, gentamicin 10 μg/disk, rifampicin 5 μg/disk, norfloxacin 10 μg/disk, ciprofloxacin 50 μg/disk, chloramphenicol 30 μg/disk, florfenicol 30 μg/disk, tetracycline 30 μg/disk, trimethoprim-sulfamethoxazole 23.75/1.25 μg/disk, amoxicillin 20 μg/disk, vancomycin 30 μg/disk, tobramycin 10 μg/disk, midecamycin 30 μg/disk, and doxycycline 300 μg/disk were tested. Interpretation of inhibition zones was carried out based on the manufacturers' and CLSI M45-A guidelines [66].

Detection Of Resistance And Virulence Genes

DNA template preparation

Bacteria cultures were grown by streaking on marine agar 2216E plate at 28 °C for overnight. Then single clone was cultured with marine broth 2216E medium at 28 °C, 200 rpm for overnight. Bacterial lysates used as templates for the PCRs were prepared as follows: 1 mL overnight bacteria was centrifuged and resuspended with 100 µL sterilized water. Then it was cooled at -80 °C for 20 min and heated at 100 °C for 15 min immediately. After having been cooled at ice for 5 min, the suspension was centrifuged for 5 min at 12000 rpm. The supernatant was separated and used as template DNA for PCR assay.

Detection of typical V. harveyi virulence genes

Eight typical V. harveyi virulence genes (incl. four quorum sensing genes cqsS, luxN, luxP, and aphA, haemolysin gene vhh, serine protease encoding gene hflK, transcription regulator encoding genes luxR, and chitinase encoding genes chiA) were detected with the multiplex PCR method that we have validated before [67]. Briefly, the PCR reaction mixture contained Premix Taq (TaKaRa Taq Version 2.0 plus dye) (Takara, Japan) 38.0 µL, each primer 0.1 µM, template DNA 80 ng,and add sterilized water to 50 µL. The reaction was performed in an automatic thermal cycler (Bio-Rad, USA) under the following optimized cycling program: an initial denaturation step of 5 min at 95 °C; 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 60 s; and a final extension at 72 °C for 10 min. The amplified PCR fragments were separated by 2.0% agarose gel which as electrophoresised with 0.5x TBE buffer at 120 V for 75 min. Reaction mixtures with sterilized water and X13SZ03 (V. harveyi 345 of which the completed genome was sequenced with accession numbers of CP025537.1 to CP025540.1) DNA as templates were served as a negative and positive controls, respectively.

Detection of atypical V. harveyi virulence genes

Six atypical V. harveyi virulence genes (incl. V. cholera virulence regulator gene $toxR_{VC}$, V. cholera haemolysin gene hlyA, V. anguillarum flagella C subunit gene flaC, V. parahaemolyticus haemolysin tdh and trh, and V. vulnificus haemolysin vvh (Ruwandeepika et al., 2010; Lorenz et al., 2017) have been detected with single PCR. Briefly, the PCR reaction mixture contained Premix Taq (TaKaRa Taq Version 2.0 plus dye) (Takara, Japan) 12.5 µL, each primer 0.4 µM, template DNA 20 ng,and add sterilized water to 25 µL. The reaction was performed in an automatic thermal cycler (Bio-Rad, USA) under the following optimized cycling program: an initial denaturation step of 5 min at 95 °C; 35 cycles of denaturation at 94 °C for 30 s, annealing at Tm for 30 s, extension at 72 °C for 60 s / kb; and a final extension at 72 °C for 10 min. The amplified PCR fragments were separated by 1.0% agarose gel which as electrophoresised with 0.5x TBE buffer at 150 V for 30 min. A reaction mixture with sterilized water as templates was served as the negative control. For each atypical virulence gene, at least five PCR products were selected for sequence and homolog analysis using Blast (NCBI) when the positive candidates more than five. Otherwise, all the PCR products were selected for sequence and homolog analysis.

Detection of antibiotic resistance genes

According to antibiotics susceptibility results, the major resistance genes to quinolones (qnrSm, qnrAm, qnrBm, qepA, oqxA, and oqxB), tetracyclines (tetO, tetS, tetW, tetQ, tetB, and tetX), sulfonamides (sul 1, sul 2, and sul 3), macrolide (ermB, and ermC), beta-lactams (blaTEM, and blaSHV), and aminoglycosides [aac(6')-Ib] were detected by single PCR with the same method as 2.3.3. In addition, an integrase gene (int) derived from the integrons of Vibrio species and the class 1 integrase gene intI1 were tested as well.

Statistical analysis

One-way ANOVA analysis (SNK test) was conducted to examine variations in average number of atypical virulence genes, antibiotic resistance, and antibiotic resistance genes among different locations. Pearson correlation coefficient was calculated to estimate the co-occurrence between the abundance of ARGs and the abundance of virulence genes, between the abundance of ARGs and antibiotic resistance, and between the genotype (absence of resistance gene) and phenotype (resistance to antibiotics). $P < 0.05$ was considered as significant difference. The Bray–Curtis distance matrix was calculated with the presence virulence genes for distance-based redundancy analysis (db-RDA) which was performed to determine the impact of antibiotic resistance on bacterial virulence. The above-mentioned statistical analyses were performed with PRIMER 6 & PERMANOVA+ [68] and IBM SPSS Statistics 19.0.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

YD conceived the study, analyzed the data, and wrote the manuscript; LX, HC, and QW performed the experiments; SL and ZG critically revised the manuscript; JF contributed the reagents. All authors read and approved the manuscript for publication.

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Not applicable.

Abbreviations

AMRB: antimicrobial-resistant bacteria; MDR:multidrug resistance; HGT:horizontal gene transfer; MLSA:multilocus sequence analysis; ARGs:antibiotic resistance genes; MGEs:mobile genetic elements; NCBI:National Coalition Building Institute; HN:Hainan; GD:Guangdong; FJ:Fujian; CLSI:Clinical and Laboratory Standards Institute; QS:quorum-sensing

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Additional Files

Additional file 1: Table S1 The antibiotic resistance pattern and the presence of virulence genes and antibiotic resistance genes in *Vibrio harveyi* isolates. xls file

Additional file 2: Table S2 The primer pair sequences used for this study. doc file

Figures

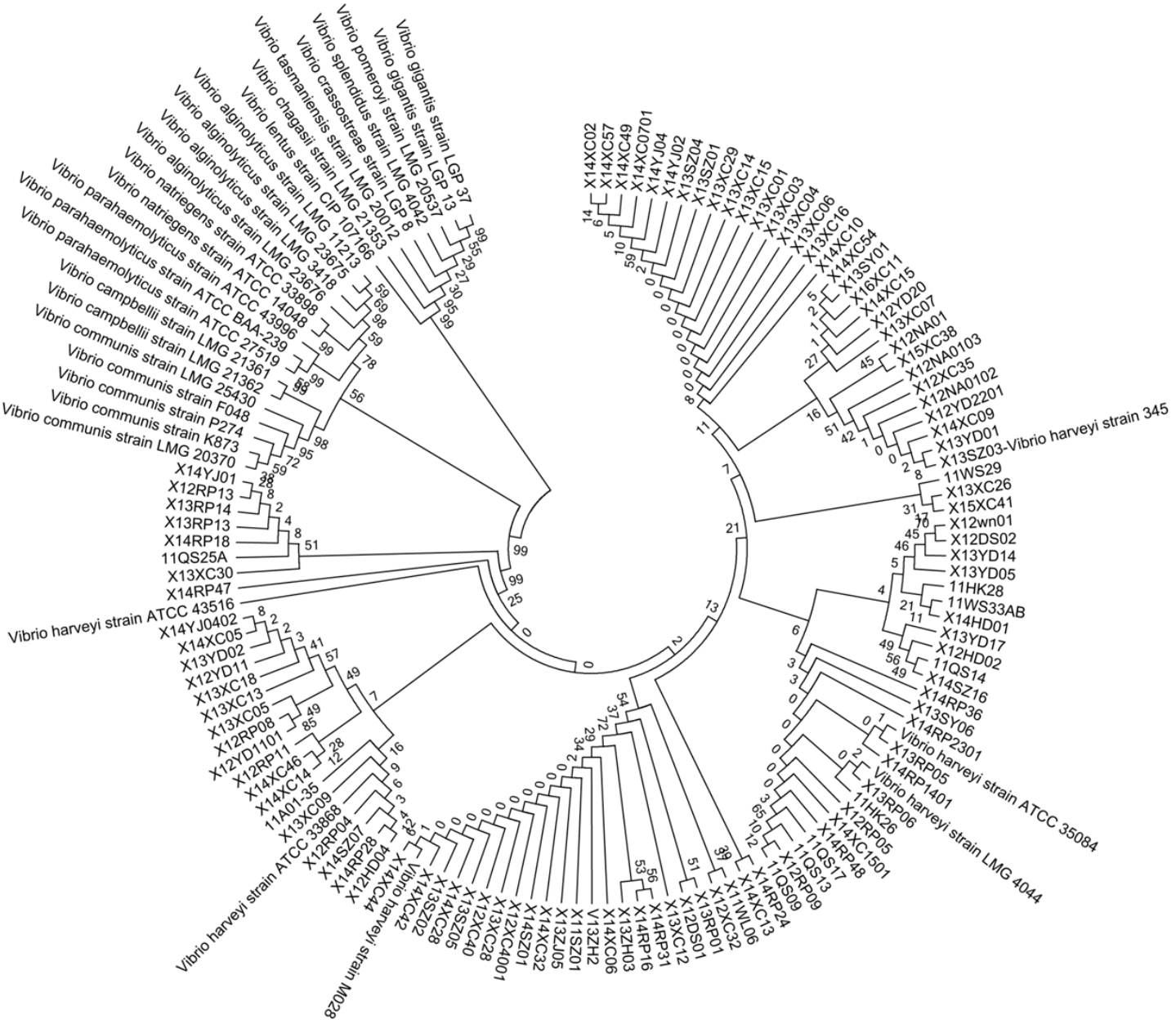

Figure 1

The phylogenetic tree of toxRVh-rctB concatenated sequences with representative microbes aligned by using ClustalW. The numbers on the branch points mean the relative bootstrap (%, Bootstrap values÷1000×100). The bar indicates 0.01 substitutions per sequence position.

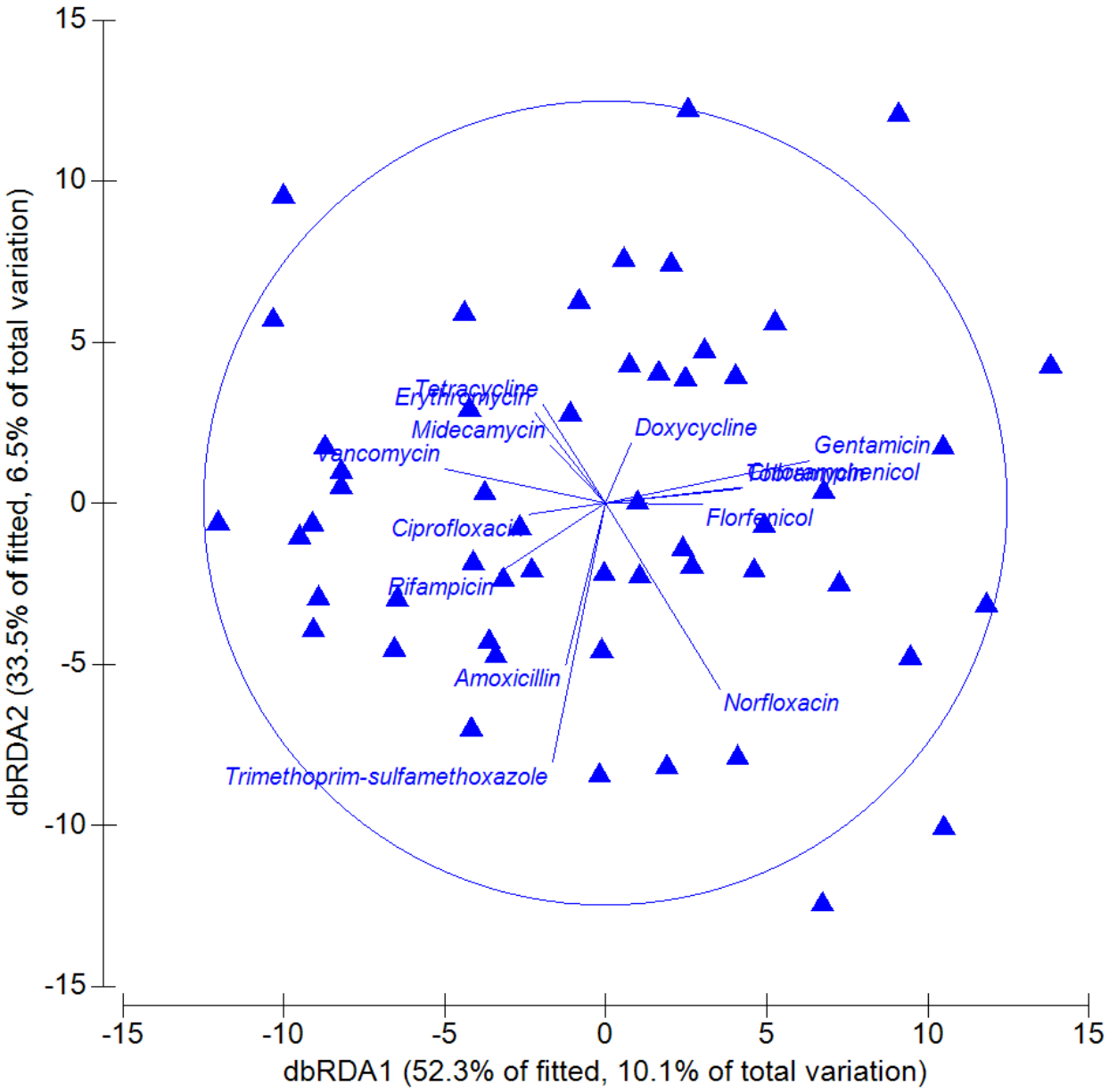

Figure 2

Distance-based redundancy analysis (db-RDA) ordination of V. harveyi isolates fitted to antibiotic resistance. The plot represents a db-RDA ordination based upon the Bray–Curtis distance of all isolations which was calculated by the presence of virulence genes.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xls
- TableS2.doc