VIBRIONACEAE DIVERSITY, MULTIDRUG RESISTANT AND MANAGEMENT

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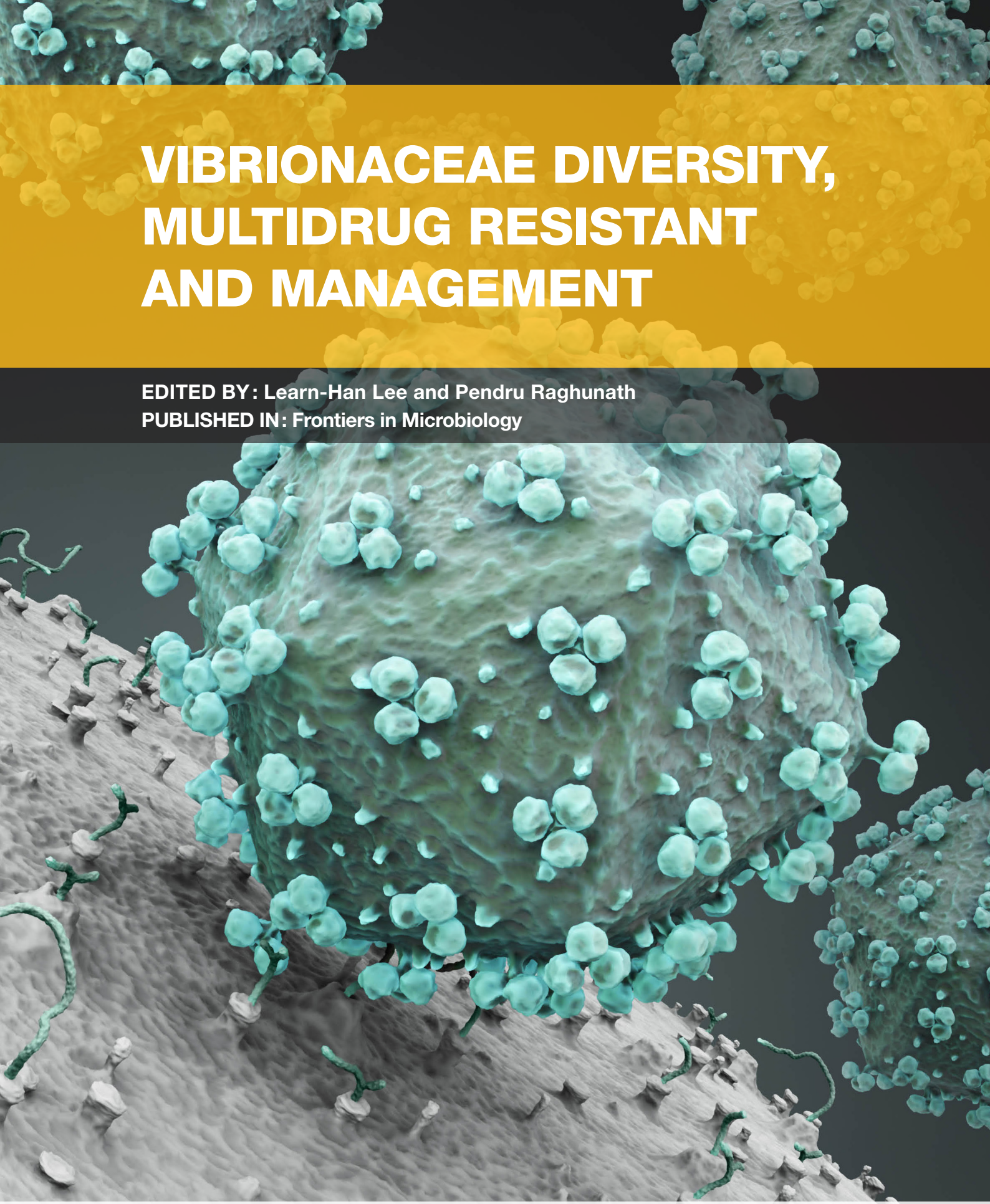

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VIBRIONACEAE DIVERSITY, MULTIDRUG RESISTANT AND MANAGEMENT

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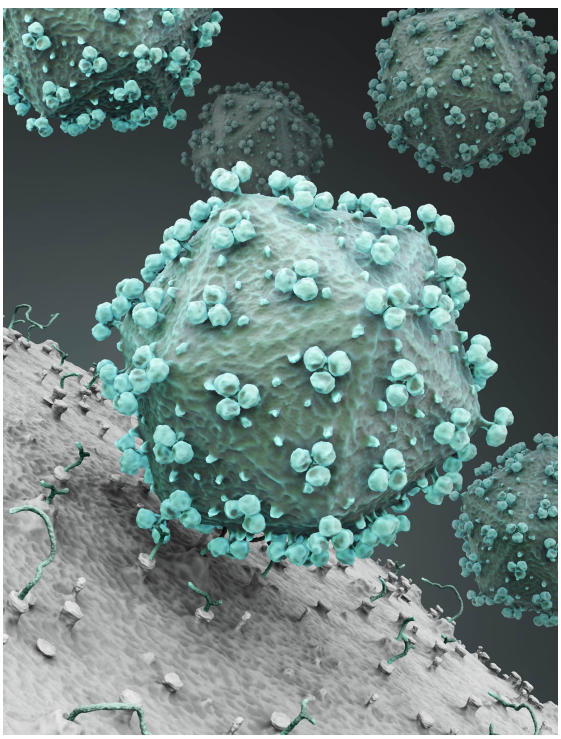

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Vibrio are Gram-negative bacteria that naturally inhabit riverine, estuarine and marine aquatic environments. Some *Vibrio* are known to be capable of causing gastroenteritis, wound infections, cholera and fatal septicemia in severe cases. Over the past decades, research on *Vibrio* has increased and has caused a great development in our knowledge of these pathogens. Focus of this research includes the discovery of emerging epidemic clones, the traits of new strains, and the occurrence of multidrug resistant strains in the ecology. Moreover, improved understandings of the prevalence, pathogenesis and evolution of *Vibrio* have revealed the significant role of these pathogens in enhancing disease transmission. The complete genomic sequences of *Vibrio* have been determined in providing a rich set of data illuminating the metabolic versatility of

the species. This book is dedicated to improving our knowledge and understanding, not solely focusing into the prevalence, detection, pathogenesis, virulence, pandemic clones and multidrug resistance, but also looking at the management of the multidrug resistance through different strategies such as non-antibiotic resistant strategies that involved the application of knowledge in bacteriophages.

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EDITORIAL
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Editorial: Vibrionaceae Diversity, Multidrug Resistance and Management

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Editorial on the Research Topic
Vibrionaceae Diversity, Multidrug Resistance and Management

The genus *Vibrio* from Vibrionaceae family were originally isolated by an Italian physician, Filippo Pacini in 1854 (Thompson et al., 2004). Presently, the genus contains a total of 142 species which are marine originated and its taxonomy is continuously being revised due to the revelation of new species (Summer et al., 2001; Igbinosa and Okoh, 2008; Sawabe et al., 2013). Some of the *Vibrio* species are identified as foodborne pathogens causing illnesses upon consumption of contaminated seafood or water (Letchumanan et al., 2014; Law et al., 2015). The three *Vibrio* species primary for foodborne diseases are *Vibrio cholera*—associated from food contamination or transmission of infection from person to person, *Vibrio parahemolyticus*—associated with food contamination, and *Vibrio vulnificus*—associated with wound infection (Letchumanan et al.). Significant efforts have been applied to analyze the genetic as well as the molecular mechanisms underlying the infection actions of *Vibrio*.

This Research Topic brings together 19 articles that features the current discoveries concerning the biology of pathogenic *Vibrio* species covering from the epidemiology studies to discovery of antibiotic resistant strains in the environment which causes immense impact on the treatment and management of illnesses. The genome and non-antibiotic management articles in this research topic provides a deeper knowledge on *Vibrio*.

As mentioned in four of the articles, *Vibrio* sp. has a well diverse epidemiology. In Japan, this ecological pathogen can be found among seawaters of coral reefs in Ishigaki. The discoveries uncovered that Ishigaki coral reefs consisted of 22 known and 12 potential novel Vibrionaceae species. The abundance of *Vibrio* sp. had a significant positive correction with the rising seawater temperature. Amin et al. presented the results that would benefit in the coral conservation and future actions. Where else, in the neighboring nation Shanghai, China, *Vibrio parahaemolyticus* has been detected as a major pathogen for foodborne gastroenteritis cases. All the *V. parahaemolyticus* isolated from patients in Shanghai hospital exhibited T3SS1 and T3SS2 genes, 7/42 positive for *trh* gene and 37/42 positive for *h* gene. Further it was revealed that these clinical isolates expressed multidrug antibiotic resistance and carried resistance genes (Li et al.). Pathogenic *Vibrios* affecting bivalves in hatcheries has been reviewed by Dubert et al. This review article provided an insight on the *Vibrio* sp. affecting the hatcheries and proposed effective measures to curb the pathogens. These *Vibrios* can be managed through proper water treatments method in hatcheries, application of antibiotics, probiosis, quorum quenching, and phage therapy (Dubert et al.). The rarely unexplored *V. vulnificus* occurrence, healthcare burden and prevention were reviewed by Heng et al. *V. vulnificus* has been reported to be infecting in many countries worldwide and caused a huge healthcare burden. Efforts to treat *V. vulnificus* infections has hampered due to the emergence of multidrug resistant strains in the environment. It was suggested that appropriate choice of

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treatment would be cefotaxime and ceftriaxone since the first line of drugs may longer be applicable to treat *V. vulnificus* infections (Heng et al.).

A remarkable feature of *V. parahaemolyticus*'s ability of sensing human bile and regulate virulence reviewed by Letchumanan et al. The role of *V. parahaemolyticus* virulence starts upon ingestion of the bacterium and exposures to bile salts. Two encoded protein, VtrA and VtrC will cooperate and form a protein complex on the surface of the membrane of the bacterial cell. This complex structure then binds to bile salts and triggers the cell to produce toxins. Upon binding of bile salts to the VtrA/VtrC complex, the cytoplasmic DNA binding domain of VtrA is activated which in turn induces VtrB to activate, resulting in the T3SS2 expression. T3SS2 virulence is secreted thus causing illness to human. The information on bile-bacteria interaction pathway is important in order to develop drugs that can suppress bacterial virulence (Letchumanan et al.).

The emergence of multidrug resistant strains has become an international health crisis as causing significant threat to human well-being (WHO, 2014; Letchumanan et al., 2015). In Bangladesh, it was reported that *V. cholerae* O1 was resistant toward ciprofloxacin and azithromycin. These two antibiotics are currently used for prophylactic treatment of *V. cholerae* infections. The reduced susceptibility to ciprofloxacin and azithromycin is alarming for cholera-endemic countries of Asia and Africa (Rashed et al.). In Malaysia, *V. parahaemolyticus* strains isolated from shellfish was reported to be resistant against second and third lines of antibiotics. Majority of the strains were resistant toward 5 antibiotics out 14 antibiotics tested. These results are a real concern and warrants a continuous surveillance on the antibiotics used in the aquaculture settings (Letchumanan et al.). Similar antibiotic resistant pattern was discovered among *V. cholerae* non-O1/non-O139 collected from Haiti waters. The highest proportion of isolates were reported to be resistant against sulfonamide, amoxicillin, and trimethoprim-sulfamethoxazole. There was isolates exhibited resistance toward erythromycin and doxycycline, the two antibiotics used to treat cholerae in Haiti. The presences of *V. cholerae* non-O1/non-O139 with resistance pattern against clinically used antibiotics may cause public health threat (Baron et al.). Pathogenic *V. cholerae* and *V. vulnificus* with antimicrobial resistance patterns has been reported in Germany. The study further revealed that resistant toward carbapenems was observed in four environmental *V. cholerae* strains studied. Carbapenems are the last line of antibiotics for the treatment of *Vibrio* infections. The occurrence of carbapenemase producing *V. cholerae* in German coastal waters is of concern and highlights the need for monitoring the use of antibiotics (Bier et al.). In Senegal, antibiotic resistant *V. cholerae* O1 was isolated during a cholera outbreak. The isolates showed resistance pattern at least four antibiotics tested including trimethoprim-sulfamethoxazole, streptomycin, trimethoprim, and sulfamethoxazole (Sambe-Ba et al.).

Likewise, the genomics of *Vibrio navarrensis* has been explored via a phylogenetic relationship by performing a single nucleotide polymorphism (SNP) analysis. Such analyses provide an in-depth overview of unique molecular makers and virulence associated genes of *Vibrio navarrensis* (Schwartz et al.). Wang et al. has applied and study the use of multiple cross displacement implication combined with gold nanoparticle-based lateral flow biosensor for detection of *V. parahaemolyticus*. Based on the findings, the method was reported to be suitable for rapid screening of *V. parahaemolyticus* in clinical, food, and environmental samples (Wang et al.).

The management of *Vibrio* spp. using non-antibiotic approach has been well addressed in two review articles in this research topic. *Streptomyces* are soil living bacteria that has been widely recognized as industrially important microorganism due to its potential in producing various antagonistic compounds (e.g., anti-biofilm, anti-quorum sensing, and anti-virulence) against *Vibrio* pathogens. *Streptomyces* can be used as probiotic and incorporated in feeds in aquaculture to protect the livestock from pathogens. Despite the fact that there are promising outcomes introduced by past researchers on the use of Streptomyces as a probiotic in aquaculture, further extensive trials are needed to establish the probiotic nature of *Streptomyces* in disease prevention and development improvement of aquaculture animals (Tan et al.). Essentially, another review article discussed about the advantages of bacteriophages as a non-antibiotic agent. Bacteriophages as a non-antibiotic approach has gained popularity recently due to the increase emergence of antibiotic resistant bacteria worldwide. The bacteriophages have various advantages such as being host specific, environmental friendly, can be effortlessly found and isolated from ecological sources, and cost effective compared to antibiotics. These phages have been proven to control and inhibit virulence of *Vibrio* sp. isolated from both clinical and environmental. The recognition and safety accreditation by US Food and Drug Administration (FDA) on phages has open the doors for phages to be commercially manufactured for human application and consumption (Letchumanan et al.).

In summary, the collection of manuscripts provided in this Research Topic offers a comprehensive view on the prevalence and antibiotic resistance profiles of *Vibrio* sp. and as well as discussed, the management methods. This issue shows the significant progress and understanding in the control of pathogenic *Vibrio* sp. and provides insights to researchers worldwide about new and promising therapies.

AUTHOR CONTRIBUTIONS

L-HL performed the literature search and writing of the manuscript. PR provided critical review and insight to improve the writing. The research project was conceptualized by L-HL.

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ORIGINAL RESEARCH
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Occurrence and Antibiotic Resistance of *Vibrio parahaemolyticus* from Shellfish in Selangor, Malaysia

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High consumer demand for shellfish has led to the need for large-scale, reliable shellfish supply through aquaculture or shellfish farming. However, bacterial infections which can spread rapidly among shellfish poses a major threat to this industry. Shellfish farmers therefore often resort to extensive use of antibiotics, both prophylactically and therapeutically, in order to protect their stocks. The extensive use of antibiotics in aquaculture has been postulated to represent a major contributing factor in the rising incidence of antimicrobial resistant pathogenic bacteria in shellfish. This study aimed to investigate the incidence of pathogenic *Vibrio parahaemolyticus* and determine the antibiotic resistance profile as well as to perform plasmid curing in order to determine the antibiotic resistance mediation. Based on colony morphology, all 450 samples tested were positive for *Vibrio* sp; however, *tox-R* assay showed that only 44.4% (200/450) of these were *V. parahaemolyticus*. Out of these 200 samples, 6.5% (13/200) were *trh*-positive while none were *tdh*-positive. Antibiotic resistance was determined for all *V. parahaemolyticus* identified against 14 commonly used antibiotics and the multiple antibiotic resistance index (MAR) was calculated. The isolates demonstrated high resistance to several antibiotics tested- including second and third-line antibiotics- with 88% resistant to ampicillin, 81% to amikacin,70.5% to kanamycin, 73% to cefotaxime, and 51.5% to ceftazidime. The MAR index ranged from 0.00 to 0.79 with the majority of samples having an index of 0.36 (resistant to five antibiotics). Among the 13 *trh*-positive strains, almost 70% (9/13) demonstrated resistance to 4 or more antibiotics. Plasmid profiling for all *V. parahaemolyticus* isolates revealed that 86.5% (173/200) contained plasmids - ranging from 1 to 7 plasmids with DNA band sizes ranging from 1.2 kb to greater than 10 kb. 6/13 of the pathogenic *V. pathogenic* strains contained plasmid. After plasmid curing, the plasmid containing pathogenic strains isolated in our study have chromosomally mediated ampicillin resistance while the remaining resistance phenotypes are plasmid mediated. Overall, our results indicate that while the incidence of pathogenic *V. parahaemolyticus* in shellfish in Selangor still appears to be at relatively reassuring levels, antibiotic resistance is a real concern and warrants ongoing surveillance.

Keywords: ***Vibrio parahaemolyticus*, shellfish, MAR index, antibiotic resistance, plasmid curing, plasmid profile**

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INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative bacterium that is widely disseminated in marine and estuarine environments worldwide (Su and Liu, 2007; Ceccarelli et al., 2013; Zhang and Orth, 2013; Letchumanan et al., 2014; Velazquez-Roman et al., 2014; Wu et al., 2014). While the majority of strains isolated from environmental sources are innocuous members of marine microbiota, a small number of *V. parahaemolyticus* strains are capable of causing human illness and are often associated with food borne gastroenteritis or diarrhea (Nair et al., 2007; Hazen et al., 2015; Raghunath, 2015). The virulent strains are discerned from avirulent strains by the presence of toxigenic genes namely, thermostable direct hemolysin (*tdh*) and/or *tdh*-related (*trh*) hemolysin genes (Letchumanan et al., 2014).

Although *V. parahaemolyticus* is commonly present in seafood, most of these isolates are regarded as non-pathogenic to human (Nishibuchi and Kaper, 1995; Velazquez-Roman et al., 2012; Raghunath, 2015). The strains isolated from environmental samples usually lack the pathogenic genes thermostable direct hemolysin (*tdh*) and/or TDH-related hemolysin (*trh*) which are responsible for causing diseases in human and marine animals (Deepanjali et al., 2005; Canizalez-Roman et al., 2011; Gutierrez West et al., 2013). Previous study reported, around 0–6% of the environmental *V. parahaemolyticus* strains carry *tdh* and/or *trh* genes (DePaola et al., 2000; Vuddhakul et al., 2000; Wong et al., 2000; Alam et al., 2002; Hervio-Heath et al., 2002; Haley et al., 2014). These two genes are considered major virulence factors of *V. parahaemolyticus* (Kaysner and DePaola, 2001; Zhang and Austin, 2005; Xu et al., 2014).

There are many methods utilized for the detection of *V. parahaemolyticus*. The standard method for detection and identification of *V. parahaemolyticus* uses microbiological media enrichments such as Thiosulfate Citrate Bile Salt (TCBS) agar, Alkaline Peptone Water (APW) along with a range of biochemical tests (Vincent et al., 2015). These methods are valuable for estimation of the total load of *V. parahaemolyticus* in a sample. This then enables estimation of potential risk for presence of pathogenic strains (Malcolm et al., 2015). Routine phenotyping and biochemical identification methods of *V. parahaemolyticus* are often complicated when the strains are isolated from seafood or marine surroundings (Nishibuchi, 2006). In order to enable rapid and accurate identification of *V. parahaemolyticus*, a combination of conventional and molecular approaches using PCR assay is often adapted by researchers. At present, pathogenic *V. parahaemolyticus* isolates in seafood and environmental samples are identified using PCR-based methods that amplify *tox-R* (the toxin operon gene that is well conserved among *V. parahaemolyticus*), *tdh* and *trh* gene sequences (Panicker et al., 2004; Yamamoto et al., 2008; Paydar et al., 2013; Law et al., 2015; Malcolm et al., 2015). PCR primers can be multiplexed in a single reaction to increase the detection limit or tailored as real-time PCR assay to provide rapid results (Grant et al., 2006; Zhang et al., 2014).

An increasing population with increased purchasing power in globally has increased the demand for and export potential of seafood; resulting in steady expansion of the Asian aquaculture industry (Rico et al., 2012). However, as aquaculture practices have intensified, the sector has been constantly challenged by aquatic animal health problems which are a major constraint to the development and expansion of the aquaculture sector (Bondad-Reantaso et al., 2005). Hence, aquaculture farmers rely on a wide range of antibiotics to prevent (prophylactic use) and treat (therapeutic use) bacterial infections in fish and other invertebrates (Cabello et al., 2013). Oxytetracycline, tetracycline, quinolones, sulphonamides and trimethoprim are among the antimicrobials permitted and utilized in the Asian aquaculture industry (Rico et al., 2012; Yano et al., 2014). The extensive use of antibiotics and other chemotherapeutics in aquaculture fields has caused the emergence of antibiotic resistant strains in the environment. Every year, more and more pathogenic *Vibrio* species have been reported to develop increasing levels of resistance toward most of the clinically used antibiotics (Letchumanan et al., 2015b).

Generally, *Vibrio* species are known to be highly susceptible to most clinically used antibiotics (Mala et al., 2014; Shaw et al., 2014; Letchumanan et al., 2015a). It is noted that most of the genetic determinants that confer antibiotic resistance are located in the plasmid. Plasmids are one of the important mediators that facilitate the transfer of antibiotic resistant genes and can be transmitted to the next generation via vertical gene transfer or exchanged with other bacteria via horizontal gene transfer (Okamoto et al., 2009; Manjusha and Sarita, 2011). Plasmid profile determination represents one of the earliest DNA based assay used for epidemiological studies and remains useful today (Meyer, 1988). Previous studies have reported that *Vibrio* sp. contain plasmids and there is a correlation between possessions of plasmid with antibiotic resistance (Molina-Aja et al., 2002; Zulkifli et al., 2009). Plasmid curing of bacteria is a possible way to eliminate the plasmids which may then allow determination of the mode of antibiotic resistance mediation. Plasmid curing protocols for *Vibrio* sp. involves chemical agents such as ethidium bromide (EB), acridine orange (AO) and sodium dodecyl sulphate (SDS), and physical agents such as treatment with ultraviolet and growth at elevated temperature (Liu et al., 2012).

Shellfish mainly resides in coastal and estuarine environments. Due to the nature of their habitat, shellfish contains diverse bacterial microbiota including *Vibrio* sp. (Romalde et al., 2014). Previous studies have found high prevalence of *V. parahaemolyticus* in shellfish in Malaysia (Zulkifli et al., 2009; Al-Othrubi et al., 2014; Sahilah et al., 2014; Tang et al., 2014; Malcolm et al., 2015). The presence of potentially pathogenic *Vibrio* species in shellfish in Malaysia highlights the need for continuous monitoring as well as consumer education on food safety. Considering these factors, the present study aimed to investigate the incidence of pathogenic and multidrug resistant strains of *V. parahaemolyticus* in shellfish by both conventional and molecular methods, as well as investigating the antibiotic resistance profiles of these organisms and to attempt to determine mediations via plasmid curing.

MATERIALS AND METHODS

Sampling

The study focused mainly on five type of shellfish; mud crab (*Scylla serrate*), flower crab (*Portunus pelagicus*), carpet clam (*Paphia textile*), hard shell clam (*Meretrix meretrix*), and mud creeper (*Cerithidea obtuse*). A total of 450 shellfish samples were purchased from three selected local wetmarkets and three local supermarkets in Selangor between January 2014 and June 2014. All the samples were sealed and transported in an ice box to the laboratory for analysis on the day of purchase.

Enumeration and Isolation of *Vibrio* sp. in Shellfish Samples

Twenty-grams of samples (without shell) were homogenized for 60 s in a stomacher (Bagmixer 400W, Interscience, St Nom, France) with 225 mL of alkaline peptone water with 2% NaCl, pH 8.5, giving a first 10^{-1} dilution. The homogenates (1:10, 1:100, and 1:1000) were analyzed by spread-plate technique for total *Vibrio* sp. counts on Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar (HiMedia, India) and incubated at 37°C for 18 h. After incubation, the total colony count and their concentrations in the original shellfish in cfu/mL was calculated.

The isolation of *V. parahaemolyticus* was carried out by incubating the homogenate at 37°C under aerobic conditions for 18 h. After incubation, a loopful of sample was streaked onto selective media, Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar (HiMedia, India). Plates were then incubated at 37°C for 18 h. Characteristic green colonies (sucrose negative) on TCBS agar were considered presumptive *V. parahaemolyticus* and picked. The colonies were purified on Tryptic Soy Agar (TSA; HiMedia, India) plates supplemented with 2% w/v sodium chloride (NaCl; Vivantis, USA) and incubated at 37°C under aerobic conditions for 18 h. A loopful of pure colony was inoculated into semi-solid nutrient agar and Tryptic Soy Broth (TSB) with 30% glycerol, incubated at 37°C for 18 h and then stored until further analysis (Zarei et al., 2012; Letchumanan et al., 2015a).

DNA Extraction

Genomic DNA of presumptive *V. parahaemolyticus* colonies was extracted using direct boiled cell lysate method (Vengadesh et al., 2012; Letchumanan et al., 2015a). In brief, *V. parahaemolyticus* colonies from semisolid nutrient agar are revived in TSB (HiMedia) with 2% w/v NaCl (Vivantis, USA) and incubated in a shaker incubator at 220 rpm for 37°C for 18 h. 1.5 mL of overnight culture suspension was subjected to centrifugation at 10,000 rpm for 5 min. The supernatant was carefully discarded, leaving the pellet dry as possible. The pellet was re-suspended in 1 mL of sterile ultrapure water, vortexed and boiled at 100°C for 7 min. The boiled cell lysate was immediately cooled on ice for 5 min before being centrifuged at 13,000 rpm for 1 min to separate the debris and DNA containing supernatant. The supernatant was carefully transferred into a new micro-centrifuge tube. The boiled cell lysate was used as the DNA template for PCR assay.

Identification of *Vibrio parahaemolyticus* using *toxR*-Based PCR Assay

PCR amplification for detection of *V. parahaemolyticus* was performed in a final volume of 20 μL, containing 2 μL of DNA template, 10 μL of 2x *Taq PLUS* PCR Smart mix 1 (SolGent^{TM}, Korea), 6 μL of sterile distilled water and 1 μL of each primer, *toxR*-F (5′-ATA CGA GTG GTT GCT GTC ATG-3′) and *toxR*-R (5′-GTC TTC TGA CGC AAT CGT TG-3′) with the expected amplicon size of 368 bp (Kim et al., 1999). The PCR amplification were performed using PCR thermocycler (Kyratec, SuperCycler Thermal Cycler, Australia) with the following cycling conditions: initial denaturation at 95°C for 4 min, 35 cycles of 94°C for 1 min, 68°C for 1 min and 72°C for 30 s, and a final elongation at 72°C for 5 min. PCR products were visualized by using 1.5% agarose gel. *V. parahaemolyticus* type strain (*V. parahaemolyticus* NBRC12711) and *V. vulnificus* type strain (*V. vulnificus* NBRC15645) was used as a positive control and negative control in every PCR reaction.

Detection of Virulence Gene

PCR amplification for detection of *V. parahaemolyticus* virulence genes, thermostable direct haemolysin (*tdh*) and thermostable-related direct haemolysin (*trh*) was performed in duplex PCR using specific primers adapted from Bej et al. (1999). The PCR was carried out in a final volume of 20 μL, containing 2 μL of DNA template, 10 μL of 2x *Taq PLUS* PCR Smart mix 1 (SolGent^{TM}, Korea), 4 μL of sterile distilled water, and 1 μL of each primer. The PCR amplifications were performed using a Thermocycler (Kyratec, SuperCycler Thermal Cycler, Australia) with the following cycling conditions: initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, and a final elongation at 72°C for 5 min. All PCR products were visualized by using 1.5% agarose gel. The *V. parahaemolyticus* type strain (*V. parahaemolyticus* NBRC12711) was used as a positive control and *V. vulnificus* type strain (*V. vulnificus* NBRC15645) was used as a negative control in every PCR reaction.

Antibiotic Susceptibility Test

Antibiotic disks (Oxoid, UK) infused with 14 antibiotics namely ampicillin (10μg), ampicillin/sulbactam (30 μg), amikacin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), chloramphenicol (30 μg), gentamicin (30 μg), imipenem (10 μg), kanamycin (30 μg), levofloxacin (5 μg), nalidixic acid (30 μg), oxytetracycline (30 μg), Sulfamethoxazole/trimethoprim (25 μg), and tetracycline (30 μg) were used in this study. The antibiotic susceptibility of *Vibrio* sp. isolates were studied using the disk diffusion method (Yano et al., 2014). The antibiotic disks were dispensed on Mueller Hilton agar (HiMedia, India) supplemented with 2% w/v NaCl (Vivantis, USA) plates with bacterial lawn. After incubation at 37°C for 18 h, the inhibition zone was measured and interpreted based on guidelines of the Clinical and Laboratory Standards Institute (CLSI) M45-A2 (Clinical Laboratory Standard Institute [CLSI], 2010).

Plasmid Profiling

Plasmid profiling was carried out following an adaptation of the method from Devi et al. (2009). Briefly, *V. parahaemolyticus* cell were grown in TSB containing 2% w/v sodium chloride and incubated at 37°C in a shaker incubator (220 rpm) for 18 h. About 1.5 mL of the culture was transferred into a micro-centrifuge tube followed by centrifugation (10,000 rpm for 2 min at 4°C). The supernatant was removed by aspiration leaving the cell pellet as dry as possible. The pellet was resuspended in ice-cold 100 µl alkaline lysis solution I (Glucose 50 mM; Tris Cl 25 mM; EDTA 10 Mm) by vigorous vortexing followed by addition of freshly prepared 200 µL alkaline lysis solution II (NaOH 2N; SDS 2% w/v). The contents were mixed by vortexing rapidly after which 150 µl ice-cold solution III (Potassium acetate 5 M: 60 ml; Glacial acetic acid 11.5 ml; dissolved in 28.5 m sterile distilled water) was added to it. The tube was closed and gently vortexed for 10 s to disperse solution III through the viscous bacterial lysate.

Then the tubes were stored in ice for 5 min before being centrifuged at 12,000 rpm for 2 min at 4°C. An equal volume of phenol-chloroform (1:1, w/v) was added to the supernatant in a fresh tube, by vortexing. The contents in the micro-centrifuge tube were centrifuged at 8,000 rpm for 3 min at 4°C and the supernatant was transferred into a fresh tube. This was repeated with chloroform: isoamyl-alcohol (24:1, v/v) for removing the phenol. The double stranded DNA was precipitated with two volumes of ethanol at room temperature, followed by vortexing before it was allowed to stand for 5 min at room temperature. The aliquot was centrifuged at 12,000 rpm for 12 min at 4°C and the supernatant was removed by gentle aspiration. The pellet of double stranded DNA was rinsed with ethanol (1 ml, 70% v/v) at 4°C and centrifuged. The supernatant was removed leaving the pellet dry as possible. The pellet was air-dried before it was re-dissolved in 30 µl ultrapure water. Electrophoresis was performed using 1% agarose gel.

Plasmid Curing

All the antibiotic resistant *V. parahaemolyticus* strains were subjected to a plasmid curing assay using AO. Isolates were grown in TSB supplemented with 0.2 mg/mL AO. The tubes were then incubated at 37°C for 24 h under constant agitation. After treatment with AO, the antibiotic susceptibility profile of the resistant phenotypes was examined. (Reboucas et al., 2011). *V. parahaemolyticus* VP152 and VP103 strains were used as a positive control in every plasmid curing assay (Letchumanan et al., 2015a).

Statistical Analysis

The experimental data was analyzed by using SPSS software version 20. Statistical analysis was performed in order to determine whether there were any significant differences in the incidence of *V. parahaemolyticus* in the varying species of crustaceans studied and also to analyze the MAR index of resistant isolates using the Anova. The significance level was set at p-value of <0.05.

RESULTS

Prevalence of *Vibrio parahaemolyticus* in shellfish

The conventional method based on the colonial appearance (green or bluish green colonies on TCBS agar) detected 450 presumptive *V. parahaemolyticus* isolates. To further confirm their identity, PCR was performed on all the presumptive *V. parahaemolyticus* isolates in order to confirm their identity by targeting species level toxin operon gene (*toxR*), 368bp. Only 44% (200/450) of the isolates were *toxR*-positive (**Table 1**). The number of *toxR*-positive isolates was similar at all sampling sites.

The density of total *Vibrio* count in the shellfish samples are shown in **Table 2**. The shellfish samples analyzed had a mean total *Vibrio* count range of 2.45–6.63 log cfu/g. A bacterial load of 5–7 log cfu/g is generally considered to be the level necessary to cause clinically significant infection in humans. Out of the 30 categories, 50% (15/30) were found to be >5 log cfu/g; however, this is with reference to *Vibrio* sp, not specifically *V. parahaemolyticus*. Of these 15 samples, eight were from wetmarkets and seven were from supermarkets. Mudcrab from all the locations sampled exceeded 5 log cfu/g. Carpet clams from the three wet markets all exceeded 6 log cfu/g while only one sample from the supermarket exceeded 5 log cfu/g. This pattern was reversed in mud creepers, however, with the supermarket samples showing higher *Vibrio* density. Overall, swimming crab showed the lowest density of *Vibrio* sp. density with four of the six sites sampled having mean densities of less than 3 log cfu/g.

Prevalence of Thermostable Direct Hemolysin *(tdh)* and TDH-Related Hemolysin *(trh)* Positive *Vibrio parahaemolyticus* Isolates

To detect pathogenic isolates, *tdh* and *trh* genes were amplified using PCR-based assay. The results of this analysis are summarized in **Table 3**. 13/200 (6.5%) *trh*-positive isolates were identified – all 13 were from shellfish samples collected from wetmarket B. Of these, six samples were mud crab, two samples were carpet clam, two samples were mud creeper and three samples were hard shell clam (**Table 1**). No *tdh*-positive *V. parahaemolyticus* isolates were detected.

Antimicrobial Susceptibilities of *Vibrio parahaemolyticus* Isolates

Vibrio parahaemolyticus isolates from shellfish samples were further tested for antimicrobial susceptibilities. All 14 antibiotics used in this study are among the antibiotics recommended by Centre for Disease Control and Prevention (CDC) for the treatment of *Vibrio* sp. infections that include fluoroquinolones (levofloxacin), cephalosporins (cefotaxime and ceftazidime),

aminoglycosides (amikacin and gentamicin) and folate pathway inhibitors (trimethoprim-sulfamethoxazole; Daniels et al., 2000; Shaw et al., 2014). The results of this test are summarized in **Table 4**. With regard to CDC recommended antimicrobial agents, the isolates exhibited resistance to ampicillin (88.0%), amikacin (81.0%), and kanamycin (70.5%). Within the third generation cephalosporin, isolates exhibited resistance to cefotaxime (73%) and ceftazidime (51.5%). Reassuringly, the isolates tested were still susceptible to several antibiotics tested, imipenem (90.0%), chloramphenicol (88.0%), tetracycline (84.0%), ampicillin/sulbactam (67.0%), levofloxacin (61.5%) and trimethoprim-sulfamethoxazole (50%). With reference to the pathogenic strains, all the thirteen *trh*-positive isolates were resistant to ampicillin, 12/13 isolates expressed resistance to amikacin and cefotaxime, and 8/13 isolates were resistant to ceftazidime.

TABLE 1 | The identification and detection of *toxR*+, *trh*+, and *tdh*+* in *Vibrio parahaemolyticus* isolates.

Sampling site	Seafood samples ($n = 450$)	Number of isolates (%)		
		Total	*toxR* + (%)	*trh* + (%)
Wetmarket A	Mud crab ($n = 15$)	15	8 (53)	0 (0)
	Swimming crab ($n = 15$)	15	11 (73)	0 (0)
	Hard shell clam ($n = 15$)	15	3 (20)	0 (0)
	Carpet clam ($n = 15$)	15	3 (20)	0 (0)
	Mud creeper ($n = 15$)	15	8 (53)	0 (0)
Total	$n = 75$	75	33 (44)	0 (0)
Wetmarket B	Mud crab ($n = 15$)	15	10 (67)	6 (60)
	Swimming crab ($n = 15$)	15	5 (33)	0 (0)
	Hard shell clam ($n = 15$)	15	4 (27)	3 (75)
	Carpet clam ($n = 15$)	15	5 (33)	2 (40)
	Mud creeper ($n = 15$)	15	12 (80)	2 (17)
Total	$n = 75$	75	36 (48)	13 (36)
Wetmarket C	Mud crab ($n = 15$)	15	9 (60)	0 (0)
	Swimming crab ($n = 15$)	15	8 (53)	0 (0)
	Hard shell clam ($n = 15$)	15	4 (27)	0 (0)
	Carpet clam ($n = 15$)	15	3 (20)	0 (0)
	Mud creeper ($n = 15$)	15	10 (67)	0 (0)
Total	$n = 75$	75	34 (45)	0 (0)
Supermarket A	Mud crab ($n = 15$)	15	6 (40)	0 (0)
	Swimming crab ($n = 15$)	15	6 (40)	0 (0)
	Hard shell clam ($n = 15$)	15	8 (53)	0 (0)
	Carpet clam ($n = 15$)	15	4 (27)	0 (0)
	Mud creeper ($n = 15$)	15	9 (60)	0 (0)
Total	$n = 75$	75	33 (44)	0 (0)
Supermarket B	Mud crab ($n = 15$)	15	7 (47)	0 (0)
	Swimming crab ($n = 15$)	15	9 (60)	0 (0)
	Hard shell clam ($n = 15$)	15	3 (20)	0 (0)
	Carpet clam ($n = 15$)	15	3 (20)	0 (0)
	Mud creeper ($n = 15$)	15	9 (60)	0 (0)
Total	$n = 75$	75	31 (41)	0 (0)
Supermarket C	Mud crab ($n = 15$)	15	6 (40)	0 (0)
	Swimming crab ($n = 15$)	15	8 (53)	0 (0)
	Hard shell clam ($n = 15$)	15	7 (47)	0 (0)
	Carpet clam ($n = 15$)	15	4 (27)	0 (0)
	Mud creeper ($n = 15$)	15	8 (53)	0 (0)
Total	$n = 75$	75	33 (44)	0 (0)

**All the V. parahaemolyticus isolates were negative for tdh gene.*

Eighty-five percentage of the isolates had multiple antibiotic resistance (MAR) index more than 0.2. Gwendelynne et al. (2005) stated that MAR indices higher that 0.2 could be a marker of contamination from high risk sources, thus indicating a potential human health risk. MAR index in this study ranged from 0.00 to 0.79, with the highest MAR index (0.79) found in a non-pathogenic isolate (SVP129) of carpet clam from supermarket B which expressed resistance to 11/14 antibiotic tested. The majority of *V. parahaemolyticus* isolates studied showed a MAR index of 0.36 (resistant to five antibiotics tested). 15% of the isolates had MAR index of 0.00–0.07, indicating the isolates were resistant to none or at least one type of the antibiotic tested.

Plasmid Profiling and Plasmid Curing of *Vibrio parahaemolyticus* Isolates

All the *V. parahaemolyticus* isolates were tested for the presence of plasmid before curing. Plasmid profiles of the 200 *V. parahaemolyticus* isolates revealed that 173/200 isolates contained one to seven plasmids ranging from 1.2 kb to above 10 kb in size while the remaining 27 isolates did not have any plasmids. 6/13 *trh*-positive *V. parahaemolyticus* isolates (**Table 3**) contained plasmids, with all six containing at least one plasmid above 10 kb. (SVP61); *trh*-positive isolate) expressed seven plasmids with sizes of 1.2, 1.75, 3, 3.1, 4, and 2 kb above 10 kb size. SVP61 was resistant to 4/14 antibiotics tested. The study revealed that (SVP129; non-pathogenic isolate) of carpet clam from supermarket B has one plasmid above 10 kb size and is resistant to 11/14 antibiotics tested.

Plasmid curing is a method which potentially enables the elimination of plasmids from the organisms being studied which can then allow the determination of antibiotic resistance mediation by retesting the organisms after plasmid curing. Antibiotic resistance of all *V. parahaemolyticus* isolates which had underdone plasmid curing was tested by antibiotic disk diffusion method. The resistance exhibited by isolates to ampicillin and tetracycline did not vary even after curing of plasmids. With reference to the 13 *trh*-positive strains, the antibiotic resistance profile of the six plasmid containing strains altered after curing while the remaining seven were unchanged. All six were ampicillin resistant initially, and after curing, one strain became susceptible to all antibiotics tested while five of the strains (SVP61, SVP54, SVP75, SVP69, VP72) remained resistant to ampicillin and became susceptible to the other antibiotics tested. This suggests that while antibiotic resistance is mediated by both

TABLE 2 | The mean of total *Vibrio* counts (log CFU/g) of each shellfish samples from respective sampling site.

Samples	Total *Vibrio* density (log CFU/g)					
	Wetmarket A	Wetmarket B	Wetmarket C	Supermarket A	Supermarket B	Supermarket C
Mud crab	5.53 ± 0.03	5.44 ± 0.12	5.39 ± 0.22	5.00 ± 0.20	5.33 ± 0.05	5.17 ± 0.48
Swimming crab	2.45 ± 0.21	4.52 ± 0.49	3.40 ± 0.40	2.29 ± 0.16	2.88 ± 1.24	2.59 ± 0.30
Hard shell clam	5.00 ± 0.69	4.90 ± 0.06	4.95 ± 0.13	4.13 ± 1.12	4.06 ± 0.12	4.00 ± 0.04
Carpet clam	6.63 ± 0.19	6.42 ± 0.04	6.50 ± 0.25	5.56 ± 0.47	3.03 ± 0.40	4.30 ± 0.43
Mud creeper	4.54 ± 0.25	5.23 ± 0.28	4.89 ± 0.55	5.86 ± 0.43	5.92 ± 0.19	5.90 ± 0.22
Total average of *Vibrio* count (log CFU/g)	4.83 ± 1.52[a]	5.26 ± 0.72[a]	5.03 ± 1.12[a]	4.57 ± 1.43[a]	4.24 ± 1.35[a]	4.40 ± 1.25[a]

Values = mean ± standard deviation (n = 15). [a] *Means with different superscripts in the same row indicate significant difference ($p < 0.05$).*

TABLE 3 | List of *trh*-positive *Vibrio parahaemolyticus* isolates.

Strains	Samples	Location	*toxR*-positive	*trh*-positive	Before plasmid curing			After plasmid curing	
					Antibiotic resistance pattern	No. of plasmid	Plasmid size	Antibiotic resistance pattern	No. of plasmids
SVP55	Mud crab	Wetmarket B	+	+	amp/ak/caz/ctx/k	None		amp/ak/caz/ctx/k	
SVP56	Mud crab	Wetmarket B	+	+	amp/ak/caz/ctx/k	None		amp/ak/caz/ctx/k	
SVP60	Mud crab	Wetmarket B	+	+	amp	None		amp	
SVP61	Mud crab	Wetmarket B	+	+	amp/ak/caz/ctx	7	1.2 kb, 1.75 kb, 3 kb, 3.1 kb, 4 kb, two above 10 kb	amp	Lost
SVP64	Mud crab	Wetmarket B	+	+	amp/ak/ctx	None		amp/ak/ctx	
SVP66	Mud crab	Wetmarket B	+	+	amp/ak/caz/ctx	None		amp/ak/caz/ctx	
SVP52	Carpet clam	Wetmarket B	+	+	amp/ak/ctx	None		amp/ak/ctx	
SVP54	Carpet clam	Wetmarket B	+	+	amp/ak/caz/ctx/k/lev	1	above 10 kb	amp	Lost
SVP73	Mud creeper	Wetmarket B	+	+	ak/ctx/k	1	above 10 kb	All susceptible	Lost
SVP75	Mud creeper	Wetmarket B	+	+	amp/ak/ctx/k	1	above 10 kb	amp	Lost
SVP69	Hard shell clam	Wetmarket B	+	+	amp/ak/caz/ctx	1	1.75 kb	amp	Lost
SVP70	Hard shell clam	Wetmarket B	+	+	amp/ak/caz/ctx/k	None		amp/ak/caz/ctx/k	
SVP72	Hard shell clam	Wetmarket B	+	+	amp/ak/caz/ctx	2	2.5 kb, one above 10 kb	amp	Lost

TABLE 4 | The antibiotic resistant profile of *V. parahaemolyticus* isolates (*n* = 200).

Class of antibiotics	Antibiotics	Concentration (μg)	No. of resistant isolates (%)	No. of intermediate isolates (%)	No. of susceptible isolates (%)
Penicillins	Ampicillin	10	176 (88.0)	10 (5.0)	14 (7.0)
	Ampicillin-sulbactum	30	38 (19.0)	28 (14.0)	134 (67.0)
Cephalosporins	Cefotaxime	30	146 (73.0)	21 (10.5)	33 (16.5)
	Ceftazidime	30	103 (51.5)	46 (23.1)	51 (25.5)
Carbapenems	Imipenem	10	1 (0.5)	18 (9.0)	181 (90.0)
Aminoglycosides	Amikacin	30	162 (81.0)	25 (12.5)	13 (6.5)
	Gentamicin	30	7 (3.5)	79 (39.5)	114 (57.0)
	Kanamycin	30	141 (70.5)	54 (27.0)	5 (2.5)
Tetracycline	Tetracycline	30	26 (13.0)	6 (3.0)	168 (84.0)
	Oxytetracycline	30	32 (16.0)	99 (49.5)	69 (34.5)
Quinolones	Nalidixic acid	30	3 (1.9)	60 (37.5)	97 (60.6)
	Levofloxacin	5	14 (8.8)	47 (29.4)	99 (61.9)
Folate pathway inhibitor	Trimethoprim-sulfamethoxazole	25	11 (6.9)	69 (43.1)	80 (50.0)
Phenicols	Chloramphenicol	30	14 (8.8)	5 (3.1)	141 (88.1)

plasmid and chromosomes in pathogenic *V. parahaemolyticus* isolates, in plasmid containing strains, aside from ampicillin resistance, most of the remaining resistance phenotypes are plasmid mediated.

SVP129 isolate contained one plasmid profile with size more than 10 kn and expressed resistance to 11/14 antibiotics tested. After plasmid curing, SVP129 isolate lost its plasmid and changed its antibiotic resistance phenotype. SVP129 isolate remained resistant to 5/14 antibiotic tested namely ampicillin, oxytetracycline, chloramphenicol, tetracycline, and sulfamethoxazole/trimethoprim. The isolate showed intermediate resistance to amikacin, ceftazidime, cefotaxime, and kanamycin, while it was susceptible to gentamycin and ampicillin/sulbactam after plasmid curing assay.

DISCUSSION

The presence of pathogenic strains of *V. parahaemolyticus* in the shellfish we studied does raise concern as these organisms are known to be a frequent cause of food-borne gastroenteritis in humans. However, while conventional colony morphology found all samples to be contaminated with *Vibrio* sp. only 44% (200/450) of these were confirmed to be *V. parahaemolyticus* based on *tox-R* assay; and only 6.5% (13/200) of these were pathogenic strains (*trh*-positive). Conventional bacteriological methods have demonstrated a highly variable occurrence of *V. parahaemolyticus* in seafood around the world. Our results are in close agreement with those reported by Zhao et al. (2011) in China, who isolated *V. parahaemolyticus* from 59.7% shellfish samples. Our study samples exhibited a mean total *Vibrio* count range of 2.45–6.63 log cfu/g. The mean total *Vibrio* count of the shellfish samples from wetmarkets were higher compared to shellfish samples from supermarkets. However, the results obtained from one-way analysis of variance (**Table 2**), showed there were no significant difference ($p > 0.05$) between the total *Vibrio* counts of the shellfish samples from all the sampling sites. The difference in the incidence of *V. parahaemolyticus* among samples from both sampling sites could possibly be contributed by the original geographic source from which the shellfish were collected, post-harvest practices and hygiene standards applied during handling, transportation and storage of seafood products. A study in Cochin, India, reported higher *Vibrio* sp. contamination in shellfish from roadside stalls compared to markets (Sudha et al., 2014). Other studies have reported lack of hygiene, improper handling, cross contamination and difference in storage temperature as the possible cause of variation in *V. parahaemolyticus* incidences in samples from supermarket (Yang et al., 2008; Tunung et al., 2010; Sudha et al., 2014).

In order to assess the actual risk to human health posed by the presence of *V. parahaemolyticus* in seafood, incidence of pathogenic strains need to be determined via detection of the toxigenic genes responsible for causing disease in humans. Of the 200 isolates tested in this study, only 13 (6.5%) were *trh*-positive and there were no *tdh*-positive strains. This incidence is similar to the results demonstrated in Malaysia by Paydar et al. (2013) who detected 6/50 (12%) *trh*-positive *V. parahaemolyticus* among the food samples tested. In another study, a much higher incidence of *tdh*-positive and *trh*-positive in shrimp and cockles samples was reported in Malaysia with twenty six isolates were *trh*-positive and eight *tdh*-positive (Al-Othrubi et al., 2014). Normally, only 1–2 % of the environmental strains harbor the *tdh* and *trh* genes (Wong et al., 2000; Alam et al., 2002; Hervio-Heath et al., 2002; Velazquez-Roman et al., 2012; Haley et al., 2014). Studies in the USA and in Japan reported that only 3% of *V. parahaemolyticus* strains isolated from seafood and environment samples were pathogenic (DePaola et al., 2003; Mahmoud et al., 2006; Abd-Elghany and Sallam, 2013). It is also reported that environmental factors including interaction with different hosts plays an important impact in the evolution of specific pathogens (Wilson and Salyers, 2003).

The continuous and extensive use of antibiotics in the aquaculture industry favors the development of a variety of resistant isolates and the dissemination of resistance genes within the bacterial population in the environment which reflects the pattern of drug use (Tendencia and Pena, 2002; Reboucas et al., 2011). All the antibiotics tested in this study are recommended antimicrobial agents used in the treatment of *Vibrio* sp. infections, including the tetracycline, levofloxacin, cefotaxime, ceftazidime, amikacin, gentamicin, and trimethoprim-sulfamethoxazole (Daniels et al., 2000; Shaw et al., 2014). Some of these antibiotics are widely used in aquaculture industry as antimicrobial agents including oxytetracycline and chloramphenicol (Dang et al., 2007). In this study, 88% of the *V. parahaemolyticus* isolates from shellfish exhibited resistance to ampicillin. Our results are in close agreement with other studies that reported resistance to ampicillin among the *V. parahaemolyticus* isolates from seafood samples (Okuda et al., 1997; Han et al., 2007; Al-Othrubi et al., 2014; Letchumanan et al., 2015a). First generation antibiotics including ampicillin are extensively used in the aquaculture thus reducing to susceptibility and resulting in low efficacy of ampicillin for *Vibrio* sp. treatment (Sudha et al., 2014).

Resistance to third generation cephalosporin, was observed in our isolates of *V. parahaemolyticus*, with 73% resistant to cefotaxime and 51.5% resistant to ceftazidime. This is slightly less compared to 80% in a study by Jun et al. (2012) studying the resistance to third generation cephalosporin among *V. parahaemolyticus* isolates from Korean seafood. Our study is in agreement with Sahilah et al. (2014) in Terengganu, Malaysia, who reported presence of *V. parahaemolyticus* isolates from shellfish to be resistant to ceftazidime and cefuroxime. However, a study by Shaw et al. (2014) reported low percentage of *V. parahaemolyticus* from USA to be resistant to cefotaxime. The discrepancies in the literature regarding the resistance phenotype of *V. parahaemolyticus* to third generation cephalosporin could possibly due to difference in test methodology or geographical variation. In the present study, high susceptibility to imipenem (90%), chloramphenicol (88.1%) and tetracycline (84%) was observed among the *V. parahaemolyticus* isolates, much in agreement with other publications (Han et al., 2007; Sahilah et al., 2014; Sudha et al., 2014).

High MAR indices were detected in this study, ranging from 0.00 to 0.79, with 85% of the isolates having a MAR index value more than 0.2. MAR indices higher than 0.2 are markers of high risk sources, which may represent a potential human health risk (Gwendelynne et al., 2005). The most frequent MAR index of the resistant *V. parahaemolyticus* isolates tested was 0.36, indicating that the strains were resistant toward five different antibiotics. The MAR index of *V. parahaemolyticus* isolates in present study varied significantly (ANOVA, $p < 0.05$) between wetmarket and supermarket. Our results are in agreement with a study by Elexson et al. (2014) in Malaysia which reported 97.2% of *V. parahaemolyticus* isolates had MAR index more than 0.2, however, a lower percentage (14.5%) with MAR index more than 0.2 was reported by Tang et al. (2014). The huge variation in the MAR index of the *V. parahaemolyticus* isolates observed in present and past studies in Malaysia may be influenced by the variance in the resistance levels depending on the source of sample collection (Khan et al., 2007; Tunung et al., 2012). Lesley et al. (2011) suggested that the difference in geographical locations may have differential selective pressures for the antibiotic resistance level.

Among the 200 *V. parahaemolyticus* isolates, only 173 isolates (86.5%) harbored between one to seven plasmid DNA bands, which range in size from 1.2 kb to above 10 kb and the rest did not exhibit the presence of plasmid DNA. The study findings correlates with results of previous studies, which have reported that *V. parahaemolyticus* harbored plasmids (Kagiko et al., 2001; Kaufman et al., 2002; Molina-Aja et al., 2002; Manjusha et al., 2005; Zulkifli et al., 2009). The isolate (SVP129) had only one plasmid above 10 kb band size and showed resistance to 11/14 antibiotics tested. With reference to *trh*-positive strains, seven of the isolates did not have any plasmid but were resistant to more than one antibiotic tested.

When submitted to plasmid curing, (SVP129) isolate lost its plasmid and altered its resistance phenotype to the antibiotics tested. The isolate remained resistant to sulfamethoxazole/trimethoprim, ampicillin, oxytetracycline, chloramphenicol, and tetracycline. SVP129 isolate showed intermediate resistant to amikacin, ceftazidime, cefotaxime, and kanamycin, while it was susceptible to gentamicin and ampicillin/sulbactam after plasmid curing assay. AO acts as an intercalating agent that inhibits plasmid replications (Letchumanan et al., 2015b). The resistance phenotype exhibited by SVP129 could be chromosomal mediated. Meanwhile, 6/13 *trh*-positive isolates lost their plasmids after curing assay and altered their resistance phenotype to amikacin, ceftazidime, cefotaxime, kanamycin, and levofloxacin (**Table 2**).

All the ampicillin resistant isolates (176/200) and tetracycline resistant isolates (26/200) remain resistant to the respective antibiotics after plasmid curing. This suggests that the resistance phenotype to ampicillin and tetracycline expressed by the isolates could be chromosomally mediated. All the ampicillin/sulbactam resistant strains lost their plasmid after the curing assay and subsequently were susceptible to ampicillin/sulbactam suggesting that the resistance was plasmid mediated. Our results are closely in agreement with other studies that reported *Vibrio* sp., strains lost their plasmids when treated with concentration of 0.2 mg/ml AO and the isolates demonstrated changes in their resistance profile (Molina-Aja et al., 2002; Barman et al., 2010; Reboucas et al., 2011; Carvalho et al., 2013; Costa et al., 2015). A study by Reboucas et al. (2011) in Brazil, reported AO was successfully used to cure multi-resistant *Vibrio* isolates from marine shrimp and concluded the ampicillin resistance strains in study are plasmid mediated. In contrast, another study reported their isolates resistance was chromosomal mediated after AO curing treatment (Costa et al., 2014). The loss of phenotype in these studies suggest that AO produce an immediate and complete inhibition of plasmid replication, thus able to act as reliable plasmid curing agent.

Plasmid curing assay may be used to eliminate bacterial plasmids and determine antibiotic resistance mediation. The assay provides vital information which would be beneficial in the global surveillance management of environmental multidrug resistance. Reducing and improving the use of antibiotics in the aquatic environment can reduce resistance and allow the antibiotic to resurface eventually as an effective therapy (Barbosa and Levy, 2000). The management of antibiotic resistance may vary depending on the resistance location, plasmidial, or chromosomal mediated resistance. Plasmidial mediated resistance can be controlled by alternating the antibiotics used in the environments. The aquaculture industry could adapt the method of switching antibiotics used in the aquatic field from time to time in order to allow withdrawal of antibiotic resistance profile in strains (Letchumanan et al., 2015b). However, it would be a challenge to control chromosomal mediated resistance because the resistant genes reside in the chromosomes of the bacteria. Hence, non-antibiotic management strategies using bacteriophages may help to control chromosomal mediated resistance. Bacteriophages are found in abundance in the aquatic environment and may play a vital role in controlling microbial populations. Many studies have demonstrated the potential of bacteriophages to control bacterial diseases and have been shown to be effective in reducing pathogen levels in aquaculture species, such as shrimp (Karunasagar et al., 2007) and finfish (Park and Nakai, 2003).

Quorum sensing and quorum quenching may be used as an alternative method to manage antibiotic resistance. This strategy has been widely proposed to control infections in aquaculture environments by detecting bacterial cell-to-cell communication thru small signal molecules of quorum sensing (Defoirdt et al., 2004). Quorum sensing is a mechanism of gene regulation in which bacteria coordinate the expression of certain genes in response to the presence or absence of small signal molecules. The bacterial communication is then stopped through quorum quenching and hence the expression of genes is disrupted (Defoirdt et al., 2008). Hence, this knowledge and information gathered would be useful to manage the emergence of antibiotic resistance.

In summary, this study showed that only 2.8% (13/450) of the seafood samples studied contained pathogenic strains of *V. parahaemolyticus*. These 13 strains were deemed pathogenic based on *trh*-positive status. These pathogenic strains all exhibited antibiotic resistance, with almost 70% (9/13) demonstrating resistance to 4 or more antibiotics tested. Out of

the 200 *V. parahaemolyticus* isolates analyzed, there was a high level of resistance to ampicillin and tetracycline as well as to some third generation cephalosporin. While the overall incidence of pathogenic strains of *V. parahaemolyticus* in Selangor is relatively reassuring, the high MAR of all the *V.parahaemolytics* tested – including the pathogenic strains- is a definite cause for concern and warrants ongoing surveillance.

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Survey on antimicrobial resistance patterns in *Vibrio vulnificus* and *Vibrio cholerae* non-O1/non-O139 in Germany reveals carbapenemase-producing *Vibrio cholerae* in coastal waters

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An increase in the occurrence of potentially pathogenic *Vibrio* species is expected for waters in Northern Europe as a consequence of global warming. In this context, a higher incidence of *Vibrio* infections is predicted for the future and forecasts suggest that people visiting and living at the Baltic Sea are at particular risk. This study aimed to investigate antimicrobial resistance patterns among *Vibrio vulnificus* and *Vibrio cholerae* non-O1/non-O139 isolates that could pose a public health risk. Antimicrobial susceptibility of 141 *V. vulnificus* and 184 *V. cholerae* non-O1/non-O139 strains isolated from German coastal waters (Baltic Sea and North Sea) as well as from patients and retail seafood was assessed by broth microdilution and disk diffusion. Both species were susceptible to most of the agents tested (12 subclasses) and no multidrug-resistance was observed. Among *V. vulnificus* isolates, non-susceptibility was exclusively found toward aminoglycosides. In case of *V. cholerae*, a noticeable proportion of strains was non-susceptible to aminopenicillins and aminoglycosides. In addition, resistance toward carbapenems, quinolones, and folate pathway inhibitors was sporadically observed. Biochemical testing indicated the production of carbapenemases with unusual substrate specificity in four environmental *V. cholerae* strains. Most antimicrobial agents recommended for treatment of *V. vulnificus* and *V. cholerae* non-O1/non-O139 infections were found to be effective *in vitro*. However, the occurrence of putative carbapenemase producing *V. cholerae* in German coastal waters is of concern and highlights the need for systematic monitoring of antimicrobial susceptibility in potentially pathogenic *Vibrio* spp. in Europe.

Keywords: antimicrobial resistance pattern, Baltic Sea, North Sea, carbapenemase, disk diffusion, broth microdilution

INTRODUCTION

The family *Vibrionaceae* within the class of Gammaproteobacteria comprises eight genera of Gram-negative, facultative anaerobic, straight, or curved rods that are mostly oxidase-positive, halophilic, and motile (Farmer and Janda, 2004). Members of this family are ubiquitously distributed in

aquatic ecosystems worldwide. They can be found as free-living bacteria and as commensals of aquatic organisms and play an important role in nutrient cycling of natural aquatic habitats. Due to their metabolic diversity and their adaptive abilities to changing environmental conditions, a seasonal, and geographical variability of total *Vibrio* populations is observed in response to climatic influences and seawater circulations (Mansergh and Zehr, 2014).

Among the *Vibrionaceae*, a number of important human pathogenic bacteria have been identified that can cause gastrointestinal infections, wound infections or septicemia. *Vibrio cholerae*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus* are considered as the most clinically relevant human pathogens within the genus *Vibrio* (Daniels and Shafaie, 2000). The latter species is widely disseminated in estuarine, marine, and coastal so surroundings and the leading cause of human intestinal infections after consumption of raw and undercooked seafood (Letchumanan et al., 2014). *V. cholerae* and *V. vulnificus* are also part of the microbial community in coastal or estuarine aquatic ecosystems with moderate salinities (Thompson and Polz, 2006).

V. cholerae is a well-known human pathogen consisting of more than 200 serogroups (Kaper et al., 1995; Lutz et al., 2013). Toxigenic *V. cholerae* of the O1 or O139 serogroup are the causative agents of cholera, an endemic disease in many Asian and African countries with symptoms of severe watery diarrhea, vomiting, and dehydration. All other serogroups designated as *V. cholerae* non-O1/non-O139 have also been linked to sporadically occurring human infections ranging from extraintestinal wound or ear infections (Huhulescu et al., 2007) to relatively mild or sometimes severe gastroenteritis (Tobin-D'Angelo et al., 2008), whereby smaller diarrheal outbreaks were also reported (Luo et al., 2013). Additionally, rarely occurring bacteremia has been described with mortality rates up to 61.5% (Petsaris et al., 2010). Since 2000, around 40 cases of *V. cholerae* non-O1/non-O139 infections in the United States have been reported to the CDC annually[1]. *V. cholerae* infections contracted in Germany were mainly ear or wound infections caused by non-toxigenic non-O1/non-O139 strains that were acquired through contact to seawater (Huehn et al., 2014). Due to the rare occurrence of *V. cholerae* non-O1/non-O139 infections, there are no official recommendations on antibiotic therapy (Petsaris et al., 2010). However, in case of bacteremia an early administration of antibiotic therapy can prevent a fatal outcome. Several case studies on *V. cholerae* non-O1/non-O139 bacteremia and wound infection exist, where fluoroquinolones and third-generation cephalosporins have been used (Huhulescu et al., 2007; Petsaris et al., 2010). But also treatment with ampicillin or last-line carbapenems has been described (Feghali and Adib, 2011; Lu et al., 2014).

V. vulnificus is known as a highly virulent pathogen. Although infections occur only sporadically, they can rapidly progress to septicemia, especially in persons with predisposing risk factors (e.g., immunocompromising conditions or chronic liver diseases resulting in elevated serum iron levels; Oliver, 2006). Foodborne infections can either result in a relatively mild gastroenteritis or in primary septicemia with mortality rates of 61% (Shapiro et al., 1998; Oliver, 2006). A second infection route for *V. vulnificus* is through open wounds exposed to seawater. Due to the high multiplication rate of the pathogen, wound infections may quickly progress to necrotizing fasciitis, which often makes surgical debridement or amputation necessary (Daniels and Shafaie, 2000). Delayed treatment promotes progression to secondary septicemia with mortality rates about seventeen per cent (Shapiro et al., 1998; Daniels and Shafaie, 2000). Surgical interventions should be considered early to prevent a fatal outcome as poor blood perfusion in necrotic tissue can impede the achievement of effective concentrations of antimicrobial agents (Chen et al., 2012). However, to avoid septicemia and a further distribution of the pathogen additional antibiotic therapy is indispensable and should be administered as early as possible. Due to the fast progression of *V. vulnificus* infections, the presence of antimicrobial resistance preventing an effective therapy can be fatal for the patient. A combination of a tetracycline with a third-generation cephalosporin or single-agent therapy with fluoroquinolones is recommended by the CDC[2], while trimethoprim-sulfamethoxazole in combination with an aminoglycoside is proposed for the treatment of pregnant women and children.

In the U.S., 95% of all seafood-related deaths can be attributed to *V. vulnificus*, whereas infections in Germany were almost exclusively wound infections occurring after contact to seawater (Oliver, 2006; Huehn et al., 2014). So far, *Vibrio* infections in Germany occur only sporadically but incidences peaked after extreme heatwaves (Huehn et al., 2014). Due to impacts of climate change, a rise in the occurrence of *V. vulnificus* and *V. cholerae* is predicted for European waters (Baker-Austin et al., 2012). Changing demography is expected to further contribute to higher incidences of *Vibrio* infections (Baker-Austin et al., 2012). In view of these forecasts and the potential severity of infections, an investigation on antimicrobial susceptibility of *Vibrio* spp. is demanded to provide guidance for medical intervention, but also for epidemiological purposes. For this reason, our study aimed to assess antimicrobial resistance prevalence among *V. vulnificus* and *V. cholerae* non-O1/non-O139 posing a public health risk for the population. Environmental isolates were obtained from German coastal and estuarine waters of the open North Sea and the intracontinental Baltic Sea. In addition, we also included isolates from clinical sources and retail seafood for comparison and to give a more comprehensive overview of antimicrobial resistance patterns of these two species in Germany. To our knowledge, this is the first study examining antimicrobial susceptibility of *V. vulnificus* and *V. cholerae* non-O1/non-O139 in Northern Europe on a large scale.

MATERIALS AND METHODS

Bacterial Strains

The strains used in this study are summarized in **Table 1** and listed in detail in Supplementary Tables S1, S2. Antimicrobial susceptibilities were determined for a total of 325 bacterial

[1] http://www.cdc.gov/cholera/non-01-0139-infections.html

[2] http://emergency.cdc.gov/disasters/vibriovulnificus.asp

TABLE 1 | Origin and source of *V. cholerae* non-O1/non-O139 (*n* = 184) and *V. vulnificus* (*n* = 141) strains included in the study.

Origin	Geographical origin	Source	Source code	No. of strains
V. cholerae				
Environmental (E) (2009–2014; *n* = 131)	Baltic Sea (BS) (*n* = 79)	Seawater (sw)	E-BS-sw	54
		Sediment (sd)	E-BS-sd	4
		Seawater/sediment (sw/sd)	E-BS-sw/sd	21
	North Sea (NS) (*n* = 52)	Bivalve mollusks (bm)	E-NS-bm	26
		Seawater (sw)	E-NS-sw	12
		Seawater/sediment (sw/sd)	E-NS-sw/sd	14
Clinical (C) (1995–2012; *n* = 18)	Travel-associated (ta) (*n* = 7)	Extraintestinal (ext)	C-ta-ext	1
		Intestinal (int)	C-ta-int	6
	Germany/Austria (G/A) (*n* = 11)	Extraintestinal (ext)	C-G/A-ext	9
		Intestinal (int)	C-G/A-int	2
Retail (R) (2008–2014; *n* = 35)	Germany (G) (*n* = 35)	Bivalve mollusks (bm)	R-G-bm	2
		Crustacean (cr)	R-G-cr	26
		Fish (fi)	R-G-fi	7
V. vulnificus				
Environmental (E) (2004–2012; *n* = 122)	Baltic Sea (BS) (*n* = 70)	Seawater (sw)	E-BS-sw	46
		Sediment (sd)	E-BS-sd	24
	North Sea (NS) (*n* = 52)	Seawater (sw)	E-NS-sw	29
		Sediment (sd)	E-NS-sd	21
		Bivalve mollusks (bm)	E-NS-bm	2
Clinical (C) (1994–2011; *n* = 19)	Denmark (D)	Extraintestinal (ext)	C-D-ext	14
	Germany (G)	Extraintestinal (ext)	C-G-ext	5

strains, including 141 isolates of *V. vulnificus* (19 clinical, 122 environmental) and 184 isolates of *V. cholerae* non-O1/non-O139 (18 clinical, 131 environmental, 35 retail). The majority of environmental strains were isolated by health authorities during the German research programs KLIWAS[3] and VibrioNet[4] between 2004 and 2014. Water and sediment samples were mostly collected at bathing sites along the Baltic Sea and North Sea coastline as well as within the estuaries of the rivers Ems and Weser (Böer et al., 2012). Environmental isolates from bivalve mollusks were obtained from coastal areas of the North Sea. Isolates from retail samples were collected by health authorities of Germany and sent to the National Reference Laboratory for Monitoring Bacteriological Contamination of Bivalve Mollusks of the Federal Institute for Risk Assessment (BfR), Germany. Clinical *V. vulnificus* and *V. cholerae* non-O1/non-O139 isolates were characterized in previous studies (Bier et al., 2013; Schirmeister et al., 2014).

DNA Extraction

DNA extraction was performed with two methods that are equally applicable for *Vibrio* species. Genomic DNA of *V. vulnificus* isolates was extracted from 1 ml of an overnight culture using the RTP Bacteria DNA Kit according to the manufacturer's protocol (STRATEC Biomedical AG, Birkenfeld, Germany).

Genomic DNA of *V. cholerae* strains was extracted using a boiling method: 1.5 ml of an overnight culture were centrifuged at 14,000 g for 4 min. The cell pellet was suspended in 300 µl TE buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 8), boiled for 10 min at 95°C, and subsequently cooled on ice. After centrifugation at 14,000 g for 2 min, a 200 µl aliquot of the supernatant was transferred to a new sterile tube. DNA preparations were stored at −20°C.

[3]http://www.kliwas.de/KLIWAS/EN/03_ResearchTasks/03_vh3/04_304/304_node.html

[4]http://www.vibrionet.de/

Species Confirmation

Species confirmation of all *V. vulnificus* and *V. cholerae* strains was carried out by species-specific *toxR* PCR amplification as previously described (Bauer and Rørvik, 2007) and in parallel by MALDI-TOF MS analysis. MALDI-TOF MS analysis was performed using a Microflex LT system mass spectrometer (Bruker Daltonik, Bremen, Germany) following the manufacturer's settings. MALDI spectra were obtained by the direct transfer method according to the manufacturer's protocol as previously described (Schirmeister et al., 2014).

Characterization of *V. cholerae* Isolates

V. cholerae isolates were characterized and subtyped via multiplex PCR targeting *rfb* sequences specific for O1 and O139 serogroups, *toxR,* and *ctxA*. PCR amplification was performed in a final volume of 25 µl with 1x PCR buffer (3 mM $MgCl_2$), 0.2 mM of each deoxynucleoside triphosphate, 0.5 µM of O1 *rfb* primers, 0.125 µM of O139 *rfb, toxR,* and *ctxA* primers, 1.5 U DreamTaq DNA polymerase (Thermo Fisher Scientific Biosciences GmbH, St. Leon-Rot, Germany), and 2 µl of genomic DNA. After an initial denaturation step at 94°C for 4 min, the cycling conditions

were the following: 30 cycles at 94°C for 30 s, 59°C for 30 s, 72°C for 30 s, followed by a final extension step at 72°C for 5 min.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility to the following 13 antimicrobial agents was determined by broth microdilution according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012a) using custom-defined microtiter plates (EUMVS2, Trek Diagnostic Systems, East Grinstead, United Kingdom): ampicillin, ceftazidime, cefotaxime, chloramphenicol, ciprofloxacin, colistin, florfenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, tetracycline, and trimethoprim. Test ranges are shown in Supplementary Table S3. Additionally, all isolates were tested for their susceptibility to amoxicillin-clavulanic acid (20/10 μg), cefepime (30 μg), imipenem (10 μg), levofloxacin (5 μg), meropenem (10 μg), and sulfamethoxazole-trimethoprim (23.75/1.25 μg) by the disk diffusion method, according to the guidelines of the CLSI using commercially available disks (Oxoid GmbH, Wesel, Germany; CLSI, 2012b).

Strains showing non-susceptibility to imipenem (zone diameter ≤ 19) were tested against an additional panel of β-lactams by broth microdilution (imipenem, ertapenem, cefepime, cefoxitin, temocillin; EUVSEC2, Trek Diagnostic Systems) or disk diffusion (aztreonam, 30 μg).

Following the guidelines of the CLSI, tests were performed with Mueller-Hinton agar and cation-adjusted Mueller-Hinton broth without supplementation of additional sodium chloride (CLSI, 2010a). *Escherichia coli* ATCC 25922 was used for quality assurance. Minimal inhibitory concentration (MIC) values and inhibition zone diameters of all strains are listed in Supplementary Tables S1, S2. Results were interpreted using the criteria summarized in Supplementary Table S3. In general, results were interpreted according to CLSI clinical breakpoints specific for *Vibrio* spp. (CLSI, 2010a), which derived from breakpoints for *Enterobacteriaceae* (CLSI, 2010b). In cases where CLSI breakpoints for *Vibrio* spp. were obsolete or not available, the latest CLSI breakpoints for *Enterobacteriaceae* were used: document M100-S25 for aztreonam, cefepime, ertapenem, gentamicin, kanamycin, imipenem, meropenem, nalidixic acid, and trimethoprim (CLSI, 2015); document Vet01-S2 for florfenicol (CLSI, 2013). Other interpretive criteria were used for colistin (EUCAST clinical breakpoints for *Enterobacteriaceae*[5] ; EUCAST, 2015), temocillin (BSAC interpretive criteria for systemic infections; Andrews, 2009), and streptomycin (based on different studies of *Vibrio* spp. and *E. coli*; National Food Institute, 2013; Shaw et al., 2014), as no CLSI breakpoints were available.

Molecular Investigation of Resistance Determinants

PCR amplification was conducted to detect specific antimicrobial resistance determinants depending on the phenotype found. Non-susceptible isolates were screened for genes mediating resistance to streptomycin (*aadA1*, *aadA2,* and *strA/B*) and β-lactams (bla_{PSE-1}, $bla_{OXA-1-like}$, $bla_{TEM-1-like}$) that are widespread in *Enterobacteriaceae* and other Gram-negative bacteria. Specifically imipenem-resistant strains (zone diameter ≤ 19) were tested for the presence of several carbapenemase and AmpC β-lactamase encoding genes. Presence of class 1 integrons was investigated by PCR amplification of the corresponding integrase gene *intI1* in all β-lactam and streptomycin non-susceptible strains.

Standard PCR reactions were performed using a Mastercycler EP gradient (Eppendorf, Hamburg, Germany) in a volume of 25 μl with 1x PCR buffer (2 mM $MgCl_2$), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.2 μM of each primer, 1.5 U DreamTaq DNA polymerase, and 2 μl of genomic DNA. After an initial denaturation step at 94°C for 4 min, the cycling conditions were the following: 30 cycles of denaturation at 94°C for 30 s, primer annealing for 30 s, and extension at 72 °C for 1 min per kb, followed by a final extension step at 72°C for 10 min.

All primer pairs, target genes, corresponding annealing temperatures, and amplicon sizes are listed in Supplementary Table S4. Enterobacterial strains carrying class 1 integrons and investigated resistance determinants (*aadA1*, *aadA2,* bla_{PSE-1}, bla_{OXA-1}, bla_{TEM-1}, bla_{NDM-1}, bla_{IMP-1}, bla_{VIM-2}, bla_{KPC-3}, bla_{OXA-48}, bla_{ACC-1}, bla_{CMY-1}, bla_{CMY-2}, bla_{DHA-1}, bla_{ACT-1}, bla_{FOX-1}, *strA,* and *strB*), as well as one *V. cholerae* non-O1/non-O139 isolate carrying a class 1 integron with the *aadA1* gene were used as positive controls. Susceptible *V. cholerae* and *V. vulnificus* strains were included as negative controls.

All streptomycin resistant *V. cholerae* (VN-3469, VN-5095, VN-10191, and VN-10192) and *V. vulnificus* (VN-0098, VN-0100, VN-0125, VN-0129) isolates were further examined for mutations in the *rpsL* gene encoding ribosomal protein S12. Streptomycin susceptible (*V. cholerae*: VN-0298, VN-2997, VN-3955, VN-4226, and *V. vulnificus*: VN-0096, VN-0274, VN-3368) and intermediate resistant strains (*V. cholerae*: VN-3944, VN-4261, and *V. vulnificus*: VN-3418, VN-3981, VN-10121) were included as controls. For specific amplification and sequencing of the whole *rpsL* gene in *V. cholerae* and *V. vulnificus*, two primer pairs Vc-rpsL-F/Vc-rpsL-R and Vv-rpsL-F/Vv-rpsL-R were designed based on published genome sequences (*V. cholerae* strains NIH41, N16961, O395, and *V. vulnificus* strains CMCP6, YJ016, MO6-24/O). Purification of PCR products was performed using the MSB® Spin PCRapace Kit (STRATEC Biomedical AG, Berlin, Germany). Sequencing was conducted on both strands through sequencing service (Eurofins MWG GmbH, Ebersberg, Germany). Electropherograms were assembled and trimmed using SeqMan Pro (v12; DNASTAR Lasergene, Madison, Wisconsin). Sequences were analyzed and compared to the sequences of reference and control strains using Accelrys Gene (v2.5, Accelrys Inc., San Diego, California).

Test for Carbapenemase Activity: Carba NP Test II/Blue-Carba Test

Strains non-susceptible to imipenem (zone diameter ≤ 19) were grown overnight at 37°C on chromID™ CARBA (bioMérieux, Nürtingen, Germany). Bacterial colonies were subsequently tested for carbapenemase activity with the improved Carba NP test II (Dortet et al., 2014) using 12 g/L imipenem/cilastatin

[5]http://www.eucast.org

(Zienam®, MSD SHARP, and DOHME GMBH, Haar, Germany) and two calibrated loops (10 µl) as bacterial inoculum to increase enzyme quantity. The Blue-CARBA test was performed as described (Pires et al., 2013) and in addition analogously to the Carba NP test II with two loops of bacterial colonies in 200 µl of the test solution in microcentrifuge tubes and supplementation of tazobactam or EDTA to inhibit class A or metallo-carbapenemases, respectively. Two *E. coli* strains positive for NDM-1 and KPC-2, respectively, as well as a KPC-3-positive *Klebsiella pneumoniae* strain served as positive controls.

Statistical Analyses

Descriptive statistics were used to analyze resistance prevalence to different antimicrobial agents (**Table 2**). Chi-square test for independence was applied with 2 × 2 contingency tables to test if observed differences displayed in **Table 3** were statistically significant (P-values ≤ 0.05). MIC_{50} and MIC_{90} were defined as the concentration at which growth of 50 and 90% of the isolates was inhibited, respectively.

RESULTS

Antimicrobial Susceptibility of Clinical and Environmental *V. vulnificus* Isolates

Fifty-seven per cent of all examined *V. vulnificus* isolates showed susceptibility to all antimicrobial agents tested (with the exception of colistin). All 141 *V. vulnificus* isolates, regardless of their origin were susceptible to quinolones, fluoroquinolones, phenicols, tetracyclines, folate pathway inhibitors, aminopenicillins with or without β-lactamase inhibitors, carbapenems, and third- and fourth- generation cephalosporins. The clinically relevant agents cefotaxime, ceftazidime, and tetracycline were among the most effective antimicrobial agents *in vitro* as they showed MIC_{90} values identical to the lowest concentration tested (**Table 4**). Non-susceptibility was exclusively observed toward aminoglycosides with 40 and 3% of all strains showing intermediate resistance (MIC 32 mg/L) and resistance (MIC 64 mg/L) to streptomycin, respectively. One clinical isolate was intermediate resistant to kanamycin (MIC 32 mg/L) while all strains were susceptible to gentamicin. The percentage of non-susceptible strains with respect to different origins is shown in **Table 3**. No significant difference was observed between clinical ($n = 19$) and environmental ($n = 122$) isolates, nor was there a significant difference between isolates from the Baltic Sea and the North Sea ($p > 0.05$, χ^2). None of the examined gene determinants encoding streptomycin resistance (*aadA1, aadA2*, *strA/B*), nor class 1 integrons were detected in streptomycin non-susceptible *V. vulnificus* isolates. Sequence analysis of the *rpsL* gene revealed that all four streptomycin resistant *V. vulnificus* isolates (VN-0098, VN-0100, VN-0125, VN-0129) as well as two susceptible (VN-0274, VN-3368) and two intermediate resistant isolates (VN-3918, VN-10121) carried one silent point mutation A-291-T compared to the three reference strains (CMCP6, YJ016, and MO6-24/O). An additional silent mutation C-351-T within the *rpsL* gene was observed in strain VN-0100.

Antimicrobial Susceptibility of *V. cholerae* non-O1/non-O139 Isolated from Clinical, Environmental and Seafood Samples

All 184 isolates investigated in this study were confirmed to be non-toxigenic *V. cholerae* non-O1/non-O139 isolates. The

TABLE 3 | Overall resistance occurrence in *V. cholerae* and *V. vulnificus* isolates with respect to different origins.

	Retail	Clinical	Environmental	North Sea	Baltic Sea
V. cholerae	($n = 35$)	($n = 18$)	($n = 131$)	($n = 52$)	($n = 79$)
Strains susceptible to all antimicrobial agents	27 (77%)	10 (56%)	86 (66%)	28 (54%)	58 (73%)
Non-susceptible strains	8 (23%)	8 (44%)	45 (34%)	24(46%)	21 (27%)
V. vulnificus		($n = 19$)	($n = 122$)	($n = 52$)	($n = 70$)
Strains susceptible to all antimicrobial agents		8 (42%)	72 (59%)	28 (54%)	44 (63%)
Non-susceptible strains		11 (58%)	50 (41 %)	24 (46 %)	26 (37 %)

TABLE 2 | Susceptibility vs. resistance occurrence (%) found among *V. cholerae* non-O1/non-O139 isolates from different origins[a].

Antimicrobial agent	Total ($n = 184$)			Retail ($n = 35$)			Clinical ($n = 18$)			Environmental ($n = 131$)			North Sea ($n = 52$)			Baltic Sea ($n = 79$)		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Amoxicillin/clavulanic acid	98	2	0	100	0	0	100	0	0	97	3	0	94	6	0	99	1	0
Ampicillin	89	0	11	89	0	11	83	0	17	90	0	10	85	0	15	94	0	6
Imipenem	97	1	2	100	0	0	100	0	0	95	2	3	94	0	6	96	3	1
Meropenem	98	2	<1	100	0	0	100	0	0	97	2	1	94	4	2	99	1	0
Nalidixic acid	99	0	1	100	0	0	89	0	11	100	0	0	100	0	0	100	0	0
Streptomycin	78	20	2	86	11	3	83	17	0	75	23	2	65	29	6	81	19	0
Trimethoprim	99	0	1	97	0	3	100	0	0	100	0	0	100	0	0	100	0	0

S, susceptible; I, intermediate resistant; R, resistant.

[a]*All strains were susceptible to ceftazidime, chloramphenicol, ciprofloxacin, cefotaxime, cefepime, florfenicol, gentamicin, kanamycin, levofloxacin, trimethoprim/sulfamethoxazole and tetracycline.*

TABLE 4 | Antimicrobial MIC distributions for the *V. cholerae* and *V. vulnificus* isolates tested.

Antimicrobial agent	Test range (mg/L)	Breakpoints[a] (mg/L)			MIC (mg/L) distribution for *V. vulnificus* ($n = 141$)			MIC (mg/L) distribution for *V. cholerae* ($n = 184$)		
		S	*I*	*R*	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range
Ampicillin	0.5–32	≤8	16	≥32	1	2	≤0.5–2	2	8	≤0.5–>32
Cefotaxime	0.06–4	≤1	2	≥4	≤0.06	≤0.06	≤0.06–0.12	≤0.06	≤0.06	≤0.06–0.12
Ceftazidime	0.25–16	≤4	8	≥16	≤0.25	≤0.25	≤0.25–0.5	≤0.25	≤0.25	≤0.25–0.5
Chloramphenicol	2–64	≤8	16	≥32	≤2	≤2	≤2	≤2	≤2	≤2
Ciprofloxacin	0.008–8	≤1	2	≥4	0.015	0.03	≤0.008–0.06	≤0.008	≤0.008	≤0.008–0.5
Florfenicol	2–64	≤4	8	≥16	≤2	≤2	≤2	≤2	≤2	≤2
Gentamicin	0.25–32	≤4	8	≥16	2	2	0.5–4	1	2	≤0.25–4
Kanamycin	4–128	≤16	32	≥64	8	16	≤4–32	≤4	8	≤4–16
Nalidixic acid	4–64	≤16		≥32	≤4	≤4	≤4–8	≤4	≤4	≤4–>64
Streptomycin	2–128	≤16	32	≥64	16	32	4–64	16	32	8–64
Tetracycline	1–64	≤4	8	≥16	≤1	≤1	≤1–2	≤1	≤1	≤1
Trimethoprim	0.5–32	≤8		≥16	1	1	≤0.5–4	≤0.5	1	≤0.5–>32

S, susceptible; I, intermediate resistant; R, resistant.

[a]*Criteria used for interpretation and corresponding references are given in Supplementary Table S3.*

majority of isolates (67%) were susceptible to all antimicrobial agents tested (with the exception of colistin). Eighteen per cent of the strains showed intermediate resistance to one or two antimicrobial agents (mostly to streptomycin) and the remaining strains (15%) showed full resistance to at least one antimicrobial agent. None of the *V. cholerae* isolates showed multidrug-resistance, defined as resistance to three or more classes of antimicrobial agents (Chen et al., 2010). Resistance profiles are shown in Supplementary Table S2, while resistance occurrence is given in **Table 2.**

As observed among *V. vulnificus*, all *V. cholerae* strains were susceptible to ciprofloxacin, chloramphenicol, florfenicol, cefotaxime, sulfamethoxazole-trimethoprim, levofloxacin, ceftazidime, cefepime, gentamicin, kanamycin, and tetracycline. Additionally, 98% of the isolates were susceptible to amoxicillin/clavulanic acid. The most effective clinically relevant agents *in vitro* were ciprofloxacin, cefotaxime, ceftazidime, and tetracycline as they showed MIC_{90} values identical to the lowest concentration tested (**Table 4**).

Similar to the *V. vulnificus* isolates, a small proportion of *V. cholerae* strains showed resistance to streptomycin (2%), while 20% of the strains were intermediate resistant. In contrast to *V. vulnificus,* the most frequent antimicrobial resistance found among all *V. cholerae* isolates was resistance to ampicillin (11%). Resistance to nalidixic acid and trimethoprim was rarely observed in two clinical isolates and in one isolate from seafood. Non-susceptibility to the carbapenems imipenem and meropenem was observed in 5 and 3% of the environmental isolates, respectively.

Clinical strains showed the highest percentage of non-susceptible strains (44%), followed by environmental strains (34%) and by strains isolated from retail seafood (23%) (**Table 3**). However, statistical analysis revealed that the observed differences to environmental isolates are not significant ($p > 0.05$, χ^2). Comparison between the geographical origin of environmental strains revealed that the percentages of strains non-susceptible to streptomycin, ampicillin, amoxicillin/clavulanic acid, meropenem, and imipenem were higher in the North Sea compared to the Baltic Sea (**Table 2**). The higher occurrence of non-susceptible *V. cholerae* strains in the North Sea (**Table 3**) was statistically significant ($\chi 2 = 5.327$, d.f. $= 1$, $p < 0.05$).

Analysis of MIC distributions revealed that susceptibilities of the two *Vibrio* species were rather similar (**Table 4**). Differences were exclusively observed for kanamycin and ciprofloxacin, where MIC_{90} values of *V. cholerae* were two and four times lower and in case of ampicillin four times higher than those for *V. vulnificus*.

Susceptibility to colistin was excluded from any statistical analysis and tables, since *V. vulnificus* and *V. cholerae* possess an intrinsic resistance to colistin, which is used for selective growth on cellobiose-polymyxin B-colistin agar (Massad and Oliver, 1987). However, five *V. cholerae* strains were highly susceptible to colistin (MICs ≤ 2 mg/L) and would therefore fail to grow on this selective agar.

Neither class 1 integrons, nor gene determinants encoding streptomycin resistance (*aadA1, aadA2*, *strA/B*) were detected among non-susceptible *V. cholerae* isolates. Sequence analysis of the *rpsL* gene revealed three silent point mutations C-198-T, A-251-T, and G-360-A in two streptomycin resistant *V. cholerae* isolates (VN-10191, VN-10192) as well as within a susceptible isolate (VN-4226). In addition, PCR amplifications to detect β-lactamase genes (bla_{PSE-1}, $bla_{OXA-1-like}$, $bla_{TEM-1-like}$) were negative in all tested *V. cholerae* isolates.

Examination of Carbapenem Non-susceptible *V. cholerae* Isolates

Among the 131 environmental *V. cholerae* non-O1/non-O139 isolates analyzed, resistance to the carbapenem imipenem (zone diameter ≤ 19) was observed in four strains (VN-2808, VN-2825,

VN-2923, and VN-2997). These strains additionally showed resistance to ampicillin, intermediate resistance to amoxicillin-clavulanic acid, as well as intermediate or full resistance to meropenem (**Table 5**). In contrast, they were susceptible to the third- and fourth-generation cephalosporins ceftazidime, cefotaxime, and cefepime. Further characterization revealed resistance to aztreonam and ertapenem, while the strains were susceptible to temocillin and intermediate resistant to cefoxitin. The four strains grew on chromID™ CARBA agar, while growth was inhibited on chromID™ OXA-48 (bioMérieux GmbH, Nürtingen, Germany), indicating the expression of carbapenem-hydrolyzing enzymes other than OXA-48 type carbapenemases. To further investigate the presence of carbapenemases, Blue-CARBA and Carba NP II tests were conducted on these strains. Imipenem-hydrolyzing activity was detected in intact cells and crude cell extracts of each of the four strains. This activity was inhibited by tazobactam but not by EDTA, suggesting the presence of Ambler class A carbapenemases rather than class B metallo-carbapenemases or class D OXA-carbapenemases (Dortet et al., 2012).

PCRs to identify genes encoding Ambler class A carbapenemases were performed. However, no products with expected sizes were observed using primers for detection of NMC-A, SME 1-3, IMI 1-3, or KPC 1-5. In the case of NMC-A, SME, and IMI, a general failure of PCR amplification cannot be excluded as no positive control strains were available.

In addition, the strains were negative for PCR-amplification of specific genes encoding Ambler class B metallo-carbapenemases (VIM 1-2, IMP, NDM-1), class D carbapenemase (OXA-48), and AmpC-β-lactamases (MOX 1-2, CMY 1-11, LAT 1-4, BIL-1, DHA 1-2, ACC, MIR-1T, ACT-1, FOX 1-5b) as well as of class 1 integrons.

DISCUSSION

Prevalence of Antimicrobial Resistance in *V. vulnificus* and *V. cholerae* non-O1/non-O139

In this study, *V. vulnificus* isolates from German coastal waters as well as of clinical origin were susceptible to quinolones, fluoroquinolones, phenicols, tetracyclines, folate pathway inhibitors, aminopenicillins with or without β-lactamase inhibitors, carbapenems, and third- and fourth-generation

TABLE 5 | β-lactam MIC values and inhibition zone diameters found in the putative carbapenemase-producers and in carbapenem susceptible isolates selected as negative controls.

	Antimicrobial agent				Strain ID of *V. cholerae* isolates						
		Breakpoints[d]			Putative carbapenemase-producers				Carbapenem susceptible controls		
		S	I	R	VN-02997	VN-02825	VN-02923	VN-02808	VN-10145	VN-00301	VN-00161
MIC values (mg/L)[a]	Ampicillin	≤8	16	≥32	>32	>32	>32	>32	2	2	2
	Cefepime	≤8	16	≥32	0.5	0.25	0.5	0.25	0.12	≤0.06	≤0.06
	Cefoxitin	≤8	16	≥32	16	16	16	16	4	4	4
	Cefotaxime	≤1	2	≥4	0.12	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06
	Ceftazidime	≤4	8	≥16	0.5	0.5	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
	Ertapenem	≤0.5	1	≥2	2	>2	>2	2	0.12	0.03	0.06
	Imipenem	≤1	2	≥4	16	>16	>16	16	1	0.5	1
	Temocillin	≤8	-	>8	4	4	4	4	4	1	1
Inhibition zone diameter (mm)[b]	Amoxicillin/ clavulanic acid	≥18	14–17	≤13	14	13.5	14	15	23	28	18
	Aztreonam	≥21	18–20	≤17	12	15	17	16	NA	NA	29
	Cefepime	≥25	19–24	≤18	28	26	28	28	34	40	30
	Ceftazidime	≥21	18–20	≤17	28	26	28	28	NA	NA	31
	Imipenem	≥23	20–22	≤19	14	15	16	15	30	36	26
	Meropenem	≥23	20–22	≤19	20	20	20	19	34	36	28
β-lactam resistance phenotype[c]					(AMC)-AMP-ATM-ETP-(FOX)-IPM-(MEM)	(AMC)-AMP-ATM-ETP-(FOX)-IPM-(MEM)	(AMC)-AMP-ATM-ETP-(FOX)-IPM-(MEM)	(AMC)-AMP-ATM-ETP-(FOX)-IPM-MEM	–	–	–

MIC, minimal inhibitory concentration; S, susceptible; I, intermediate resistant; R, resistant; NA, not assessed; AMC, amoxicillin/clavulanic acid; AMP, ampicillin; ATM, aztreonam; ETP, ertapenem; FOX, cefoxitin; IPM, imipenem; MEM, meropenem.

[a] *MIC values obtained by broth microdilution.*

[b] *Inhibition zone diameter obtained by disk diffusion.*

[c] *Results against non-β-lactams are not displayed; intermediate resistance is shown in brackets.*

[d] *Criteria used for interpretation with corresponding references are given in Supplementary Table S3.*

cephalosporins. Non-susceptibility was exclusively observed toward aminoglycosides; predominantly streptomycin and sporadically kanamycin. Similar observations were made by Han et al. (2007), who reported total susceptibility with comparable MIC_{90} values to chloramphenicol, ampicillin, ceftazidime, cefotaxime, ciprofloxacin, gentamicin, and tetracycline among *V. vulnificus* isolates from oysters of the Louisiana Gulf coast, USA (Han et al., 2007). In a recent study of *V. vulnificus* isolates from the Chesapeake Bay, USA (Shaw et al., 2014), the highest percentage of resistance was also observed against streptomycin. However, a large percentage of intermediate resistant strains to chloramphenicol (78%) and sporadically non-susceptibility to β-lactams was also reported in that study (Shaw et al., 2014). Compared to our study, higher percentages of non-susceptible strains to ampicillin, tetracycline, nalidixic acid, trimethoprim, and especially to the aminoglycosides streptomycin and gentamicin were observed in a study of 151 *V. vulnificus* isolates from South Carolina, USA, while resistance to chloramphenicol and meropenem was under one per cent (Baker-Austin et al., 2009).

Among *V. cholerae* non-O1/non-O139 isolated from clinical, environmental, and seafood samples in Germany, no multidrug-resistance was observed and the majority (67%) of isolates were susceptible to all antimicrobial agents tested. Full resistance was most frequently found toward ampicillin (11%) and streptomycin (2%). In addition, a considerable proportion of isolates showed intermediate resistance to streptomycin (20%). Resistance to nalidixic acid and trimethoprim was only sporadically found in isolates from clinical and seafood samples, respectively. While numerous studies on antimicrobial resistance of toxigenic *V. cholerae* O1, O139 strains have been published, data on *V. cholerae* non-O1/non-O139 are less frequent. A recent study showed similar antimicrobial resistance patterns among environmental *V. cholerae* non-O1/non-O139 isolates from the Chesapeake Bay, USA (Ceccarelli et al., 2015). No multidrug-resistant isolates were detected and resistance to β-lactams was found in some isolates. In one large-scale study from India on antimicrobial susceptibility of *V. cholerae* non-O1/non-O139 isolates, the highest percentage of resistant strains was also seen for ampicillin (88%) and streptomycin (85%) though with a considerably higher frequency (Kumar et al., 2009). In contrast to our study, Kumar et al. (2009) reported a high prevalence of multidrug resistance and only a small percentage of strains were susceptible to all ten antimicrobial agents tested (12%).

Several PCR analyses were performed to reveal the underlying molecular mechanisms responsible for the observed non-susceptibility to streptomycin, ampicillin, and imipenem.

Resistance to streptomycin is often mediated by enzymatic inactivation through adenylation by aminoglycoside (3″) adenyltransferases (*aadA* genes) or through phosphorylation by aminoglycoside phosphotransferases (*strA*/*strB* genes; Shaw et al., 1993; Tsai et al., 2014). However, none of the *Vibrio* isolates was positive for amplification of *aadA1*, *aadA2,* or *strA/B* genes that are commonly found in *Enterobacteriaceae* and that have already been identified in *V. cholerae* (Hochhut et al., 2001; Sá et al., 2010; Yu et al., 2012). Ribosomal alterations resulting from mutations in the *rpsL* gene encoding ribosomal protein S12 can be another cause of streptomycin resistance (Shaw et al., 1993; Tsai et al., 2014). However, amino acid sequences of ribosomal protein S12 were identical in all investigated strains, irrespective of the streptomycin resistance phenotype. Observed single nucleotide polymorphisms within the *rpsL* gene of some resistant as well as of some susceptible isolates were silent mutations. This indicates that other streptomycin inactivating enzymes or other resistance mechanisms, such as decreased permeability or other ribosomal alterations (e.g., mutations in the *rrs* gene encoding the 16S ribosomal RNA) may be responsible for the observed phenotype (Shaw et al., 1993; Tsai et al., 2014).

Likewise, all β-lactam non-susceptible *Vibrio* isolates were negative for amplification of bla_{PSE-1}, $bla_{OXA-1-like}$, and $bla_{TEM-1-like}$ genes encoding common β-lactamases in *Enterobacteriaceae* and other Gram-negative bacteria. This suggests that resistance may be encoded by other β-lactamase genes which are known to show a high diversity in Gram-negative bacteria. β-lactam resistance may also be mediated by other mechanisms such as reduced permeability, increased efflux, or target alterations (e.g., reduced affinity or increased amount of penicillin-binding protein (PBP); Foster, 1983).

Carbapenemase Producing *V. cholerae*

Antimicrobial susceptibility patterns as well as growth patterns on different selective media indicated the presence of a β-lactamase with carbapenem hydrolyzing activity in four environmental *V. cholerae* non-O1/non-O139 isolates from the Baltic Sea and the North Sea. The expression of carbapenemases was confirmed by positive Blue-CARBA and positive Carba NP II tests, which specifically detect imipenem hydrolyzing activity (Dortet et al., 2012; Pires et al., 2013). Inhibition of carbapenemase activity by tazobactam but not by EDTA suggested the presence of Ambler class A carbapenemases rather than class B metallo-carbapenemases or class D OXA-carbapenemases (Dortet et al., 2012). However, we found no evidence for the presence of specific genes encoding Ambler class A carbapenemases.

The four strains showed an exceptional resistance profile: They were non-susceptible to aminopenicillins, carbapenems, cefoxitin, and aztreonam and were only slightly inhibited by the β-lactamase inhibitor clavulanic acid. However, they were fully susceptible to third- and fourth-generation cephalosporins as well as to temocillin. The observed resistance to aminopenicillins coupled with susceptibility to extended spectrum cephalosporins may indicate the presence of an OXA-type carbapenemase (Ambler class D) reviewed by Walther-Rasmussen and Høiby (2006). However, with some exceptions, e.g., OXA-23, these enzymes are generally not inhibited by tazobactam, as seen for the four strains in the Carba NP II and Blue-CARBA tests and generally don't mediate resistance to aztreonam (Walther-Rasmussen and Høiby, 2006).

So far, the identity of the enzyme responsible for imipenem hydrolyzing activity in the four strains remains unclear, as none of the examined carbapenemase and AmpC-β-lactamase genes could be detected. It cannot be excluded that in addition to a carbapenem hydrolyzing enzyme other resistance mechanisms,

such as reduced affinity of PBPs, porin alterations resulting in decreased membrane permeability or active efflux systems, either alone or in combination may also contribute to the observed phenotype (Walther-Rasmussen and Høiby, 2006; Queenan and Bush, 2007; Nordmann et al., 2012). Carbapenem-resistant *V. cholera*e have already been reported in other studies. NDM-1 carbapenemase was detected in a *V. cholerae* O1 El Tor Ogawa strain isolated from a 2-year old child (Mandal et al., 2012) as well as in *V. cholerae* isolated from seepage water in India (Walsh et al., 2011). Furthermore, increasing resistance to carbapenems was recently described among *V. cholerae* O1 or O139 strains isolated between 1986 and 2012 in southwest China (Gu et al., 2014).

CONCLUDING REMARKS

In this study, antimicrobial agents recommended as first choice agents for the treatment of *V. vulnificus* and *V. cholerae* non-O1/non-O139 infections such as fluoroquinolones, tetracyclines, and extended spectrum cephalosporins were found to be effective *in vitro* against both species. However, the administration of aminopenicillins, carbapenems, or aminoglycosides for treatment of *V. cholerae* non-O1/non-O139 infections, which has been reported in few studies (Daniels and Shafaie, 2000; Feghali and Adib, 2011; Lu et al., 2014) should be considered carefully, as non-susceptibility was most frequently observed against ampicillin and streptomycin and sporadically to carbapenems. For *V. vulnificus*, non-susceptibility was exclusively observed to the aminoglycosides streptomycin and kanamycin. However, gentamicin was effective against both species and could be an aminoglycoside of choice for the treatment of children and pregnant woman, as was also suggested by others (Shaw et al., 2014).

We report the detection of carbapenemase producing *V. cholerae* from different locations of the German coast line (North Sea and Baltic Sea) representing an environmental reservoir of carbapenem resistance. An entry into the sea resulting from sanitary pollution or human recreational activities cannot be excluded, but seems not likely as vibrios are indigenous bacteria of the marine environment and not intestinal commensals of humans or terrestrial animals. The strains displaying carbapenemase activity showed resistance to an unusual pattern of β-lactams. Therefore, characterization of the underlying genetic background is necessary to identify the responsible genes e.g., using whole genome sequencing as the most promising approach. Further investigations on the mobility as well as on the location of encoding genes are also needed, since location on mobile genetic elements would imply a higher risk for interspecies spread. Carbapenems are last line antimicrobial agents for treatment of multidrug-resistant Gram-negative bacteria and are of high therapeutic value (Nordmann et al., 2012). The occurrence of putative carbapenemase producing *V. cholerae* in the North and Baltic Sea is therefore of great concern and highlights the need for systematic monitoring of antimicrobial susceptibility in potentially pathogenic *Vibrio* spp. in Europe.

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SUPPLEMENTARY MATERIAL

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The First Temporal and Spatial Assessment of *Vibrio* Diversity of the Surrounding Seawater of Coral Reefs in Ishigaki, Japan

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Coral reefs perform a major role in regulating marine biodiversity and serve as hotspot for highly dynamic and diverse microbiomes as holobionts. Corals around Ishigaki, however, are at risk due to tremendous stressors including elevation of seawater temperature, eutrophication and so on. However, no information is currently available on how *Vibrio* diversity fluctuates spatially and temporally due to environmental determinants in Ishigaki coral reef ecosystems. The aim of this study is to elucidate spatiotemporal *Vibrio* diversity dynamic at both community and population levels and to assess the environmental drivers correlated to *Vibrio* abundance and diversity. The *Vibrio* community identified based on *pyrH* gene phylogeny of 685 isolates from seawater directly connecting to Ishigaki coral holobionts consisted of 22 known and 12 potential novel *Vibrionaceae* species. The most prominent species were *V. hyugaensis*, *V. owensii* and *V. harveyi* followed by *V. maritimus/V. variabillis*, *V. campbellii*, *V. coralliilyticus,* and *Photobacterium rosenbergii*. The *Vibrio* community fluctuations, assessed by PCoA with UniFrac distance and clustering with Euclidean distance were varied less not only by year but also by site. Interestingly, significant positive correlation was observed between rising seawater temperature and the abundance of *V. campbellii* ($r = 0.62$; $P < 0.05$) whereas the opposite was observed for *V. owensii* ($r = -0.58$; $P < 0.05$) and the C6 group of *V. hyugaensis* ($r = -0.62$; $P < 0.05$). AdaptML-based microhabitat differentiation revealed that *V. harveyi*, *V. campbellii*, *P. rosenbergii,* and *V. coralliilyticus* populations were less-ecologically distinctive whereas *V. astriarenae* and *V. ishigakensis* were ecologically diverse. This knowledge could be important clue for the future actions of coral conservation.

Keywords: vibrios, diversity dynamics, coral reef, seawater, environmental determinants

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INTRODUCTION

Coral reef ecosystem consists of a flexible consortium of eukaryotic and prokaryotic organisms as a holobiont and are rich in compounds for interacting each other as cross talks, protection of their territory and as triggers for these symbiotic association dynamics (Kooperman et al., 2007; Shnit-Orland and Kushmaro, 2009; Kvennefors et al., 2012). Coral hosts large, diverse and specific

microbial populations, which have both important beneficial and harmful roles for the host (Ritchie and Smith, 2004; Rosenberg et al., 2007). More specifically, corals provide three habitats for bacteria (the surface mucus layer, coral tissue, and calcium carbonate skeleton) each of which harbors a distinct bacterial population (Koren and Rosenberg, 2006). Among the bacterial populations, vibrios have been recognized as important members of coral holobionts (Rohwer and Kelly, 2004; Vezzulli et al., 2010, 2012; Baker-Austin et al., 2013; Moreira et al., 2014; Rubio-Portillo et al., 2014; Munn, 2015). Some vibrios may establish mutualistic partnership with corals by releasing nutrients and secondary metabolites (Ritchie, 2006; Chimetto et al., 2008, 2009), while others play a major role in the disruption of coral health (Munn, 2015).

Coral and coral reefs are in steep decline worldwide due to the combined effects of various stressors such as global warming, pollution, overfishing and infectious diseases (Hughes et al., 2003; Weil et al., 2006; Dinsdale et al., 2008; Vezzulli et al., 2012; Baker-Austin et al., 2013). Rising seawater temperature related to global climate change is a big threat to coral health and is ultimately linked to increasing coral disease such as bleaching events (Tout et al., 2015). Impressive progress has been reported regarding characterization of the coral microbiota over the last decade with its altered composition often correlated to the appearance of signs and diseases and/or bleaching that ultimately suggest a link between microbes, global coral health and stability of reef ecosystems (Krediet et al., 2013). Among them, coral vibriosis is a well-known disease, of which occurrences are greatly influenced by increasing seawater temperature (Vezzulli et al., 2012; Baker-Austin et al., 2013; Munn, 2015). In particular, at warm temperatures, *V. shiloi* expresses a cell-surface adhesion protein that is required for bacterial attachment to the coral surface and simultaneously expresses Toxin-P that ultimately inhibits photosynthesis of the coral-endosymbiotic algae and super-oxide dismutase required for survival of this pathogen inside the coral (Rosenberg et al., 2007; Munn, 2015).

The coral reef ecosystem in Ishigaki Island in southwestern Japan is surrounded by well-developed fringing corals with a variety of mangroves and sandy or rocky shores (Sano, 2000; Shimomura and Naruse, 2015). Recently, these reefs have been severely stressed due to typhoons, coral bleaching and soil pollution. Red soil derived turbid seawater in Okinawa, Ishigaki, and Iriomote coral reef seawater adversely affects the photosynthesis of coral symbiont algae (Zooxanthelae) and also interfered with the settlement of coral larvae on the ocean bed which ultimately influenced coral damage (Kakuma and Kamimura, 2011). Due to this, eutrophication including inflow of red soil is an important threat to Ishigaki corals. More specifically, accumulation of red soil and high nutrient inputs due to land development since 1972, have severely affected the Ishigaki coral reef (Hasegawa, 2011). Rivers from the northern part of Ishigaki coral reef, originating from agricultural watershed may have detrimental effects on corals because exposure to silt and nutrient rich sediments may badly stress corals (Weber et al., 2006). In addition, run-off from soils after typhoons can easily damage coral as well (Harii et al., 2014). Presently, several reports are available regarding coral-microbiota from different parts of the world (Rohwer et al., 2002; Wild et al., 2004; Rosenberg et al., 2007; Chimetto et al., 2009; Alves et al., 2010; Krediet et al., 2013; Chimetto Tonon et al., 2015) but little is known about the Ishigaki coral reef microbial communities.

Therefore, the aims of this study are to characterize the *Vibrio* diversity spatially and temporally, to elucidate which environmental determinants [temperature or dissolved organic carbon (DOC)] correlate the *Vibrio* diversity dynamics and to characterize antimicrobial resistance profiles of potential coral pathogens.

MATERIALS AND METHODS

Water Sampling and Isolation of Vibrios

Seawater samples were collected in 2012, 2013, and 2014 (June–July) from five sites (**Figure 1**) off the Ishigaki coral reef, Okinawa, Japan. The sampling sites were Miyara (24°20.5489′ N, 124°13.0408′ E), Osaki (24°25.4171′ N, 124°04.4956′ E), Taketomi (24°20.5260′ N, 124°05.6443′ E), Sekisei outer (24°21.7557′ N, 124°02.7190′ E), and Iriomote (24°23.208′ N, 123°55.681′ E) at depths of 3–4 m. More specifically, these seawater samples were within 2 m of coral holobionts and were collected by SCUBA diving and stored in clean containers and/or sterilized tubes. No specific permissions were required for the water sampling activities in these locations. The seawater samples were then brought back to the laboratory for bacterial isolation. A total of 0.2 mL of the seawater sample was directly spread on to thiosulfate-citrate-bile salt-sucrose (TCBS) agar plate (Nissui Pharmacy, Tokyo, Japan) and incubated at 25°C for 48 h. After incubation, numbers of individual colonies were counted manually to estimate 'viable *Vibrio* counts.' All colonies except those showing lack of growth were purified twice on the TCBS agar plates. The purity was checked using the ZoBell 2216E agar plate at 25°C. These strains were preserved in cryo-vials using the ZoBell broth supplemented with 20% (v/v) glycerol at −80°C.

Molecular Phylogenetic Affiliation on the Basis of *pyrH* Gene Sequences to *Vibrionaceae* Species Groups

Molecular phylogenetic affiliation of all *Vibrio* isolates ($n = 685$) was performed on the basis of *pyrH* (uridylate kinase) gene sequences. A 550 bp of *pyrH* gene was amplified using pyrH80F and pyrH530R primers (Sawabe et al., 2007) as a colony suspension or a purified DNA as a PCR template. Genomic DNA was purified using a Promega Wizard Genomic DNA extraction system according to the protocol provided by the manufacturer. The PCR cycle consisted of an initial denaturation step at 95°C for 180 s and 30 cycles of an denaturation (95°C for 60 s), an annealing step (50°C for 60 s) and an extension step (72°C for 60 s). The PCR products were analyzed on 1.5% agarose gel and the PCR products producing a single band on agarose gels were purified using Promega Gel and PCR purification system (Promega, Madison, WI, USA). Approximately, 50 ng of template was directly sequenced using the pyrH80F or pyrH530R

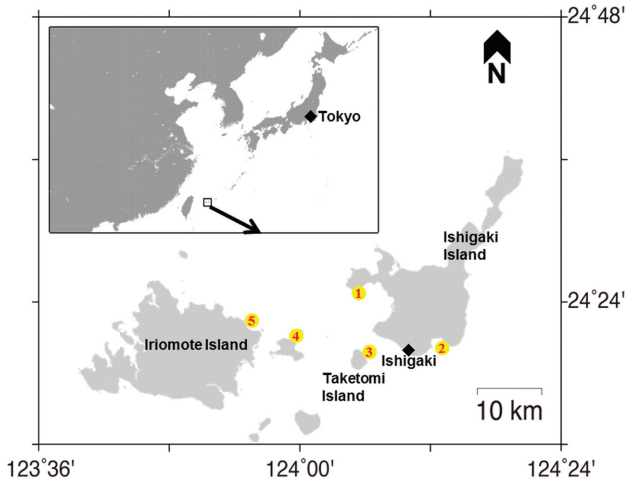

FIGURE 1 | Map showing the sampling locations. Sites are marked with a yellow circle. (1) Osaki; (2) Miyara; (3) Taketomi; (4) Sekisei outer; (5) Iriomote in Ishigaki coral reef ecosystem.

primer and a BigDye terminator sequencing kit version 3.1 (Life Technologies, Carlsbad, CA, USA) according to the protocol recommended by the manufacturer. DNA sequencing was performed with an Applied Biosystems model 373A automated sequencer. The same primers (pyrH80F or pyrH530R) were used for the sequencing.

Using our well-curated *pyrH* gene sequences, which could cover more than 145 known described species of *Vibrionaceae*, molecular phylogenetic affiliations of Ishigaki *Vibrio* isolates were performed. To retain the *pyrH* tree topology within *Vibrionaceae*, *pyrH* gene sequence of *Escherichia coli* W3110 (NC_010473) was included in the dataset as an out group. The sequences were aligned using Clustal X version 2.1 (Larkin et al., 2007) and the alignment was checked by eye and corrected manually. The position used for the affiliation corresponded to positions 171 to 543 in the *V. cholera* O1 Eltor N16961 (AE003852). *pyrH* gene phylogeny was reconstructed by neighbor-joining method (Saitou and Nei, 1987) with bootstrap values of 500 replicates implemented to MEGA programs version 6.06 (Tamura et al., 2013). Evolutionary distances were corrected using the Jukes-Cantor method. Clustering to a described species with more than 98.4% *pyrH* gene similarity was used for the final affiliation for species identification of Ishigaki coral reef isolates. For the affiliation of closed species in clusters harboring *V. harveyi*, *V. owensii*, *V. hyugaensis*, *V. communis*, *V. campbellii,* and *P. damselae* subsp. *piscicida*, a >99.7% cutoff was adopted to affiliate species. All *pyrH* and 16S rRNA genes sequences used in this study have been deposited in BaMBa under data package identification number pmeirelles.20.1. To further affiliate unassigned *pyrH* clusters to new species candidates, 16S rRNA gene sequences of 38 representative strains from *pyrH* unassigned clusters were obtained according to Al-saari et al. (2015). In brief, the amplification primers (24F and 1509R) used for PCR amplification gave a 1.5 kb long PCR product and corresponded to positions 25–1521 in the *E. coli* sequence. A 99.7% cut-off was used for the final affiliation of Ishigaki coral reef isolates to the new *Vibrionaceae* species candidate.

Inferred Habitat Associations of *Vibrio* Community

To infer habitat associations of the *Vibrio* community in Ishigaki coral reef during a 3-years (2012–2014) assessment, PCoA based on unweighted UniFrac distance, hierarchical cluster analysis of *Vibrio* community composition and AdaptML was performed.

UniFrac distance was calculated using 658 *pyrH* gene sequences of *Vibrio* isolates and Quantitative Insights Into Microbial Ecology (QIIME) 1.9 software package (Caporaso et al., 2010) according to Yamazaki et al. (2016) except the use of *pyrH* gene phylogenetic tree. Relationships among *Vibrio* communities in accordance with sampling sites and years were visualized using PCoA. Hierarchical clustering analyses were performed with R Development Core Team (2011), except where indicated. Abundance and multivariate figures were plotted with packages ggplot2 and reshape (Wickham, 2007, 2009). The hierarchical cluster shown in **Figure 3B** was constructed using Ward's minimum variance method (performed using "hclust" R function), which aims at finding compact, spherical clusters, on a Euclidian dissimilarity matrix (calculated using "dist" R function). *Vibrio* diversity indices (Shannon entropy and Shannon evenness [i.e., Hill's Ratio]) and richness were calculated with the vegan R package (Oksanen et al., 2005).

The mathematical model (AdaptML), which uses a Hidden Markov Model, was used to predict the phylogenetic bounds of ecologically distinct *Vibrio* populations and their habitat composition (distribution among environmental categories). 'Clusters of vibrios' sequences were obtained using the software AdaptML as described previously (Hunt et al., 2008). In brief, the software combines genetic information embedded in sequence-based phylogenies and information about the ecology, herein the source and place of isolation, in order to identify genetically and ecologically distinct bacterial populations. AdaptML algorithms can account for environmental parameter discretization schemes and are based on the model concept of habitat (a place and related features that determines microbial distribution). Habitats are characterized by discrete probability distributions describing the likelihood that a strain adapted to a habitat will be sampled from a given ecological state, i.e., at a particular location in the water column). A maximum likelihood model is used for the evolution of habitat association on the tree (Hunt et al., 2008). The habitat-learning and clustering steps of AdaptML were performed using the default settings. Confident assignments are shown to fit ecological populations predicted by the model. The model threshold value was set at 0.05 and *E. coli* W3110 was used as an out-group. The bootstrap percentages analysis were rerun 100 times with the same phylogenetic tree to verify the stability of the predictions. The circular tree figure was drawn using online iTOL software (Letunic and Bork, 2007). To prevent numerical instabilities in AdaptML's maximum likelihood computations, branches with zero length were assigned to the minimal observed non-zero branch length: 0.001. Clades supported in 80% of bootstraps are shown.

Correlation between *Vibrionaceae* Species Groups and Environmental Determinants

Seawater environmental determinants including temperature and DOC were assessed. Water temperature was measured directly using a thermometer. DOC was determined using the method described by Grasshoff et al. (2009). Pearson correlation between each *Vibrio* abundance and environmental determinant was calculated using Microsoft Excel software.

RESULTS

Diversity of Vibrios in Ishigaki Coral Reef Seawater

Our investigation of *Vibrio* diversity in Ishigaki coral reef seawater using *pyrH* gene phylogeny discovered a total of 44 clusters (Supplementary Table S1; **Figure 2**); 32 clusters were affiliated to 22 known *Vibrionaceae* species including 10 *V. hyugaensis* and 2 *P. damselae* subsp. *piscicida* subclusters,

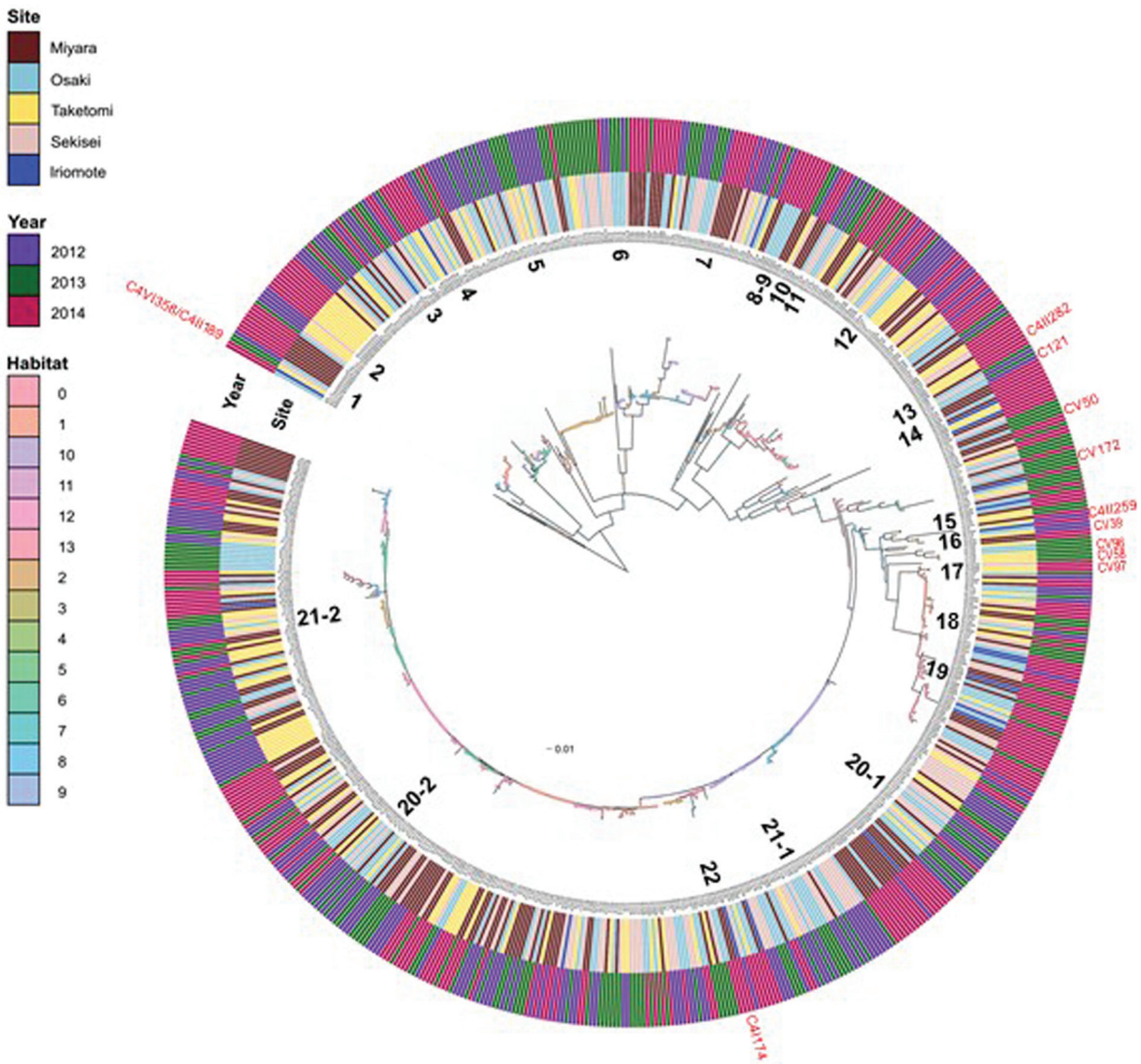

FIGURE 2 | Inferred habitats predicted by AdaptML model using *pyrH* gene sequences of *Vibrio* isolates from Ishigaki coral reef seawater. Inner and outer ring indicate sampling sites and sampling years respectively. Predicted habitats are shown as nodes on the tree (habitat legend matches the colored circles). The closest named *Vibrio* species to numbered *Vibrio* populations' are as follows- (1) *P. aphoticum*; (2) *V. astriarenae*; (3) *V. ishigakensis*; (4) *V. pelagius*; (5) *P. rosenbergii*; (6) *V. mediterranei*; (7) *V. variabillis/maritimus*; (8) *V. nigripulchritudo*; (9) *V. orientalis*; (10) *V. tubiashii*; (11) *V. alginolyticus*; (12) *V. coralliilyticus/neptunis*; (13) *V. ponticus*; (14) *V. azurius*; (15) *V. alfacsensis*; (16) *V. orientalis*; (17) *P. damsella* subsp. *piscicida*; (18) *V. harveyi*; (19) *V. campbellii*; (20-1) *V. owensii*-1; (20-2) *V. owensii*-2; (21-1) *V. hyugaensis*; (21-2) *V. hyugaensis*; (22) *V. communis*. Red marked strains, outside of the outer ring indicate the positions of putative new species candidates.

and the other 12 were affiliated to novel species candidates in *Vibrionaceae*. A total of 658 strains were identified as known *Vibrionaceae* species to *V. alfacsensis*, *V. alginolyticus*, *V. astriarenae*, *V. azureus*, *V. campbellii*, *V. communis*, *V. coralliilyticus/V. neptunis*, *V. harveyi*, *V. hyugaensis*, *V. ishigakensis*, *V. mediterranei*, *V. nigripulchritudo*, *V. orientalis*, *V. owensii*, *V. ponticus*, *V. pelagius*, *V. rotiferianus*, *V. tubiashii*, *V. variabillis/maritimus*, *Photobacterium aphoticum, P. damselae* subsp. *piscicida,* and *Photobacterium rosenbergii*.

However, 26 strains belonged to C121 ($n = 5$), CV39 ($n = 2$), CV50 ($n = 4$), CV58 ($n = 1$), CV96 ($n = 1$), CV97 ($n = 1$), CV172 ($n = 1$), C4I174 ($n = 4$), C4II189, C4II259 ($n = 1$), C4III282 ($n = 3$) and C4V358 clusters which were not affiliated to any known vibrios species (**Figure 2**; Supplementary Table S1). *V. hyugaensis* was the largest group of the *Vibrio* community in Ishigaki coral reef seawater ($n = 200$, 29.2%) and was mostly diversified into 10 different *pyrH* clusters (Supplementary Table S1). The occupation rates of each *V. hyugaensis* cluster were C71 (2.0%), C68 (4.5%), C6 (7.6%), C16 (1.9%), C49 (1.9%), C58 (3.7%), C46 (6.0%), C156 (0.1%), C164 (1.3%), and C4I100 (0.1%). *V. owensii,* a potent coral pathogen, was present in 21.6% of the total isolated vibrios. The third most abundant *Vibrio* group was *V. variabilis/maritimus* which was recorded in 8.8% of the isolated vibrios. Other prominent vibrios were *P. rosenbergii* (6.0%), *V. campbellii* (5.3%), and *V. harveyi* (4.5%). Some other recognized potential coral pathogens, *V. coralliilyticus* ($n = 49$, 7.2%) and *V. alginolyticus* ($n = 5$) were also found. Less significant amounts of identified vibrios (Supplementary Table S1) were *V. ponticus* ($n = 3$, 0.4%), *V. pelagius* ($n = 9$, 1.3%), *V. tubiashii* ($n = 5$, 0.7%), *V. rotiferianus* ($n = 3$, 0.4%), *P. aphoticum* ($n = 3$, 0.4%) and two recent newly described species, *V. astriarenae* (3.4%) and *V. ishigakensis* (4.2%). *V. nigripulchritudo* ($n = 1$), *V. alfacsensis* ($n = 1$), *V. orientalis* ($n = 1$), *V. azureus* ($n = 1$), and *V. mediterranei* ($n = 1$) were less highlighted vibrios.

Vibrio Community Dynamics

Unweighted FastUniFrac analysis revealed no-apparent grouping of *Vibrio* communities not only site by site but also year by year (**Figure 3A**). However, a simple cladogram constructed on the basis of relative abundances in the *Vibrio* community illustrated two apparent clusters in the *Vibrio* communities of Iriomote 2014, Osaki 2014, Sekisei 2014, Taketomi 2014 and Miyara 2013 and those of Taketomi 2012, Miyara 2014, Sekisei 2012, and Osaki 2013 (**Figure 3B**). Signatures of the former and the latter communities showed significant abundances ($P < 0.05$) of *V. campbellii*, *V. hyugaensis* (C6 group), and *V. owensii* respectively. Vibrios from Sekisei 2013 and Osaka 2012 were sub-clustered using a relative higher abundance of *P. rosenbergii*.

Correlation between Environmental Variables and *Vibrio* Abundances

Vibrio campbellii abundance significantly increased ($r = 0.62$; $P < 0.05$) along with rising seawater temperature whereas *V. owensii* ($r = -0.58$; $P < 0.05$) and the C6 group of *V. hyugaensis* ($r = -0.62$; $P < 0.05$) abundances significantly decreased with an increase in seawater temperature (**Table 1**). Moreover, the correlation assessment between *Vibrio* abundances and DOC availability illustrated (**Table 1**) that *P. rosenbergii* and the *V. hyugaensis* (C6 group) increased along with increased DOC whereas *V. owensii*, *V. campbellii,* and *V. coralliilyticus* showed the opposite results.

Ecologically Distinct Populations among *Vibrio* Isolates

The AdaptML analysis showed that the *Vibrio* isolates are distributed in eight habitat spectra (H-0, H-1, H-2, H-6, H-8, H-10, H-12, and H-13; **Figures 2** and **4**). Habitats are the part of an ecosystem (a spectrum of environment types) from where microbial populations are isolated. *V. owensii*, *V. harveyi*, *V. hyugaensis* (C71, C46, C6, C16, C49, C58, and C68 groups), *V. campbellii*, *V. ishigakensis*, *V. variabillis/V. maritimus*, *V. coralliilyticus/V. neptunis*, *P. rosenbergii,* and *V. pelagius* were observed from all the sites although *V. alfacsensis*, and *V. nigripulchritudo* were found only from Taketomi and Miyara respectively (**Figure 4**). Another two vibrios, *V. azureus* and *V. mediterranei* were specific for Osaki and one more single *Vibrio*, *V. orientalis* was recorded solely from Sekisei reef site. The spatiotemporal distribution of *V. campbelli* was likely to support the temperature dependent abundance from 2014 in these study areas (**Figure 4**). From all the predicted habitat spectra, H-10 and H-12 showed the strains which were isolated from all the sampling sites and sampling years with major isolates from Sekisei-2012 (41.7%) and Miyara-2014 (41.2%), respectively. Other habitats (H-0, H-1, H-2, H-6, H-8, H-13) showed the isolates (Supplementary Figure S1) from several sampling sites and years but not from all. The major isolates of H-0, H-1, H-2, H-6, H-8, and H-13 were obtained from Osaki-2014 (34.9%), Taketomi-2014 (34.9%), Osaki-2012 (20.2%), Osaki-2013 (33.7%), Miyara-2014 (78.6%), and Taketomi-2012 (55%) respectively.

The adapted predominant *Vibrio* species (Supplementary Table S2) in H-0 were *V. harveyi* (9.7%), *V. campbellii* (94.4%), *V. ishigakensis* (6.9%), *V. variabillis/V. maritimus* (11.7%), *V. coralliilyticus/V. neptunis* (32.7%), and *V. pelagius* (44.4%). The highlighted vibrios in H-1 were *V. harveyi* (80.6%) and *V. hyugaensis* C6 group (42.3%), *V. hyugaensis* C58 group (48.0%), *V. hyugaensis* C46 group (26.8%) and *V. astriarenae* (43.5%). H-2 comprised mostly of *V. owensii* (8.8%), *V. hyugaensis* C16 group (76.9%) and *P. rosenbergii* (97.6%). Two prevalent vibrios in H-6 were *V. owensii* (24.3%) and *V. ishigakensis* (44.8%). The most abundant vibrios in H-8 were *V. owensii* (16.2%) and *V. variabillis/V. maritimus* (28.3%). The dominant vibrios in H-10 were *V. owensii* (17.6%), *V. hyugaensis* C68 group (32.3%), *V. hyugaensis* C46 group (56.1%), *V. ishigakensis* (31.0%) and *V. variabillis/V. maritimus* (41.7%). In H-12 the preeminent vibrios were *V. owensii* (29.1%) and *V. hyugaensis*-C6 (30.8%). H-13 was found to contain *V. coralliilyticus*–*V. neptunis* (40.8%) and *V. astriarenae* (30.4%). Moreover, several potential novel vibrios (CV39, CV58, CV96, CV97) and one opportunistic coral pathogen *V. alginolyicus* (100%) were retrieved specifically from H-2.

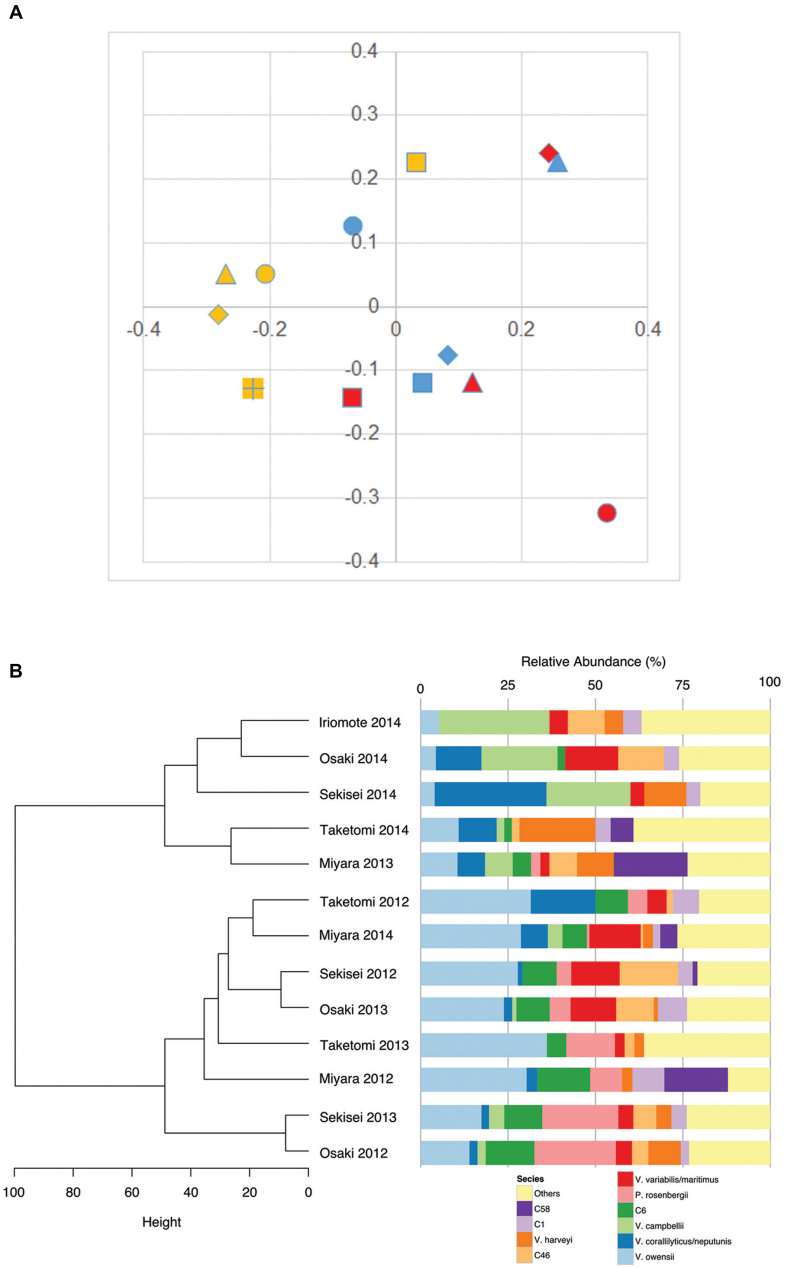

FIGURE 3 | *Vibrio* community diversity dynamics in Ishigaki coral reef seawater. (A) Multidimensional scaling plot (FastUnifrac:PCoA), isolates indicating colors and shapes: blue, 2012; red, 2013; yellow, 2014; dot, Taketomi; square, Miyara; diamond, Osaki; triangle, Sekisei; square (+), Iriomote; **(B)** dendrogram clustering of vibrios according to their relative abundances.

AdaptML could also estimate the evolutionary history of ecological differentiation (Hunt et al., 2008). Interestingly, the recent ongoing adaptive radiations (with a shallow branch: meaning frequently changing adaptations) were observed as more likely to be in *V. astriarenae*, *V. ishigakensis*, *V. mediterranei*, *V. variabilis/V. maritimus*, *V. coralliilyticus/V. neputunis,* and *V. communis/V. owensii/V. hyugaensis*, however, no such radiations were observed in *V. campbellii*, *V. harveyi,* and *P. rosenbergii* (**Figure 2**).

DISCUSSION

Ishigaki coral reef ecosystems are known to be the largest and the most diverse reef ecosystems in Japan, but are in now extremely vulnerable. In this era of global warming, the surrounding seawater of coral reefs are one of the most important ecological niches as well as heat carriers. It is worth investigating the evolution of spatiotemporal *Vibrio* dynamics in oceanic fields not only for the conservation of fragile coral reefs but also

TABLE 1 | Correlation coefficient (*r*) and significant levels (*P*-values) obtained from the correlation analysis between major *Vibrio* abundances and environmental variables.

Vibrios	Environmental parameter	Correlation coefficient (*r*-value)	Significance (*P*-value)
V. owensii	Temperature	−0.58	0.038
V. campbellii	Temperature	0.62	0.023
P. rosenbergii	Temperature	−0.39	0.18
V. coralliilyticus	Temperature	0.16	0.60
V. hyugaensis (C6)	Temperature	−0.62	0.0013
V. owensii	DOC	−0.58	0.64
V. campbellii	DOC	−0.55	0.13
P. rosenbergii	DOC	0.60	0.086
V. coralliilyticus	DOC	0.25	0.52
V. hyugaensis (C6)	DOC	0.54	0.13

for public health and aquaculture issues (Rubio-Portillo et al., 2014; Tout et al., 2015). We performed a 3 years survey to elucidate *Vibrio* diversity using well-curated *pyrH* gene sequence set to achieve fine scale discrimination (Thompson J.R. et al., 2005; Thompson et al., 2005a, 2007; Tall et al., 2013; Chimetto Tonon et al., 2015). The *pyrH* gene set allows us not only to affiliate *Vibrio* isolates to known described *Vibrionaceae* species or currently unknown new species candidates but also to elucidate the structure of regional populations of specific species and how they have diverged. Our study demonstrated that the *Vibrio* community of the coral surrounding seawater consisted of at least 22 known described species and 12 undescribed species candidates (26 isolates) which is likely to be a more diversified *Vibrio* community compared to those reported from tropical and temperate coral reef environments (Chimetto et al., 2008; Raina et al., 2009; Alves et al., 2010; Chimetto Tonon et al., 2015). Unfortunately, we did not assess the Ishigaki coral reef microbiota due to lack of coral samples, the seawater around Ishigaki coral reefs is likely to share major *Vibrionaceae* communities of reported microbiomes *V. communis*/*V. owensii*, *V. mediterranei* (*V. shiloi*), *V. harveyi*, *V. alginolyticus*, *V. campbellii*, *V. maritimus*/*V. variabilis*, *V. tubiashii*, *V. coralliilyticus*, *V. pelagius* consisting of coral holobionts (Alves et al., 2010; Chimetto Tonon et al., 2015). High abundance of *Harveyi* clade (*V. hyugaensis*, *V. communis, V. owensii, V. harveyi, V. campbellii*) and lower abundance of *Splendidus* clade species (*V. pelagius*) are the specific clues to help define subtropical Ishigaki coral reef microbiota (Hunt et al., 2008; Tall et al., 2013). *Coralliilyticus* clade species (*V. coralliilyticus, V. neptunis*), *Mediterranei* clade species (*V. mediterranei*, *V. maritimus, V. variabillis*) and *P. rosenbergii* are secondary significant members (>5% constitution) of the Ishigaki coral reef seawaters.

The availability of at least 12 novel species candidates (CV172, CV50, C4I174, CV96, CV58, CV39, CV97, C4II282, C121, C4II259, C4II189, and C4V358) in the Ishigaki coral reef ecosystem suggests important evidence of *Vibrionaceae* evolution. Relevant subsequent studies (Thompson et al., 2005b; Rosenberg et al., 2007; Reis et al., 2009; Moreira et al., 2014; Chimetto Tonon et al., 2015) reported that coral and surrounding seawater are prime sources in isolating novel vibrios and is an important habitat for studying the microevolution of vibrios. Comparing previously recorded vibrios from coral and reef ecosystems (Chimetto et al., 2009; Alves et al., 2010; Chimetto Tonon et al., 2015) we found mostly diversified populations including numerous vibrios unique to our studied reef locations. The retrieved unique *Vibrio* populations were *V. hyugaensis*, *V. ishigakensis*, *V. astriarenae*, *P. rosenbergii*, *P. damsella* subsp. *piscicida*, *P. aphoticum*, *V. nigripulchritudo*, *V. alfacsensis,* and *V. azureus* suggesting that Ishigaki coral reef locations are hotspots for versatile and dynamic *Vibrio* populations and may serve as diversified ecological niches.

This spatiotemporal survey also revealed that the community and population level dynamics in the surrounding seawater of coral holobionts in Ishigaki reef ecosystems can determine generalists and specialists (**Figures 2** and **4**). *V. owensii* and *V. hyugaensis* populations were considered to be the dominant generalist populations in the coral seawater ecosystems. The other prevalent generalists (Supplementary Table S1; **Figure 2**) in Ishigaki reef seawater were *V. variabillis*, *V. harveyi*, *V. campbellii*, *P. rosenbergii*, *V. coralliilyticus*, *V. astriarenae,* and *V. ishigakensis*.

Numerous previous reports suggested that vibrios from *Harveyi* clade appear to have ecologically diversified passively by invading new niches (Chimetto et al., 2008; Preheim et al., 2011; Chimetto Tonon et al., 2015). The observed diversities of *V. owensii* and *V. hyugaensis* populations were likely to be higher than those of *V. harveyi* and *V. campbellii* (**Figure 2**). *V. owensii* was isolated from cultured crustaceans in Australia as a member of *Harveyi* clade (Cano-Gomez et al., 2010). This species was also isolated from *Acropora* white syndrome lesions in American Samoa and is considered to be a putative coral pathogen (Ushijima et al., 2012; Wilson et al., 2012) and was also isolated from '*Montipora capitata*' diseased-coral (Tissue loss disease: *Montipora* White Syndrome) in Hawaiian coral reefs. *V. hyugaensis* was isolated from a seawater sample collected in Miyazaki prefecture in Japan (Urbanczyk et al., 2015). It is a luminous *Vibrio* and several light-producing bacteria were reported from seawater samples taken from different sites in Miyazaki prefecture between 2010 and 2012 (Urbanczyk et al., 2014).

Specialist *Vibrio* populations in Ishigaki reef seawater showed site-specific distributions (Supplementary Table S1). *V. azureus* and *V. mediterranei* were isolated specifically from Osaki whereas, *V. nigripulchritudo*, *V. alfacsensis,* and *V. orientalis* were noted as region-specific in Miyara, Taketomi and Sekisei, respectively. Bacteria as coral holobionts may be host-specific or may display spatial variability which might show protective activities against pathogens or other detrimental agents by occupying entry niches and space (as competitors) or by production of antibiotic compounds (Rohwer et al., 2002; Mullen et al., 2004; Kelman et al., 2006; Ritchie, 2006). Moreover, the mathematical model AdaptML generated the environmental grouping (Supplementary Table S2) of the Ishigaki reef seawater *Vibrio* populations and detected both ecologically restricted and ecologically distinctive (Hunt et al., 2008) populations. Two

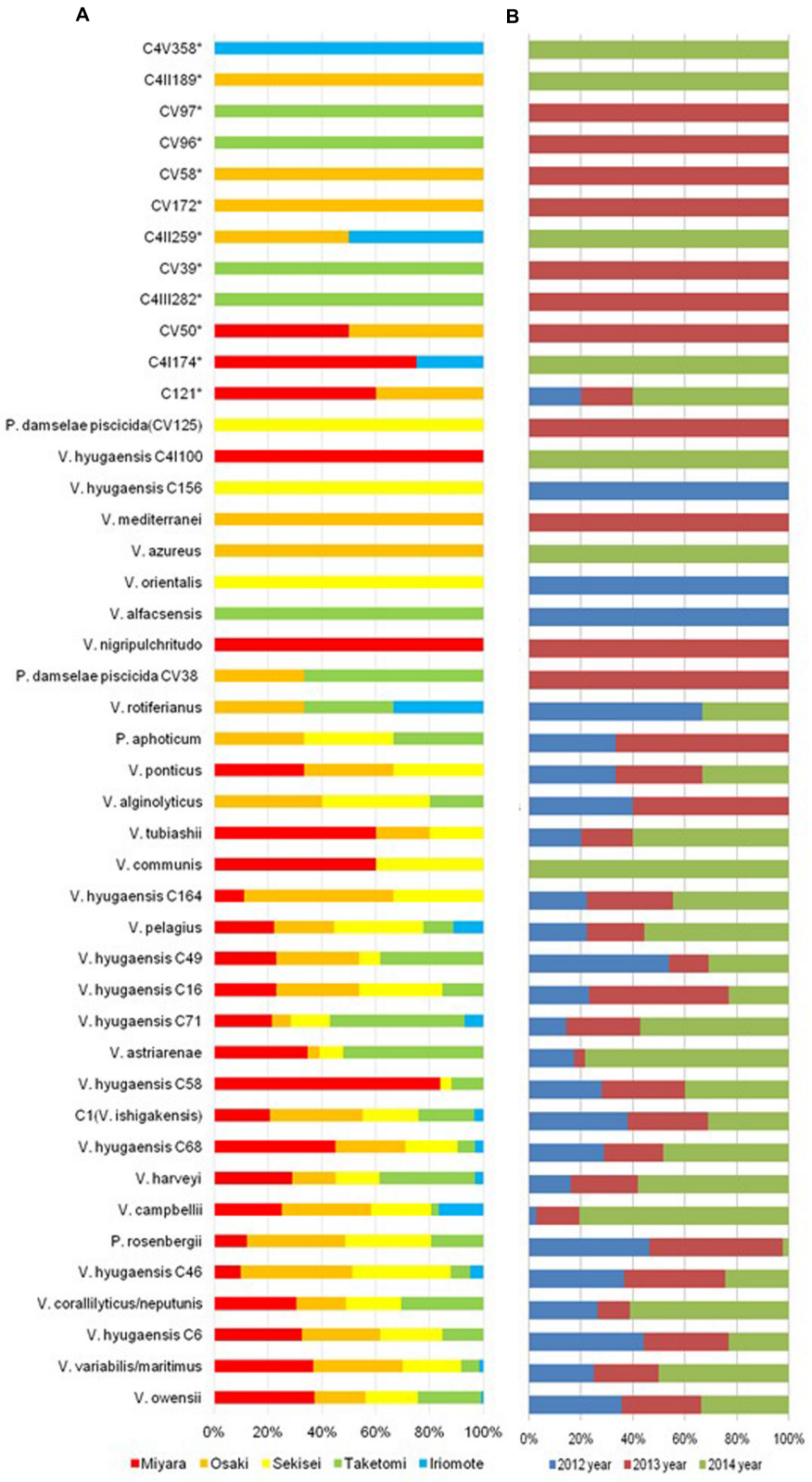

FIGURE 4 | Spatiotemporal *Vibrio* population dynamics in Ishigaki coral reef seawater. (A) Spatial *Vibrio* population dynamics (red, brown, yellow, green, sky blue colors indicate sampling sites of Miyara, Osaki, Sekisei Outer, Taketomi, and Iriomote respectively); **(B)** Temporal *Vibrio* population dynamics (blue, violate, and green colors indicate sampling year of 2012, 2013, and 2014 accordingly).

proposed novel vibrios isolated from this location *V. astriarenae*, *Agarivorans* clade (Al-saari et al., 2015) and *V. ishigakensis*, *Halioticoli* clade (Gao et al., 2016) were found in more ecologically diverse populations (**Figure 2**). The former is agarolytic whereas the latter is non-motile and alginolytic. Benthic flora might determine the specific niches for these species.

One of the more intriguing findings disclosed by this study was the availability of versatile and dominant putative coral pathogenic vibrios, *V. owensii* and *V. coralliilyticus* (Supplementary Table S1; **Figure 2**) occupied 21.6 and 7.2%, respectively in our total analyzed vibrios. This may be due to higher global oceanic temperatures. Temperature influenced pathogenic potential of coral pathogenic vibrios may lead to an increase in incidences of diseases in marine environments. Swimming motility, swarming motility and protease activity have been shown to be related to the virulence of *V. owensii* (Ushijima et al., 2012). According to Kimes et al. (2012) for *V. coralliilyticus*, an increase in seawater temperature to more than 27°C plays a direct role in triggering virulence genes and other factors related to host degradation, secretion, antimicrobial resistance, and motility. The mechanism of virulence includes motility and chemotaxis that involve post-colonization with subsequent production of coral tissue damaging 'zink-metalloproteases' (Ben-Haim et al., 2003). Recently, Tout et al. (2015) demonstrated that raised-seawater temperature increase natural *Vibrio* abundances, particularly the notorious coral pathogen *V. coralliilyticus*, in coral ecosystems. However, our findings on *V. coralliilyticus* abundance were not significantly correlated to seawater temperature rises. Beyond the previously mentioned potential coral pathogens, we also identified *V. harveyi* (4.5%) and *V. alginolyticus* (0.7%) from the seawater. *V. alginolyticus* was found only in the single habitat H-2 suggesting that this bacterium may have a new ecological niche-partitioning in Ishigaki reef seawater. Other results associated with coral pathogenic vibrios (Luna et al., 2010; Zhenyu et al., 2013; Munn, 2015) show that most of them are opportunistic in nature and show their virulence under certain environmental conditions by overwhelming the host-defense, overgrowth and tissue destruction.

Environmental variables, particularly temperature are considered to be the prominent triggering factor in *Vibrio* ecology, population dynamics, physiological stress response and evolution (Sato et al., 2009). Elevated temperatures and high loads of nutrients particularly and DOC have direct impacts on coral holobiont microbial communities and ultimately make them diverse, abundant and even niche or host specific (Kline et al., 2006; Paz et al., 2007). Other findings similarly demonstrate that elevated nutrients (i.e., phosphate, nitrate, ammonia) and DOC in coastal waters have been a cause of reef decline (Bruno et al., 2003; Kline et al., 2006). In our assessment, only specific *Vibrionaceae* populations correlated with environmental parameters (e.g., temperature, DOC). *V. campbellii* abundance was significantly correlated to rises in seawater temperatures, *V. owensii* and C6 group of *V. hyugaensis* showed significant negative correlations with increasing seawater temperature. The optimum growth temperature of *V. hyugaensis* was reported to be 26°C and it could not grow at 37°C, which might reflect its low tolerance to high temperatures. These findings corroborated previous reports that thermal anomalies and nutrient-rich environment including DOC are well-known ecological triggers, involved in proliferation and increased abundance of holobiont microbial communities in coastal environments globally (Ducklow et al., 1986; Wild et al., 2004; Barott and Rohwer, 2012) along with reef ecosystems in southern Japan (Goto et al., 2010). Roder et al. (2014) also noted that coral associated bacterial communities including potential pathogens emerge and dominate during environmental stress and the shift of bacterial community may be a direct effect of temperature on growth of specific members of the microbial community (Garren et al., 2014).

CONCLUSION

Our curated data set of *Vibrionaceae pyrH* gene sequences allows us to perform the first characterization of *Vibrio* diversity and assessment of spatiotemporal diversity dynamics of *Vibrio* populations both at community and population levels along with the relationship to environmental determinants from Ishigaki coral reef seawater. The determined *Vibrio* community of seawater directly connecting to Ishigaki coral holobionts included at least 22 known described species and 12 as yet undescribed species, which are more diversified compared to reported tropical and temperate reef vibrios. Several vibrios such as, *V. campbellii*, *V. owensii* and the C6 group of *V. hyugaensis* abundances seem to correlate with seawater temperature. *V. owensii* and C6 group of *V. hyugaensis* show significant negative correlations with increasing seawater temperature but *V. campbellii* is positively significant in this regard. This study also demonstrates the presence of most of the globally recognized opportunistic potential coral-pathogenic vibrios (*V. owensii, V. harveyi*, *V. alginolyticus*). This pioneering study on Ishigaki coral reef seawater vibrios both at community and population levels will be the important gateway for further deep research on vibrios in coral and reef systems.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AA, FT, and TomooS. Performed the experiments: AA, GF, NA, PM, YY, and SM. Analyzed the data: AA, GF, NA, PM, YY, and TomooS. Contributed reagents/materials/analysis tools: AA, GF, NA, PM, YY, SM, FT, TokoS, and TomooS. Wrote the paper: AA and TomooS. Critical review, ideas and suggestion given during manuscript preparation: GF, NA, PM, YY, SM, FT, and TokoS.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01185

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Antimicrobial Susceptibility of Autochthonous Aquatic *Vibrio cholerae* in Haiti

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We investigated the antimicrobial susceptibility of 50 environmental isolates of *Vibrio cholerae* non-O1/non-O139 collected in surface waters in Haiti in July 2012, during an active cholera outbreak. A panel of 16 antibiotics was tested on the isolates using the disk diffusion method and PCR detection of seven resistance-associated genes (*strA/B*, s*ul1/2, ermA/B,* and *mefA*). All isolates were susceptible to amoxicillin-clavulanic acid, cefotaxime, imipenem, ciprofloxacin, norfloxacin, amikacin, and gentamicin. Nearly a quarter (22.0%) of the isolates were susceptible to all 16 antimicrobials tested and only 8.0% of the isolates ($n = 4$) were multidrug-resistant. The highest proportions of resistant isolates were observed for sulfonamide (70.0%), amoxicillin (12.0%), and trimethoprim-sulfamethoxazole (10.0%). One strain was resistant to erythromycin and one to doxycycline, two antibiotics used to treat cholera in Haiti. Among the 50 isolates, 78% possessed at least two resistance-associated genes, and the genes *sul1*, *erm*A, and *str*B were detected in all four multidrug-resistant isolates. Our results clearly indicate that the autochthonous population of *V. cholerae* non-O1/non-O139 found in surface waters in Haiti shows antimicrobial patterns different from that of the outbreak strain. The presence in the Haitian aquatic environment of *V. cholerae* non-O1/non-O139 with reduced susceptibility or resistance to antibiotics used in human medicine may constitute a mild public health threat.

Keywords: *Vibrio cholerae* non-O1/non-O139, antimicrobial resistance, Haiti, aquatic environment, cholera

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INTRODUCTION

Reports on clinical strains of *Vibrio cholerae* O1 resistant to commonly used antibiotics are on the rise (Garg et al., 2000, 2001; Ghosh and Ramamurthy, 2011; Harris et al., 2012). Antibiotic resistance is a global health concern because resulting infections can be more difficult to treat. The increase in resistance to antimicrobial drugs can result either from the accumulation of genetic mutations, following exposure of the circulating bacteria to antibiotics during the medical treatment of epidemics, or from the acquisition of resistance genes, through the mobilization and exchange of a variety of genetic elements. In the case of *V. cholerae*, self-transmissible mobile genetic elements may harbor an SXT constin (a large conjugative element), which may confer resistance to sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin (Hochhut et al., 2001).

Surprisingly, data on the susceptibility of environmental isolates of *V. cholerae* non-O1/non-O139 are still scarce (Kumar et al., 2009; Bier et al., 2015; Bhuyan et al., 2016). However, knowledge on the prevalence of antimicrobial resistance in these serogroups is of global health interest for two reasons.

First, contrary to O1/O139, non-O1/non-O139 serogroups are detected worldwide in all types of waters: freshwater, estuarine, saline water, and wastewater. Aquatic environments may provide an ideal setting for the acquisition and dissemination of antibiotic resistance: (i) they are frequently affected by anthropogenic activities (Marti et al., 2014); (ii) they contain autochthonous bacterial flora that may harbor resistance-associated genes; (iii) they bring bacteria from different origins (human, livestock, etc.) into contact with each other; (iv) they can contain antimicrobials or biocides which may select for resistant bacteria. Wastewater constitutes a hot spot for the emergence of antimicrobial resistance (Rizzo et al., 2013), particularly for *V. cholerae*, which has a dual life cycle (intestinal and aquatic); (Schoolnik and Yildiz, 2000). Therefore, due to its specific genetic abilities, and its ecological characteristics, *V. cholerae* may be an important vehicle of transmission of resistance genes in all aquatic environments either within bacterial species or between bacterial genera. In countries with endemic cholera, these autochthonous aquatic serogroups of *V. cholerae* can also co-infect cholera cases, providing an opportunity for the exchange of antimicrobial resistance genes with clinical strains of serogroup O1 in the human intestinal lumen. Furthermore, the intestinal epidemic clones circulating in the environment can theoretically exchange resistance genes with the autochthonous populations of *V. cholerae*.

Second, some clones of *V. cholerae* non-O1/non-O139 from aquatic environments may be opportunist pathogens, which can cause gastro-intestinal or extra-intestinal infections (bacteremia, septicemic, otitis, etc.) (Lukinmaa et al., 2006; Deshayes et al., 2015). Throughout the world, the number of cases of *V. cholerae* non-O1/non-O139 infections are rising (Baker-Austin et al., 2013); moreover, reports of very severe infections are becoming more frequent, with even one death recently reported in Austria (Hirk et al., 2016). In Haiti, *V. cholerae* non-O1/non-O139 has been shown to be a local intestinal infectious pathogen. Very early in the on-going Haitian cholera outbreak (November 2010), a microbiological investigation of 81 clinical cases of acute diarrhea with varying severity from 18 towns showed that *V. cholerae* non-O1/non-O139 was isolated from 28% of the stool specimens, either alone ($n = 17$) or in co-culture with toxigenic *V. cholerae* O1 ($n = 5$). The latter cases were confirmed cholera cases, as were 39 other cases with the presence of toxigenic *V. cholerae* O1 alone (Hasan et al., 2012). However two years later (from April 2012 to March 2013), systematic testing of profuse watery diarrhea cases was carried out in four hospitals that had cholera treatment facilities. Among the 1616 specimens of stools tested in culture, 60% were positive for toxigenic *V. cholerae* O1, one gave a non-toxigenic isolate of *V. cholerae* O1, but none were positive for *V. cholerae* non-O1/non-O139 (Steenland et al., 2013). Other studies on stools collected from patients during the Haiti outbreak found only serogroup O1 isolates (Talkington et al., 2011; Katz et al., 2013). These results are not contradictory: non-O1/non-O139 serogroups can cause a diarrheal disease that is generally less severe than cholera and do not particularly have epidemic potential (Menon et al., 2009). Nevertheless, the pathogenic clones of *V. cholerae*, whether they are agents of cholera or of milder infections, can circulate via aquatic environments and some authors even speculate that toxigenic clones of *V. cholerae* are autochthonous to estuaries and rivers systems worldwide as are other clones (Colwell, 1996).

We therefore investigated the antimicrobial susceptibility of 50 environmental isolates of non-toxigenic *V. cholerae* collected in various surface waters in Haiti in July 2012, during the active cholera epidemic (Baron et al., 2013). The aim of this study was to document, in this context, the susceptibility of strains belonging to non-O1/non-O139 serogroups to 16 antibiotics used and compare it to those of the Haitian epidemic strain.

MATERIALS AND METHODS

Collection of Strains

In a previous study, water samples, including wastewater, were collected from 35 stations described in Baron et al. (2013). *V. cholerae* was detected by culturing samples on TCBS (thiosulfate citrate bile sucrose) agar (Difco, BD Biosciences, Le pont de Claix, France) after enrichment in peptone alkaline saline water (41°C ± 1 for 16–24 h; Muic, 1990). Presumptive identification of *V. cholerae* was given to all sucrose-fermenting isolates that were able to grow on nutrient agar without added NaCl, and that tested positive for oxidase (Baron et al., 2007). Presumptive *V. cholerae* were isolated from 27 of the 35 stations sampled, but isolates from six stations could not be regrown (**Table 1**).

Fecal contamination (FC) was determined using Petrifilm™ Select *Escherichia coli* (Département Microbiologie Laboratoires 3M Santé, Cergy, France). Based on the FC level (**Table 1**), the 21 sampled stations were divided into two groups. The high FC (HFC) group for which the density of *E. coli* was at least 10^4 CFU/100 mL included five stations that were all wastewaters. The low FC (LFC) group, for which the *E. coli* density level was $<10^4$ CFU/100 mL, included Trou Caïman Lake (Station 3) and Etang Saumâtre Lake (Stations 4 and 5; **Table 1**). Conductivity was assessed at the laboratory with a field conductometer (Hanna HI-99301, Grosseron, Nantes, France).

Confirmation and Characterization of *V. cholerae*

Agglutination using a polyclonal antibody specific to the O1 surface antigen (Bio-Rad, Marnes-la-Coquette, France) was performed on presumptive *V. cholerae* isolates at the Haiti National Public Health Laboratory. A saline solution was used as a control to identify self-agglutinating isolates. One isolate per enrichment and per station was conserved and sent to the ANSES-Laboratory of Ploufragan-Plouzané for further analysis. The identification of presumptive *V. cholerae* was confirmed by PCR (Nandi et al., 2000). The genes coding for the O1 and O139 surface antigens (*rfb*) were assessed with PCR using O1- and O139-specific primers (Hoshino et al., 1998; **Table 2**). The cholera toxin gene *ctxA* was screened using PCR (Hoshino et al., 1998; Nandi et al., 2000).

TABLE 1 | Distribution of the 50 isolates of confirmed *V. cholerae* non-O1/non-O139 and characteristics of the 27 stations (see Baron et al., 2013 for correspondence).

Sampling stations				Water characteristics			Number of isolates
Department	Town	Location	ID[a]	Salinity (‰)[b]	Fecal contamination:		
					E. coli /100 mL	Group[d]	
West (Metropolitan area)	Port-au-Prince	Martissant (street wastewater)	2	ND[c]	ND	HFC	2
West (Metropolitan area)	Carrefour	Mariani (wastewater in the river)	32	0.21	106,000	HFC	NG[e]
West (Metropolitan area)	Carrefour	Mariani (river shore)	33	0.20	35,000	HFC	2
West (Metropolitan area)	Carrefour	Mariani (river shore)	34	0.21	46,000	HFC	3
West (Metropolitan area)	Carrefour	Mariani (macrophyte lagoon)	35	25.02	12,400	HFC	NG
West (Metropolitan area)	Carrefour	Mariani (macrophyte lagoon)	36	10.72	50,000	HFC	NG
Artibonite	Gonaives	Small canal of wastewater	19	2.09	91,000	HFC	4
Artibonite	Gonaives	Large canal of wastewater	24	1.40	36,000	HFC	3
West	Thomazeau	Trou Caïman Lake	3	1.27	ND	LFC	3
West	Thomazeau	Etang Saumâtre Lake (shore)	4	4.90	ND	LFC	3
West	Thomazeau	Etang Saumâtre Lake (far from the shore)	5	5.62	ND	LFC	2
Artibonite	Saint Marc	Etang Bois-Neuf	18	12.41	2000	LFC	5
Artibonite	Saint Marc	Pont-Sondé (Artibonite River)	6	0.14	4800	LFC	4
Artibonite	Grande Saline	Main canal 1	17	0.15	2800	LFC	2
Artibonite	Grande Saline	Main canal 2	16	0.15	3600	LFC	2
Artibonite	Grande Saline	Drouin—main canal 3 (point-of-use)	7	0.15	2100	LFC	2
Artibonite	Grande Saline	Artibonite River estuary 1	9	0.14	2100	LFC	1
Artibonite	Grande Saline	Artibonite River estuary 2	10	0.15	3100	LFC	1
Artibonite	Grande Saline	Basin 1	14	0.75	1000	LFC	1
Artibonite	Grande Saline	Basin 2	15	0.27	<100	LFC	4
Artibonite	L'Estère	L'Estère (river)	25	0.15	300	LFC	1
Artibonite	L'Estère	L'Estère (small canal)	26	0.15	400	LFC	1
Artibonite	L'Estère	L'Estère (large canal)	27	0.14	300	LFC	1
Artibonite	L'Estère	L'Estère (roadside)	28	0.37	4700	LFC	NG
Artibonite	Desdunes	Route de Desdunes (small canal)	29	0.26	200	LFC	NG
Artibonite	Desdunes	Route de Desdunes (large canal)	30	0.52	300	LFC	3
Artibonite	Desdunes	Route de Desdunes (rice field)	31	0.30	<100	LFC	NG

[a] *ID, identification number of the station;*
[b] *salinity: fresh water <0.5‰; brackish water 0.5–16‰;*
[c] *ND, no data;*
[d] *HFC, high fecal contamination: E. coli >10^4 CFU/100 mL; LFC, low fecal contamination: E. coli ≤10^4 CFU/100 mL;*
[e] *NG, isolate from the National Health of Public Health in Haiti did not grow in the ANSES laboratory.*

Antibiotic Resistance Profiles

The susceptibility of *V. cholerae* isolates was tested using the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2010a) for the 16 following antimicrobial agents: ampicillin, amoxicillin-clavulanic acid, cefotaxime, imipenem, chloramphenicol, nalidixic acid, ciprofloxacin, norfloxacin, amikacin, gentamicin, streptomycin, tetracycline, doxycycline, sulfonamide, trimethoprim-sulfamethoxazole, and erythromycin (**Table 3**). *E. coli* ATCC 25922 served as a positive control. The CLSI interpretative criteria for disk diffusion susceptibility testing of *Vibrio* spp. (CLSI, 2010a) were used when available. For nalidixic acid, norfloxacin, amikacin, and streptomycin, the interpretative criteria for *Enterobacteriaceae* were used (CLSI, 2016; **Table 3**). No criteria were available for erythromycin or doxycycline; therefore, the distribution of the inhibition diameters was recorded and interpretation was based on obtained distribution plots. The separation between wild-type (microorganisms without acquired resistance mechanisms) and non-wild-type populations (microorganisms with acquired resistance mechanisms) was determined by visual inspection of the diameter distribution (Hombach et al., 2014). Wild-type populations were considered as susceptible populations and non-wild-type as resistant populations. The few intermediate results were categorized as resistant for this study.

Genes associated with resistance to streptomycin (*strA* and *strB*) and to sulfonamide (*sul1* and *sul2*)-which may be associated with the presence of *V. cholerae* SXT-as well as genes associated with erythromycin resistance (*ermA*, *ermB,* and *mefA*)-an antimicrobial agent used in Haiti for cholera treatment-were detected using PCR (Pei et al., 2006; Popowska et al., 2012; Di Cesare et al., 2013). Class 1, 2, and 3 integrons were screened

TABLE 2 | List of primers used in this study.

Targeted gene	Primer name	Primer sequence (5′–3′)	T°C[a]	Amplicon size (bp)	References
ermA	ermA1 ermA2	TAACATCAGTACGGATATTG AGTCTACACTTGGCTTAGG	54	139	Di Cesare et al., 2013
ermB	ermB1 ermB2	CCGAACACTAGGGTTGCTC ATCTGGAACATCTGTGGTATG	54	200	Di Cesare et al., 2013
mef	mef1 mef2	AGTATCATTAATCACTAGTGC TTCTTCTGGTACTAAAAGTGG	54	348	Di Cesare et al., 2013
sul1	Sul1-1 Sul1-2	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	65	162	Pei et al., 2006
sul2	Sul2-1 Sul2-2	TCCGGTGGAGGCCGGTATCTGG CGGGAATGCCATCTGCCTTGAG	57.5	190	Pei et al., 2006
O139 *rfb*	O139-1 O139-2	AGCCTCTTTATTACGGGTGG GTCAAACCCGATCGTAAAGG	55	449	Hoshino et al., 1998
O1 *rfb*	O1-1 O1-2	GTTTCACTGAACAGATGGG GGTCATCTGTAAGTACAAC	55	192	Hoshino et al., 1998
ctxA	ctx Ats ctx A	CTCAGACGGGATTTGTTAGGCACG TCTATCTCTGTAGCCCCTATTACG	64	301	Nandi et al., 2000
ompW	ompW ts ompW ta	CACCAAGAAGGTGACTTTATTGTG GAACTTATAACCACCCGCG	64	588	Nandi et al., 2000
strA	strA-F strA-R	GAGAGCGTGACCGCCTCATT TCTGCTTCATCTGGCGCTGC	57	862	Popowska et al., 2012
strB	strB-F strB-R	GCTCGGTCGTGAGAACAATC AGAATGCGTCCGCCATCTGT	54	859	Popowska et al., 2012

[a]*Annealing temperature.*

using PCR (Barraud et al., 2010). All primer pairs, target genes, corresponding annealing temperatures, and amplicon sizes are listed in **Table 2**.

RESULTS

Collection of Isolates

The collection was composed of 50 isolates of *V. cholerae* from 21 stations (**Table 1**): 36 (72.0%) were isolated from the 16 LFC stations and 14 from the 5 HFC stations (for description of stations see Baron et al., 2013). None of the 50 *V. cholerae* isolates belonged to O1 or O139 serogroups nor produced the cholera toxin (i.e., the *ctxA* gene was not detected).

Antibiotic Resistance Phenotypes

All tested isolates were susceptible to seven antibiotics of the tested panel: amoxicillin/clavulanic acid, cefotaxime, imipenem, ciprofloxacin, norfloxacin, amikacin, and gentamicin. Eleven isolates (22.0%) were susceptible to all 16 tested antibiotics (three isolates out of 14 from the HFC group and eight out of 36 from the LFC group). The highest proportions of resistant isolates were observed for sulfonamide (70.0%), ampicillin (12.0%), and trimethoprim-sulfamethoxazole (10.0%; **Table 4**).

One isolate, VCH126 from HFC station 19, showed a smaller inhibition zone (15 mm) for doxycycline than the 49 other isolates (**Figure 1B**) and was resistant to tetracycline. Given that the results from the tetracycline disk are typically used to predict susceptibility to doxycycline (Centers for Disease Control Prevention, 1999), we concluded that VCH126 was resistant to doxycycline. VCH3, isolated from LFC station 2, showed an inhibition zone of 7 mm for erythromycin (**Figure 1A**); this isolate was declared resistant to erythromycin.

Among the 39 isolates that were resistant to at least one antibiotic, 12 different profiles were observed. The dominant profile was resistance to sulfonamide only ($n = 26/39$; 66.7%), and the 11 other profiles were represented by only one or two isolates (**Figure 2**). Antimicrobial resistance was not significantly different between LFC and HFC stations (Fisher exact test; $p = 0.91$).

Multidrug resistant isolates are defined as isolates which are resistant to at least three different antimicrobial classes (Magiorakos et al., 2012). Four isolates (10.5%), VCH3, VCH126,

TABLE 3 | Interpretative criteria used to determine antimicrobial susceptibility with the disk diffusion test in *Vibrio cholerae* isolates.

Antimicrobial class	Antimicrobial agent	Abbreviation	Disk content (μg)	Zone diameter interpretative criteria (mm)			References
				Susceptible	Intermediate	Resistant	
ß-lactams	Ampicillin	AM	10	≥17	14–16	≤13	CLSI, 2010a
	Amoxicillin-clavulanic acid	AMC	20/10	≥18	14–17	≤13	CLSI, 2010a
	Cefotaxime	CTX	30	≥26	23–25	≤22	CLSI, 2010a
	Imipenem	IPM	10	≥23	20–22	≤19	CLSI, 2010a
Phenicols	Chloramphenicol	C	30	≥18	13–17	≤12	CLSI, 2010a
Aminoglycosides	Amikacin	AMK	30	≥17	15–16	≤14	CLSI, 2010a
	Gentamicin	GEN	10	≥15	13–14	≤12	CLSI, 2010a
	Streptomycin	STR	10	≥17	13–16	≤12	CLSI, 2016
Quinolones	Ciprofloxacin	CIP	5	≥21	16–20	≤15	CLSI, 2010a
	Nalidixic acid	NA	30	≥19	14–18	≤13	CLSI, 2016
	Norfloxacin	NOR	10	≥17	13–16	≤12	CLSI, 2016
Folate pathway inhibitors	Sulfonamide	SSS	300	≥17	13–16	≤12	CLSI, 2010a
	Trimethoprim-sulfamethoxazole	SXT	1.25/23.75	≥16	11–15	≤10	CLSI, 2010a
Tetracyclines	Tetracycline	TET	30	≥15	12–14	≤11	CLSI, 2010a
	Doxycycline	DO	30	–	–	–	
Macrolides	Erythromycin	ERY	15	≥17	13–16	≤12	CLSI, 2016

Interpretative criteria specific for Vibrio spp., including V. cholerae described in CLSI document M45 3rd edition (CLSI, 2010a) are adapted from those for Enterobacteriaceae M100 25S (CLSI, 2010b). For streptomycin, norfloxacin and nalidixic acid breakpoints described for Enterobacteriaceae in M100 26S (CLSI, 2016) were used. No breakpoints were available for doxycycline or for erythromycin.

VCH85, and VCH55, were multidrug-resistant (**Table 4**). They displayed four different profiles (**Figure 2**). The four isolates were all resistant to streptomycin and sulfonamide, but none were resistant to nalidixic acid. Two isolates (VCH3 and VCH126), from stations 2 and 19, were resistant respectively to six and four classes of antimicrobials. They were both resistant to the antimicrobials belonging to the classes of folate pathway inhibitors (trimethoprim-sulfamethoxazole, sulfonamide), phenicols, tetracycline, and aminoglycosides. These phenotypic resistances may be conferred by genes that are frequently associated with the presence of an SXT element. Nevertheless, the class 1 integron integrase gene was detected in one isolate (VCH3) only. VCH3 was also resistant to erythromycin and ampicillin, but susceptible to doxycycline. VCH126 was also resistant to doxycycline.

Antibiotic Resistance Genotypes

Seven resistance-associated genes were screened on the 50 isolates, regardless of the resistance profile. We chose to screen for resistance associated with streptomycin (*strA*/*B*) and sulfonamide (*sul1*/*2*), because these genes can be present in the SXT constin, and with erythromycin (*ermA*/*B*, *mefA*) which is used in Haiti for cholera treatment.

Among the 50 isolates, 78% possessed at least two resistance-associated genes (**Figure 3**). The genes *sul1*, *ermA,* and *strB* were detected in the four multidrug-resistant isolates. Three of these isolates harbored five resistance-associated genes and one harbored four genes (**Figure 3**). The *sul1* gene was detected in 41 (82%) isolates. The *sul2* gene was detected only in three isolates (6.0%) and always in association with *sul1*; these isolates were resistant to sulfonamide. The *strA* gene was detected only in three isolates (6.0%) and always in association with *strB*; these three isolates were resistant to streptomycin. In contrast, the two other isolates that were resistant to streptomycin did not harbor either *strA* or *strB*. *StrB* gene was detected in 11 other isolates that were susceptible to streptomycin. *ErmA* gene was detected in 90.0% of the isolates, while only one strain (VCH3) was resistant to erythromycin; this strain harbored only *ermA*. *Mef* A was detected in only one strain (VCH90) which also carried the *ermA* gene, but was susceptible to erythromycin (**Table 4**).

DISCUSSION

Only few studies have investigated the presence of resistance-associated genes in *V. cholerae* non-O1/non-O139 strains in association with phenotypic susceptibility (Raissy et al., 2012; Bier et al., 2015; Bhuyan et al., 2016). One study was carried out on 184 *V. cholerae* non-O1/non-O139 strains of clinical and environmental origin (water and fish), and showed that 11 were resistant to ampicillin, but all were sensitive to the other

TABLE 4 | Phenotypic and genotypic profiles of susceptibility in the 50 strains of *V. cholerae* non-O1/non-O139.

			Phenotypic antimicrobial suceptibility[c]										Molecular determinants[d]							
Strain	**Station**	**Group[a]**	**Resistance profile**	**SSS**	**STR**	**E[b]**	**DO[b]**	**TET**	**AM**	**NA**	**SXT**	**C**	***sul1***	***sul2***	***strA***	***strB***	***ermA***	***ermB***	***mefA***	**Integron**
VCH3	2	HFC	C–SXT–AM–TET–SSS–STR–E	R	R	**7***	24	R	R	S	R	R	+	–	+	+	+	–	–	+
VCH126	19	HFC	C–SXT–TET–SSS–STR–DO	R	R	22	**15***	R	S	S	R	R	+	+	+	+	+	–	–	–
VCH85	25	LFC	C–SXT–SSS–STR	R	R	18	25	S	S	S	R	R	+	+	+	+	+	–	–	–
VCH55	17	LFC	AM–SSS–STR	R	R	21	29	S	R	S	S	S	+	–	–	–	–	–	–	–
VCH23	6	LFC	AM–SSS	R	S	22	29	S	R	S	S	S	+	–	–	+	+	–	–	–
VCH28	7	LFC	AM–SSS	R	S	16	27	S	R	S	S	S	+	–	–	–	+	–	–	–
VCH59	18	LFC	AN–SSS	R	S	22	31	S	S	R	S	S	+	–	–	–	+	–	–	–
VCH12	3	LFC	SSS–STR	R	R	18	27	S	S	S	S	S	+	–	–	–	+	–	–	–
VCH113	33	HFC	SXT–SSS	R	S	18	27	S	S	S	R	S	+	–	–	–	+	–	–	–
VCH13	5	LFC	SSS	R	S	18	29	S	S	S	S	S	+	–	–	+	+	–	–	–
VCH66	18	LFC	SSS	R	S	21	26	S	S	S	S	S	+	–	–	–	+	–	–	–
VCH72	19	HFC	SSS	R	S	20	27	S	S	S	S	S	+	–	–	–	–	–	–	–
VCH4	4	LFC	SSS	R	S	20	27	S	S	S	S	S	+	–	–	–	+	–	–	–
VCH31	9	LFC	SSS	R	S	19	28	S	S	S	S	S	+	–	–	–	+	–	–	–
VCH107	30	LFC	SSS	R	S	18	27	S	S	S	S	S	–	–	–	+	+	–	–	–
VCH5	4	LFC	SSS	R	S	17	29	S	S	S	S	S	+	–	–	+	+	–	–	–
VCH30	7	LFC	SSS	R	S	23	28	S	S	S	S	S	+	–	–	–	–	–	–	–
VCH41	15	LFC	SSS	R	S	21	28	S	S	S	S	S	–	–	–	–	+	–	–	–
VCH45	15	LFC	SSS	R	S	18	29	S	S	S	S	S	+	–	–	–	+	–	–	–
VCH52	16	LFC	SSS	R	S	21	31	S	S	S	S	S	–	–	–	–	+	–	–	–
VCH57	17	LFC	SSS	R	S	22	28	S	S	S	S	S	+	–	–	–	–	–	–	–
VCH65	18	LFC	SSS	R	S	21	28	S	S	S	S	S	–	–	–	–	+	–	–	–
VCH76	24	HFC	SSS	R	S	20	29	S	S	S	S	S	+	–	–	–	+	–	–	–
VCH79	24	HFC	SSS	R	S	20	29	S	S	S	S	S	+	–	–	–	+	–	–	–
VCH81	24	HFC	SSS	R	S	23	29	S	S	S	S	S	+	–	–	–	+	–	–	–
VCH90	27	LFC	SSS	R	S	17	29	S	S	S	S	S	+	+	–	+	+	–	+	–
VCH104	30	LFC	SSS	R	S	21	29	S	S	S	S	S	–	–	–	+	–	–	–	–
VCH2	2	HFC	SSS	R	S	18	28	S	S	S	S	S	–	–	–	+	+	–	–	–
VCH9	3	LFC	SSS	R	S	22	29	S	S	S	S	S	+	–	–	–	+	–	–	–
VCH102	30	LFC	SSS	R	S	19	32	S	S	S	S	S	+	–	–	–	+	–	–	–
VCH112	33	HFC	SSS	R	S	21	29	S	S	S	S	S	+	–	–	–	+	–	–	–
VCH48	15	LFC	SSS	R	S	23	30	S	S	S	S	S	+	–	–	–	+	–	–	–
VCH50	16	LFC	SSS	R	S	22	31	S	S	S	S	S	+	–	–	–	+	–	–	–
VCH117	34	HFC	SSS	R	S	21	30	S	S	S	S	S	+	–	–	–	+	–	–	–
VCH146	4	LFC	SSS	R	S	23	30	S	S	S	S	S	+	–	–	–	+	–	–	–
VCH17	5	LFC	AM	S	S	24	30	S	R	S	S	S	+	–	–	+	+	–	–	–

(Continued)

TABLE 4 | Continued

Strain	Station	Group[a]	Phenotypic antimicrobial suceptibility[c]									Molecular determinants[d]							
			Resistance profile	SSS	STR	E[b]	DO[b]	TET	AM	NA	SXT	C	*sul1*	*sul2*	*strA*	*strB*	*ermA*	*ermB*	*mefA*	Integron
VCH26	6	LFC	AM	S	S	21	31	S	R	S	S	S	+	−	−	−	+	−	−	−
VCH69	19	HFC	AN	S	S	22	29	S	S	R	S	S	+	−	−	−	+	−	−	−
VCH61	18	LFC	SXT	S	S	23	26	S	S	S	R	S	−	−	−	−	+	−	−	−
VCH22	6	LFC	S	S	S	20	32	S	S	S	S	S	+	−	−	+	+	−	−	−
VCH7	3	LFC	S	S	S	20	30	S	S	S	S	S	+	−	−	−	+	−	−	−
VCH34−1	10	LFC	S	S	S	19	32	S	S	S	S	S	−	−	−	+	+	−	−	−
VCH40	14	LFC	S	S	S	19	30	S	S	S	S	S	−	−	−	−	+	−	−	−
VCH44	15	LFC	S	S	S	20	30	S	S	S	S	S	+	−	−	−	+	−	−	−
VCH116	34	HFC	S	S	S	20	30	S	S	S	S	S	+	−	−	−	+	−	−	−
VCH62	18	LFC	S	S	S	22	32	S	S	S	S	S	+	−	−	−	+	−	−	−
VCH70	19	HFC	S	S	S	21	34	S	S	S	S	S	+	−	−	−	+	−	−	−
VCH89	26	LFC	S	S	S	14	30	S	S	S	S	S	−	−	−	−	+	−	−	−
VCH131	34	HFC	S	S	S	21	32	S	S	S	S	S	+	−	−	+	+	−	−	−
VCH20	6	LFC	S	S	S	17	31	S	S	S	S	S	+	−	−	−	+	−	−	−

SSS, sulfonamide; STR, streptomycin; E, erythromycin; DO, doxycycline; TET, tetracycline; AM, ampicillin; NA, nalidixic acid; SXT, trimethoprim-sulfamethoxazole and C, chloramphenicol; R, resistant; S, susceptible; all intermediate results were considered as resistant.

[a]Based on the fecal contamination level, the 21 sampled sites were divided into two groups. High fecal contamination (HFC) with E. coli $>10^4$ CFU/100 mL; low fecal contamination (LFC) with E. coli $\leq 10^4$ CFU/100 mL.

[b]For doxycycline and erythromycin, as no interpretative criteria was available, the diameter of inhibition is given. indicates that the isolate was resistant to this antimicrobial agent.*

[c]All the isolates were susceptible to amoxicillin/clavulanic acid, amikacin, gentamicin, cefotaxime, ciprofloxacin, imipenem and norfloxacin, and were not included in this table.

[d]+, gene/integron was detected by PCR; -, gene/integron was not detected by PCR.

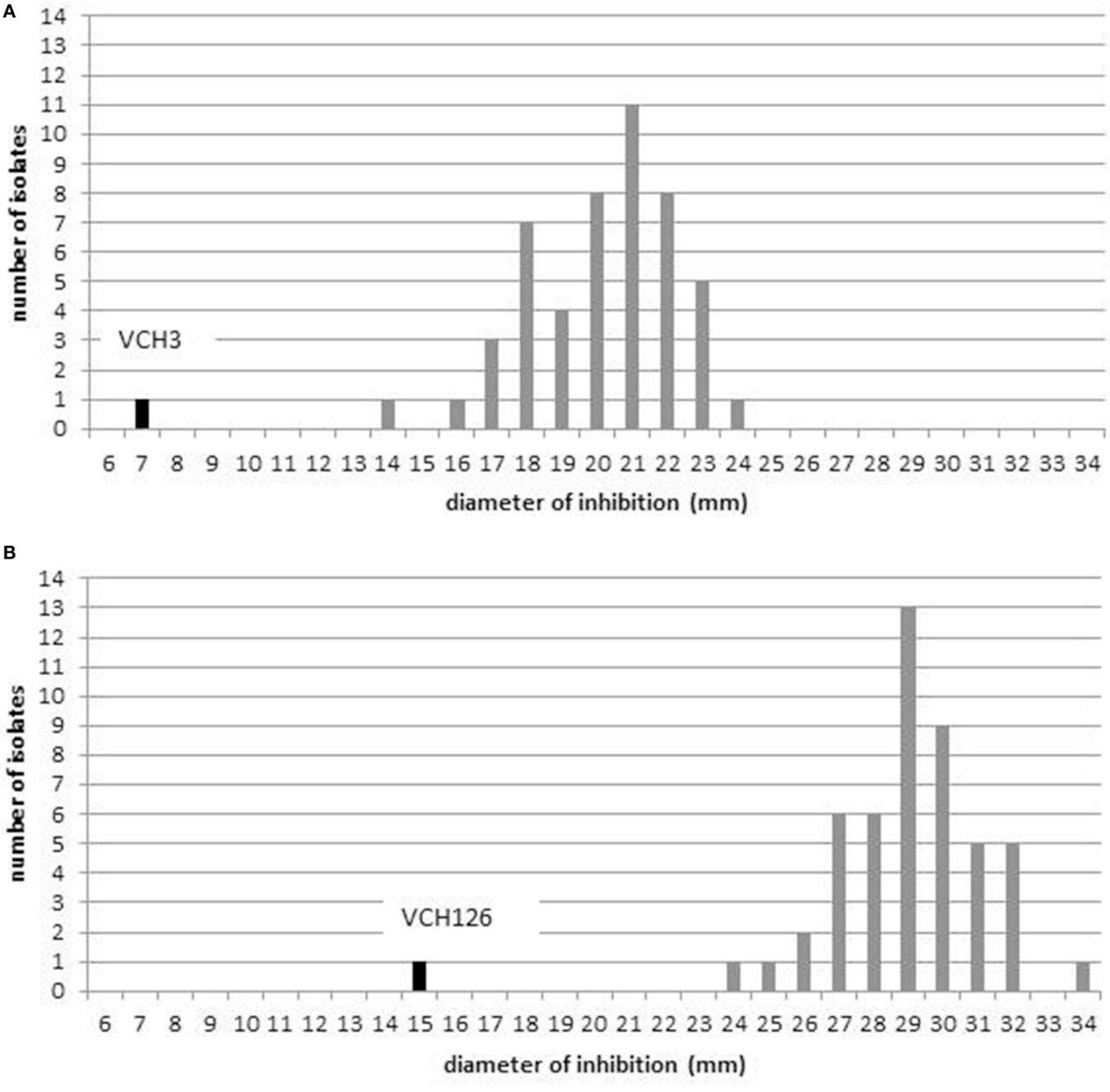

FIGURE 1 | Distribution of diffusion zone diameters: (A) erythromycin (15 μg); (B) doxycycline (30 μg).

beta-lactam antibiotics tested, with the exception of four strains resistant to carbapenems (Bier et al., 2015). However, no beta-lactamase-coding genes were found. The other study screened for three resistance-associated genes (*ermB*, *strA,* and *sul2*) on three strains of *V. cholerae* isolated from seafood in Iran. Only the *strA* gene was detected in one isolate which was resistant to streptomycin, amikacin, and gentamicin (Raissy et al., 2012).

In this study, we detected *strA* in three isolates which were resistant to streptomycin but susceptible to the other aminoglycosides tested. The *ermA* gene was not detected, but the *ermB* gene was detected in 90% of *V. cholerae* isolates, although only one isolate showed a resistance phenotype. This gene, which codes for the methylation of the target, is frequently detected in *Staphylococcus* spp. On the contrary, one strain possessed both the *ermA* gene and the *mefA* gene, which codes for an efflux pump, another mechanism of resistance. Nonetheless, this isolate presented a susceptible phenotype (zone of inhibition = 17 mm).

Our study indicates that the PCR and susceptibility testing approaches are complementary, because they show the presence of genes associated with resistance in susceptible strains and also their absence in resistant strains. This indicates that the link between genes and phenotypes is probably complex.

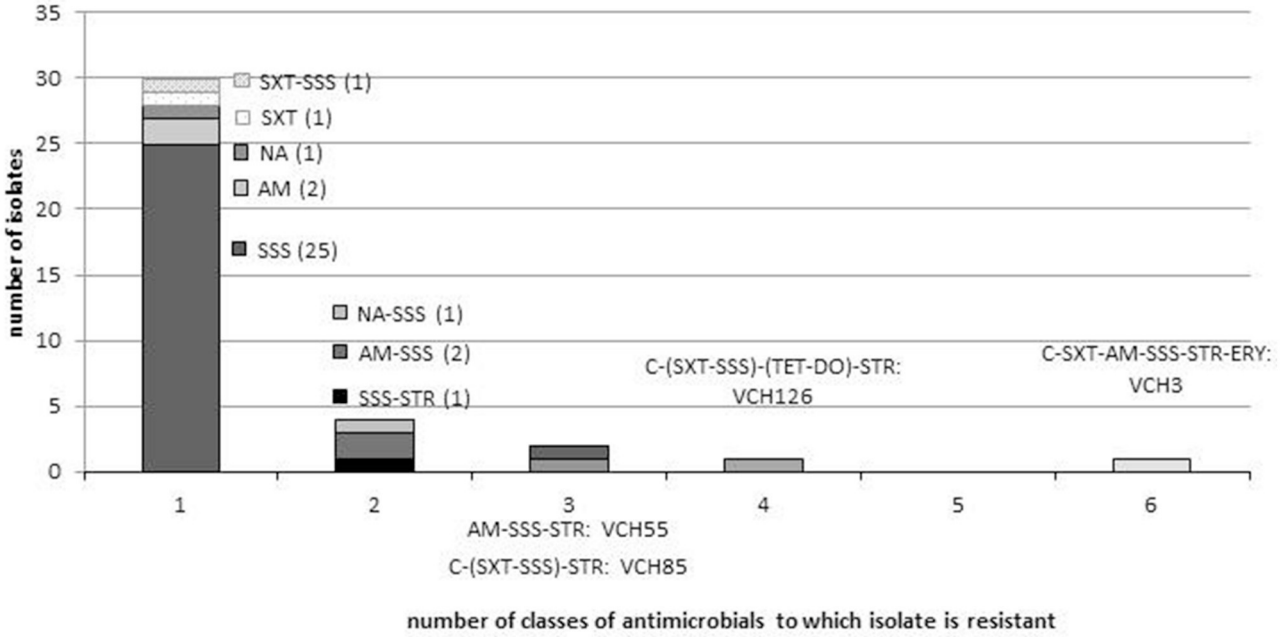

FIGURE 2 | Distribution of resistance profiles in *V. cholerae* non-O1/non-O139 isolates. The number of isolates for each resistance profile is indicated in brackets, and isolate identity is indicated only for the four multidrug-resistant strains. AM, Ampicillin; C, chloramphenicol; NA, nalidixic acid; STR, streptomycin; TET, tetracycline; DO, doxycycline; SSS, sulfonamide; SXT, trimethoprim-sulfamethoxazole; ERY, erythromycin. SXT and SSS belong to the class of folate inhibitor pathway and DO and TET to the tetracycline class.

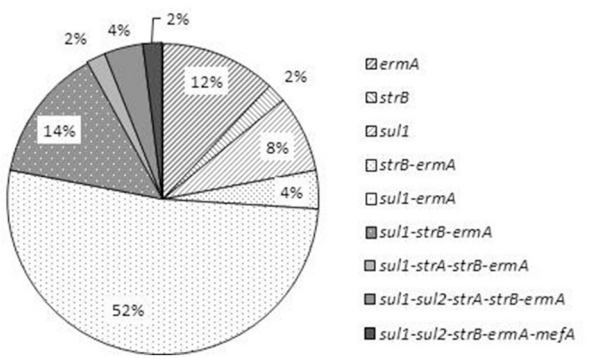

FIGURE 3 | Distribution of the profiles of resistance-associated genes in the 50 *V. cholerae* non-O1/non-O139 isolates.

Interestingly, the multidrug-resistant isolates were those that harbored the highest number of resistance-associated genes. Even if a larger panel of associated resistance genes could be investigated, it would not be exhaustive.

Screening for resistance genes is just a first step: it provides information on the reservoir of resistance genes in the autochthonous aquatic *V. cholerae* non-O1/non-O139 population in our collection of isolates. The next step requires the localization of these genes (chromosome, plasmid, ICE) to estimate their potential for dissemination more precisely. Some of these resistance-associated genes are associated with mobile genetic elements. The *sul1/2* genes have been detected in class 1 integrons or/and in plasmids in *V. cholerae* O1 (Dalsgaard et al., 2001; Iwanaga et al., 2004; Ceccarelli et al., 2006) and in non-O1/non-O139 serogroups (Dalsgaard et al., 2000). The *strA/B* genes are associated with the class 1 integron as well as the SXT element (Hochhut et al., 2001; Iwanaga et al., 2004) in *V. cholerae* O1. In this study, a class 1 integron was detected in only one strain (VCH3), which showed the expected multidrug resistance (chloramphenicol, trimethoprim-sulfamethoxazole, ampicillin, tetracycline, and sulfonamide) that can be harbored by the SXT/R391 integrative and conjugative element (ICE). In Haiti, Ceccarelli et al. (2013) described the presence of two types of SXT/R391 ICEs displaying different genetic organizations, one in O1 serogroup strains (ICEVchHai1) and another (ICEVchHai2) in some clinical isolates of non-O1/non-O139 serogroups (Ceccarelli et al., 2013). ICEVchHai2 lacks the antibiotic resistance cluster typically inserted in variable region 3. It remains to be seen whether VCH3 harbors an ICE and, if so, which genetic organization it possesses.

Considering the Haitian situation, we compared the susceptibility profiles of the non-O1/non-O139 isolates with those of *V. cholerae* O1 of either clinical or environmental origin, which have been published since the beginning of the cholera outbreak in October 2010 (Sjölund-Karlsson et al., 2011; Talkington et al., 2011; Alam et al., 2014, 2015; Folster et al., 2014). Our results clearly indicate that the non-O1/non-O139 *V. cholerae* isolated from surface waters showed phenotypical antimicrobial patterns different from those of the epidemic strain.

Two collections of toxigenic *V. cholerae* O1 strains have been tested for antimicrobial susceptibility: 122 laboratory-confirmed *V. cholerae* O1 clinical isolates, recovered by the National Public Health Laboratory in Haiti from October 2010 to January 2011 (Sjölund-Karlsson et al., 2011) and 17 toxigenic *V. cholerae* O1 isolates collected from surface waters in the West Department from April 2013 to March 2014 (Alam et al., 2015). These *V. cholerae* O1 strains, regardless of their origin (clinical or environmental) all displayed the same multidrug resistance profile: resistance to streptomycin, sulfamethoxazole, trimethoprim-sulfamethoxazole, and nalidixic acid. The 50 *V. cholerae* non-O1/non-O139 strains collected in our study displayed 12 different profiles of resistance but only 8.0% of them were multidrug resistant. Two multidrug-resistant strains isolated from wastewater were resistant to four and six families of antibiotics. None of the four multidrug-resistant non-O1/non-O139 isolates studied displayed the phenotypical profile of the O1 serogroup. In the Alam et al. (2015) study, all 17 isolates of *V. cholerae* O1 from aquatic environments were susceptible to doxycycline, tetracycline, chloramphenicol, and ampicillin. In contrast, among the non-O1/non-O139 isolates in this study, some were resistant to doxycycline (1 strain), tetracycline (2 strains), chloramphenicol (3 strains), and ampicillin (6 strains), and one was resistant to erythromycin.

In another collection of 1029 *V. cholerae* O1 strains collected from 18 towns in Haiti from April 2012 to March 2013, the 115 *V. cholerae* tested by CDC Atlanta (Steenland et al., 2013) showed 100% susceptibility to ampicillin and to tetracycline, whereas a fraction of our *V. cholerae* non-O1/non-O139 were resistant to ampicillin and tetracycline (respectively 12.0 and 4.0%).

These differences of susceptibility profile between the strains of *V. cholerae* non-O1/non-O139 studied and the profile of O1 epidemic strains of Haiti could be partly linked to a difference in the history of exposure to antibiotics. Given that *V. cholerae* non-O1/non-O139 serogroups isolates studied were collected in aquatic environment, we could rise the hypothesis that they have not experienced the same selection pressure as the toxigenic *V. cholerae*. Therefore, because doxycycline, tetracycline, and erythromycin are currently used in the treatment of diarrheal diseases in Haiti, the resistance acquired here by aquatic *V. cholerae* non-O1/non-O139 to these antibiotics may be due to selection pressure on local enteropathogenic bacteria. Accordingly, among the four multidrug-resistant isolates of *V. cholerae* non-O1/non-O139 detected in this study, the two strains harboring resistance to the most antibiotics (four and six different classes of antibiotics, respectively, for VCH126 and VCH3) came from raw wastewater (VCH 126 in Gonaives and VCH3 in Martissant) and these two strains were resistant to two of the three antibiotics used locally: VCH126 was resistant to tetracycline and to doxycycline and VCH3 was resistant to erythromycin and tetracycline but not to doxycycline.

In a previous study, Katz et al. discovered that the epidemic clone is poorly transformable by horizontal gene transfer, and they found no evidence that environmental strains have played any role in its evolution (Katz et al., 2013). This could explain that the susceptibility profile of the epidemic strain has not changed since the beginning of the outbreak, except the single observation of the variant 2012EL-2176 isolated from a clinical case in 2012. That single variant showed the typical resistance phenotype of the outbreak strain, but additional resistance to ampicillin had been acquired and the minimum inhibitory concentration of tetracycline had become intermediate (Folster et al., 2014). Resistance to ampicillin and tetracycline were both found in our non-O1/non-O139 isolates.

Nevertheless, to have information about the possibility of genetic exchanges between non-O1/non-O139 *V. cholerae* isolates and the O1 epidemic clone, deeper genetic investigations are necessary for example by whole genome sequencing, determination of the MLST profile or comparison of mutations on targeted genes (*ctxB*, *QRDR*, etc.).

The prevalence of antibiotic resistance in non-O1/non-O139 *V. cholerae* in an endemic zone of cholera has also been studied in Sanitpur, Assam, North East India (Bhuyan et al., 2016). A collection of 107 strains of *V. cholerae* (among which a single strain of O1 Ogawa) was isolated from 38 water samples (river Brahmapoutra and its tributaries, canal, tea garden) before the rainy season and during monsoon (flooding) in 2012 and 2014. Antibiotic susceptibility was tested by the same disk diffusion method, allowing comparisons with our results. The dominant resistance to sulfonamide in Haïti (70%) was consistent with the dominant resistance to sulfamethoxazole (around 60%). Resistance to two antibiotics was much more frequent in Assam than in Haïti: streptomycin (respectively around 50 *vs*. 10%), and nalidixic acid (around 50 *vs*. 4%). Resistance to three antibiotics was slightly more frequent in Assam than in Haïti: Ampicillin (around 35 *vs*. 12%), Erythromycin (around 15 *vs*. 2%), and tetracycline (around 10 vs. 4%). Two antibiotics gave opposite results: resistance to chloramphenicol was present in Haiti (6%) and absent in Assam; on the contrary, resistance to ciprofloxacin was present in Assam (5%) and absent in Haïti. These differences may be linked to sampling fluctuations but could also be linked to different choices in the two countries for diarrheal diseases and severe cholera treatment.

Thus, in cholera endemic context, the presence of *V. cholerae* non-O1/non-O139 with reduced susceptibility or resistance to antibiotics used for human medicine in the aquatic environment may constitute a mild public health threat. Although the risk of therapeutic failure for infections by a few multidrug-resistant strains seems limited (and the incidence of such infections is still unknown in Haiti), the screening for non-O1/non-O139 serogroups should be included in surveillance programs on diarrhea outbreaks. Moreover, the hypothesis that the aquatic population of *V. cholerae* may constitute a reservoir of resistance genes, supplied by genetic exchanges *in situ* with enteric bacteria such as *E. coli*, which do not survive in aquatic environments, still needs to be investigated.

CONCLUSION

In the context of a cholera outbreak in Haiti, non-O1/non-O139 *V. cholerae* in surface waters showed antimicrobial patterns different from the epidemic strain: there were few

multidrug-resistant strains and a high diversity of resistance profiles, including resistance to doxycycline, tetracycline and erythromycin. In contrast, according to the literature, all the clinical and environmental isolates of toxigenic serogroup O1 in Haiti were susceptible to these three antibiotics or had reduced susceptibility (tetracycline) and all presented the same specific multidrug-resistance profile.

However, the presence of acquired resistance to antibiotics among the autochthonous *V. cholerae* non-O1/non-O139 in the aquatic environment can be construed as the result of a local selection pressure on enteric bacteria in Haiti, where the use of antibiotics is not strictly regulated.

Further research is required to address this public health issue. The ability of the autochthonous aquatic population of *V. cholerae* non-O1/non-O139 to acquire and transfer resistance genes, especially in wastewaters, needs to be investigated. In addition, the contribution of the non-O1/non-O139 serogroups to co-infections of cholera cases, or to diarrhea cases in Haiti, should be documented as part of national surveillance programs of gastro-intestinal infections.

AUTHOR CONTRIBUTIONS

SB, contributed to the design of the work, performed the field study, contributed the acquisition, the analysis and the interpretation of the data, participated to the assays, and wrote the paper. JL, contributed to the design of the work, performed the field study and the interpretation of the data, participated to the assays, and wrote the paper. EL, contributed the acquisition, the analysis and the interpretation of the data for the work, participated to the assays, and revised the paper. RP, contributed to the design of the work, revising the work and final approval of the version to be published. SR, contributed to the design of the work, revising the work and final approval of the version to be published. EJ, contributed to the analysis and the interpretation of the data, and revised the paper. IK, contributed to the analysis and the interpretation of the data, and revised the paper. JB contributed to the acquisition of the data and revised the manuscript.

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Identification of Atypical *El Tor V. cholerae* O1 Ogawa Hosting SXT Element in Senegal, Africa

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Vibrio cholerae O1 is the causative agent of cholera with classical and El Tor, two well-established biotypes. In last 20 years, hybrid strains of classical and El Tor and variant El Tor which carry classical *ctx*B have emerged worldwide. In 2004–2005, Senegal experienced major cholera epidemic with a number of cases totalling more than 31719 with approximately 458 fatal outcomes (CFR, 1.44%). In this retrospective study, fifty isolates out of a total of 403 *V. cholerae* biotype El Tor serovar Ogawa isolates from all areas in Senegal during the 2004–2005 cholera outbreak were randomly selected. Isolates were characterized using phenotypic and genotypic methods. The analysis of antibiotic resistance patterns revealed the predominance of the S-Su-TCY-Tsu phenotype (90% of isolates). The molecular characterization of antibiotic resistance revealed the presence of the SXT element, a self-transmissible chromosomally integrating element in all isolates. Most of *V. cholerae* isolates had an intact virulence cassette (86%) (*ctx*, *zot*, *ace* genes). All isolates tested gave amplification with primers for classical CT, and 10/50 (20%) of isolates carried classical and El Tor *ctx*B. The study reveals the presence of atypical *V. cholerae* O1 El Tor during cholera outbreak in Senegal in 2004–2005.

Keywords: *Vibrio cholerae*, O1 virulence, antibioresistance, SXT element, Senegal

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INTRODUCTION

Cholera is an epidemic diarrheal disease caused by toxigenic *Vibrio cholerae,* serogroup O1 or O139. There are two biotypes in the serogroup O1, classical and El Tor. The seventh pandemic of cholera were due to *V. cholerae* O1, biotype El Tor, began in Celebes (Islands) in 1961 and spread in West African countries in the early 1970s while the fifth and the sixth pandemics of cholera were caused by the classical biotype (Kaper et al., 1995). For over a decade, Africa has been the continent most affected by cholera in terms of the number of individuals infected and the frequency of outbreaks recorded[1]. In 2004–2005, cholera outbreaks occurred in eight countries in Africa with 125,082 cases and 2,230 deaths, CFR 1.78 (WHO, 2006). However, in some areas, the CFR exceeded 10%. In Senegal, the cholera outbreak has caused 31,719 cases with approximately

[1]http://www.who.int/wer

458 deaths (CFR, 1.44%) and the most affected regions were Diourbel and Dakar; the index case was a young Guinean living in a populous district in Dakar (Manga et al., 2008). Smaller scale epidemics have been reported from 2006 to 2008. However, no cases have been reported since July 2010, this is probably due to the monitoring implemented by the Senegalese Ministry for Health (Global Task Force on Cholera Control, 2006).

The pathogenicity of *V. cholerae* O1 and O139 isolates depends on a combination of factors including the coordinated expression of virulence factors, and the secretion of cholera toxin (CT). Molecular analysis of *V. cholerae* revealed the presence of two genetic elements in the genome of pathogenic strains: the lysogenic bacteriophage (CTXø), which hosts at least six genes including toxin genes: (*ctxAB*, *ace*, and *zot* encoding, respectively A and B subunits CT, accessory enterotoxin, and zonula occludens toxin), and the *Vibrio cholerae* pathogenicity island (VPI), which carries genes for the pilus colonization factor, toxin coregulated pilus (TCP) (Pearson et al., 1993; Waldor et al., 1997). Strains named "atypical El Tor" have traits of both classical and El Tor (Nair et al., 2002). Recently, several atypical El Tor strains have been reported, including Matlab variants (Safa et al., 2006), Mozambique variants (Ansaruzzaman et al., 2004), altered El Tor (Nair et al., 2006), and hybrid El Tor strains, harboring the classical CT allele *ctxB1* (Goel et al., 2008; Safa et al., 2008). Safa et al. (2010) proposed the term "atypical El Tor" for all *V. cholerae* O1 El Tor that harbor classical traits.

Vibrio cholerae O1 strains isolated in Africa are known to be resistant to many antibiotics (Ceccarelli et al., 2006; Quilici et al., 2010). Drug-resistance of bacteria is mainly linked to the mobilization and the dissemination of resistance genes through genetic determinants such as plasmids, integrons, and transposons. The SXT element is a self-transmissible mobile genetic element belonging to the family of integrating and conjugative elements (ICEs) that originally was discovered in a *V. cholerae* O139 isolate from India (MO10) which is resistant to streptomycin (Sm), trimethoprim (Tm), sulfamethoxazole (Su), and chloramphenicol (Wozniak et al., 2009; Ceccarelli et al., 2011b). The SXT element is always integrated into the 5′ end of the chromosomal gene *prfC* and able to replicate with the host chromosome (Waldor et al., 1997). Capture and spread of antibiotic resistance determinants by integrons is an effective route of antimicrobial resistance dissemination among Gram-negative bacteria (Mazel, 2006). Several of integrons have been described based on integrase gene. The class 1 integron is widely spread among *V. cholerae* isolates with various types of resistance gene cassettes (Dalsgaard et al., 2001; Ceccarelli et al., 2006). Class 4 integron named superintegrons is a component SXT element found in several bacteria in particular in *V. cholerae*. Superintegrons are the ancestors of multiresistant integrons (Mazel, 2006). Integrons and ICEs have been found in *V. cholerae* isolated in Mozambique, Iran, and India and they have largely contributed to the spread of antibiotic resistance (Amita et al., 2003; Adabi et al., 2009; Pugliese et al., 2009).

In Senegal, there is little data available on the genetic determinants of virulence and antibiotic-resistance among epidemic *V. cholerae* isolates. Despite the description of multiresistant isolates during the lastest cholera outbreaks in Senegal in 1994 (Aidara et al., 1998) and in 2004–2006 (Manga et al., 2008), the molecular mechanisms of antibiotic resistance have never been studied.

The objective of this work was to characterize the genetic determinants of virulence and antibiotic-resistance in *V. cholerae* O1 isolated during the latest cholera outbreak in Senegal.

MATERIALS AND METHODS

Bacterial Isolates

A total of 403 *V. cholerae* serogroup O1 strains were isolated in different areas in Senegal between November 2004 to May 2005 from patients with acute diarrhea. A sampling of fifty isolates were randomly selected to represent the most affected population: Dakar (39); Diourbel (09); Kaolack (01); Louga (01) (**Figure 1**).

All isolates were identified with the API 20E (Biomérieux, Marcy l'Etoile, France) and serotyped using anti-Ogawa, anti-Inaba antisera.

Susceptibility to Antibiotics

Antimicrobial susceptibility testing was performed using the Kirby Bauer disk diffusion method on Müller Hinton agar. The following antibiotics were tested: ampicillin (AM, 10 μg), amoxicillin-clavulanic acid (AMC, 20 μg/10 μg), cefotaxime (CTX, 30 μg), streptomycin (S, 10 μg), tetracycline (TCY, 30 UI), chloramphenicol (CHL, 30 μg), nalidixic acid (NA, 30 μg), pefloxacin (5 μg), trimethoprim (T, 5 μg), sulfamethoxazole (Su, 200 μg), trimethoprim-sulfamethoxazole (TSu, 1.25 μg/23.25 μg). The diameter of inhibition zones was interpreted following the CLSI recommendations for enterobacteria[2].

DNA Extraction

Total DNA was obtained by using QIAamp DNA Mini Kit (Qiagen SA, Courtaboeuf, France).

PCR Amplifications

Detection of Antibiotic Resistance and Virulence Molecular Markers

PCR analysis was performed for detection of genetic determinants of antibiotic resistance and virulence. Amplification was carried out and virulence with primers described elsewhere (Ploy et al., 2000; Hochhut et al., 2001; Shi et al., 2006), and GenBank accession number AF 099172. For the detection of virulence genes, primers used as those described by Ogawa et al. (1990), Keasler (1993), and Shi et al. (1998) (**Table 1**) and GenBank accession numbers (AF262318, GQ485654). Chromosomal integration was detected by amplification of the right SXT element chromosome junction (*attP-prfC* gene sequence) (**Table 1**).

All amplified DNA fragments were resolved by conventional electrophoresis in 1% agarose gel, stained with ethidium bromide and visualized under UV light.

[2]http://www.clsi.org/

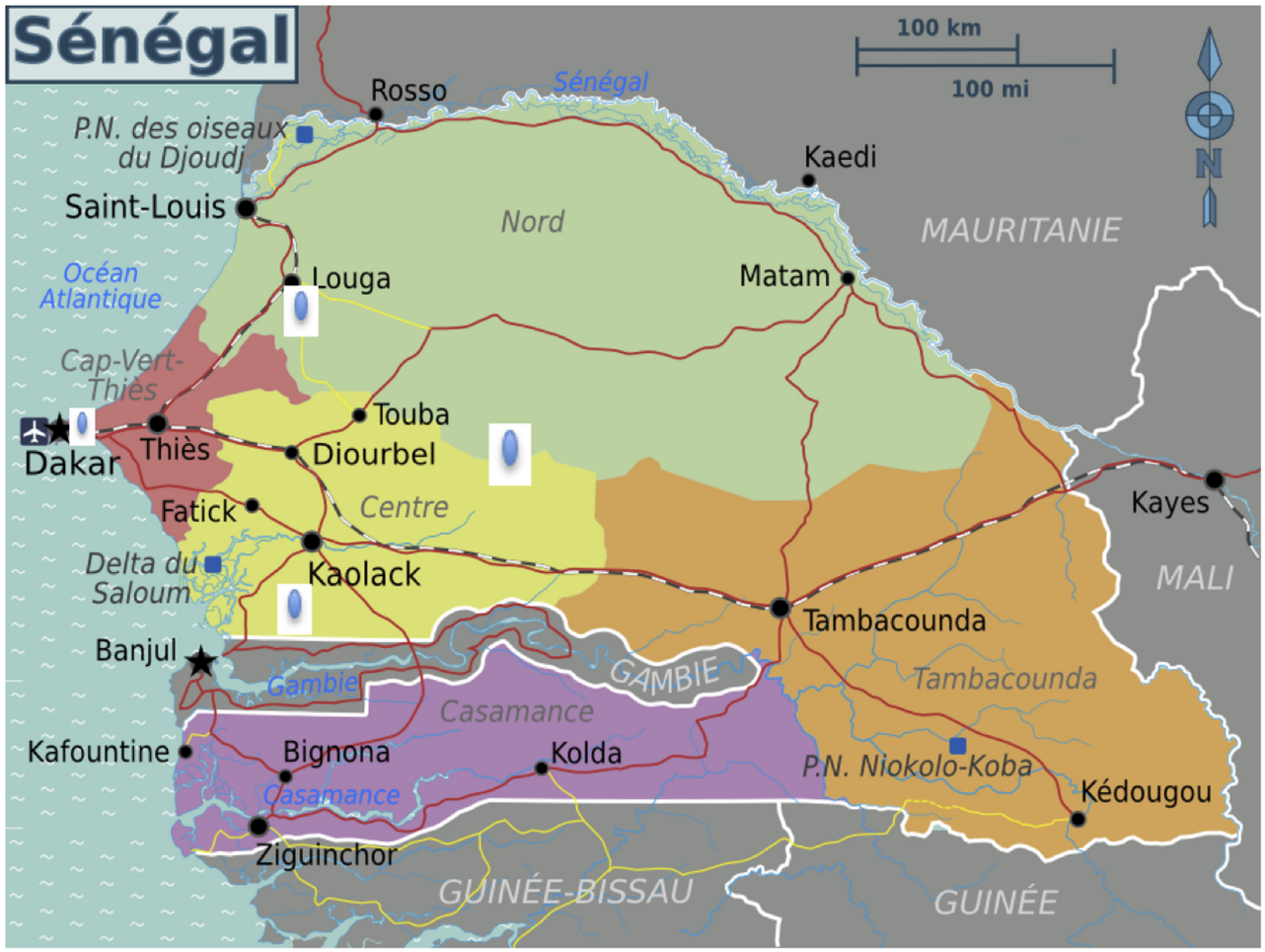

FIGURE 1 | Origin of isolates (Dakar: 39; Diourbel: 09; Kaolack: 01; Louga: 01).

ctxB Typing by MAMA PCR

Mismatch Amplification Mutation Assay (MAMA) based PCR was performed to detect the presence of *ctxB* classical and or El Tor biotype *V. cholerae* O1 isolates, using specific primers described elsewhere (Morita et al., 2008).

Cloning and Sequencing

The integrase SXT fragment (int_{SXT}) was purified with the QIAquick kit (Qiagen SA, Courtaboeuf, France), and cloned with the pGEMT vector (Promega, Madison, WI, USA), transformed into XL1-Blue competent cells (Stratagene, Garden Grove, CA, USA).

The insert of the recombinant plasmid was sequenced with dye terminator on ABI Prism automatic sequencer as described by the manufacturers. The sequences were analyzed by nucleotide BLAST search at the National Center for Biotechnology Information (NCBI) website[3]. Primers were used for cloning and sequencing are listed on **Table 1**.

Conjugation Experiments

Conjugation experiments were used to transfer resistance determinants from *V. cholerae* O1 isolates into nalidixic acid-resistant *E. coli* C1 strain. Mating experiments were carried out by mixing volumes of Luria Bertani (LB) broth in a ratio 2:1 overnight culture of donor and recipient strains. The cultures were transferred to LB agar plates containing trimethoprim (32 µg/ml), sulfamethoxazole (160 µg/ml), and nalidixic acid (50 µg/ml). To confirm the transfer of antibiotic resistance genes, transconjugants were tested for sensitivity to antibiotic and by PCR.

This study was carried out in accordance with the recommendations of Senegalese Ethical Committee, with informed consent from all subjects. The isolates used in this study were taken for the purposes of research, and the protocol was approved by the Senegalese Ethical Committee (N°0046/MSAS/DRPS/CNERS)[4].

[3]https://blast.ncbi.nlm.nih.gov/Blast.cgi

[4]www.healthresearchweb.org/fr/senegal/ethics_1057

TABLE 1 | Primers used in this study.

Primer sequence (5′–3′)	Primers	Target gene	Reference
ACA TGT GAT GGC GAC GCA CGA	intI1L	*intI1*	Ploy et al., 2000
ATT TCT GTC CTG GCT GGC GA	intI1R		
CAC GGA TAT GCG ACA AAA AGG T	intI2L	*intI2*	Ploy et al., 2000
GTA GCA AAC GAG TGA CGA AAT G	intI2R		
GCC TCC GGC AGC GAC TTT CAG	intI3L	*intI3*	Ploy et al., 2000
ACG GATCTGCCAAACCTGACT	intI3R		
GTG TTC GCG AAT TTA TGC	Int4-1	*intI4*	Shi et al., 2006
ACG GGA TAA TGG GCT TAA	Int4-2		
GCT GGA TAG GTT AAG GGC GG	SXT1	int_{SXT}	Hochhut and Waldor, 1999
CTC TAT GGG CAC TGT CCA CAT TG	SXT2		
CAA GCG GAA AAA AAT CCA TA	SXT-R1F	*SXT*	Pugliese et al., 2009
AGAGTCAACTGCGGTCAGAG	SXT-R1R	*prfC*	
CTCAGACGGGATTTGTTAGGCACG	ctxA-1	*ctxA*	Keasler, 1993
TCTATCTCTGTAGCCCCTATTACG	ctxA-2		
GGG CGA GAA AGG ACG C	Zot-1	*zot*	Shi et al., 1998
CCT TGT AGC GGT AGC TCG	Zot-2		
TAA GGA TGT GCT TAT GAT GGA CAC CC	Ace-1	ace	Shi et al., 1998
CGT GAT GAA TAA AGA TAC TCA TAG G	Ace-2		
CAC GAT AAG AAA ACC GGT CAAGAG	TcpA-F	*tcp* (Classical)	Ogawa et al., 1990
ACC AAA TGC AAC GCC GAA TGG AGC	TcpA-R		
GAA GAA GTT TGT AAA AGA AGA ACA C	TcpA-F	*tcp* (El Tor)	Ogawa et al., 1990
GAA AGG ACC TTC TTT CAC GTT G	TcpA-R		
ATC AG TGA TTC AAT CAT TC	RstC-F	*RST*(Classical)	AF 262318
ATT TAAGAG TTG AGA GAG AT	RstC-R		
AGA ATG TCT TAT CAG CAT AC	RstET-F	*RST*(El Tor)	
TAG CCA CCC AAA GAA AGG CA	RstET-R		
GATGGCAGCTTGCCGCAACCTC	SXT-X	int_{SXT}	This study
GGAATTCGGCAGTCAAGGCAGAGGGC	SXT-X-M	int_{SXT}	This study
CATCAGAAGTATAGAAATCTGACTG	SXT-X-2	int_{SXT}	This study
TGTACGATCATTGAAATAAAAAGACC	SXT-X-3	int_{SXT}	This study
GGAATTCGCGTTGCTGATCCGCAGCTTT	SXT-1-M	int_{SXT}	This study
CGGGATCCGTTGTAGACCAACTTTTAACGTATAC	SXT-I-3-M	int_{SXT}	This study
CCAGCTATTGAGCTGATTGAACTG	SXT-I	int_{SXT}	This study

RESULTS

Antimicrobial Susceptibility

In this retrospective study, we show that isolates were resistant to at least four antibiotics including streptomycin (S), trimethoprim (T), sulfamethoxazole (Su), trimethoprim-sulfamethoxazole (Tsu). The analysis of antibiotic resistance patterns reveals three phenotypes: S-Su-T-TSu (90%), S-Su-T-TSu-C (8%), S-Su-T-TSu-AM (2%). All isolates were resistant to trimethoprim-sulfamethoxazole but susceptible to tetracyclines.

Detection of Genetic Determinants of Antimicrobial Resistance: Detection of Integrons, Resistance Genes, and SXT Element

All isolates were negative for class 1, 2, and 3 integrons. A 900 bp PCR product of the *intI4* gene was obtained for all isolates.

The amplification of SXT integrase revealed an amplicon size of 3 kb in all isolates, different from the expected size, i.e., 592 bp. The SXT integrase from senegalese isolates was identical to a fragment of *V. cholerae* KN14, isolated in Kenya GenBank accession number (AB535680). The integration of the SXT element in the chromosome was highlighted by the amplification of the right SXT element-chromosome junction (*attP-prf*C) which producted a 785 bp PCR product.

To identify genes hosted by the ICE circulating in Senegal, PCR analysis revealed the presence of the following antibiotic resistances genes (*sulI*, *floR*, *strA*, and *dfrA1*), except *dfr18* gene.

Detection of Virulence Markers

The *ctxB, zot, ace* genes in the CTX element were present, respectively, in 98, 92, and 88% of isolates. All isolates gave positive results for *tcpA* (classical and El Tor) (**Figure 2**) and for bacteriophage *rst*R repressor gene of the El Tor and classical types. The detection of virulence genes revealed the presence of the genome of filamentous bacteriophage CTXø.

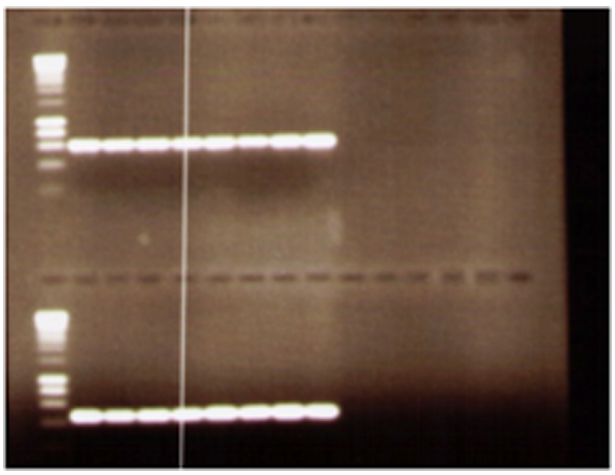

FIGURE 2 | Agarose gel electrophoresis of examples of PCR products of *Vibrio cholerae* O1 isolates using *tcpA* (classical and El Tor).

ctxB Typing

All isolates tested gave amplification with primers for classical CT allele *ctxB1*, and 10/50 (20%) of isolates gave amplicons with primers specific for classical and El Tor CT allele *ctxB1*.

Conjugation Experiments

Mating experiments revealed transfer of the resistance determinants to chloramphenicol, streptomycin, sulfamethoxazole, and trimethoprim by conjugation. To identify a possible ICE-mediated resistance, transconjugants were tested for SXT-related elements. Antimicrobial susceptibility testing of *V. cholerae* transconjugants showed that resistance profiles were expressed by each of the transconjugants. The PCRs for detection of the int_{SXT} element-integrase gene gave an amplicon of the same size of 3 Kbp.

DISCUSSION

Senegalese isolates are still susceptible to quinolones while resistance to nalidixic acid or reduced sensitivity to a fluoroquinolone was described in Africa and India (Quilici et al., 2010; Ismail et al., 2013; Kutar et al., 2013).

Our results showed that class 1, 2, and 3 integrons were not involved in the spread of resistance among Senegalese *V. cholerae* O1 isolates, even though they have been detected in Mozambican *V. cholerae* O1 isolates and other Gram-negative enteric bacteria in Senegal (Hochhut and Waldor, 1999; Dalsgaard et al., 2001; Gassama et al., 2004; Gassama-Sow et al., 2006). According to Mazel (2006), the *IntI4* gene associated with superintegron is characterized by a large number of gene cassettes, closely associated with genome evolution rather than the capture of antibiotic resistance.

All genes (*sulI*, *floR*, *strA*, and *dfrA1*) excepted *dfrA18 gene* were found in our isolates suggesting that the SXT isolated in Senegal is closely related to the SXT^{ET}. Kenyan isolates also lacked the *dfrA18* gene (Kiiru et al., 2009). Since the emergence of SXT in *V. cholerae* O139, several studies on *V. cholerae* O1 have found this ICE as responsible for the dissemination of antibiotic resistance in Africa and Asia (Dalsgaard et al., 2001; Amita et al., 2003; Opintan et al., 2008; Adabi et al., 2009; Pugliese et al., 2009). ICEs of the SXT/R391 family are usually found in atypical O1 El Tor *V. cholerae* epidemic strains; they confer a narrow antibiotic resistance profile (Wozniak et al., 2009). Conjugation experiments revealed that although the isolates carry the SXT/R391-like elements which confers resistance to streptomycin, trimethoprim sulfamethoxazole and chloramphenicol, they lack multiple resistant integrons.

Further studies are needed to characterize and completely sequenced the SXT-related ICE in senegalese isolates.

Vibrio cholerae O1 strains isolated during the lastest outbreak (2004–2005) in Senegal were "atypical" as appointed by Safa et al. The presence of these atypical isolates may explain the disease severity After 2001, atypical *V. cholerae* O1 strains have emerged in India and spread worldwide, particularly in Africa. Indeed, atypical *V. cholerae* O1 strains were described in Mozambique (B33) (Ansaruzzaman et al., 2004), and Angola (Ceccarelli et al., 2011a). The appereance of atypical strains in Senegal is enigmatic, and suggest that probably these new strains followed the same West African path used by cholera to enter Africa in the early 1970s. The presence of int_{sxt} identical to a fragment of *V. cholerae* KN14 could confirm this hypothesis. The global replacement of El Tor prototype by atypical strains indicates the evolution of *V. cholerae* O1. Our study revealed that atypical strains are also in the process of replacing El Tor strains; this phenonenon has been described in Eastern Africa (Ceccarelli et al., 2011a). This global replacement is believed to be due to unknown environmental factors and phages contribution (Faruque and Mekalanos, 2012).

CONCLUSION

Based to our results, atypical *V. cholerae* O1 El Tor strains were responsible for cholera outbreak in Senegal in 2004–2005.

According to our study integrons were not involved in the spread of resistance among senegalese isolates of *V. cholerae* O1 even though they have been detected in other Gram-negative enteric bacteria in Senegal. However, the detection of the SXT element in all isolates, irrespective of their resistance phenotypes, could have a clinical significance and should be monitored to avoid dissemination in other bacteria. The understanding of the basis of antimicrobial resistance patterns could inform guidelines for empirical treatment to reduce injudicious antimicrobial use. Further studies should be conducted to characterize the SXT element identified in Senegalese *V. cholerae* isolates. The genetic changes occurred in *V. cholerae* O1 El Tor strains need to be monitored to prevent severe cholera outbreaks in Africa.

AUTHOR CONTRIBUTIONS

BS-B participated in the molecular genetic studies and drafted the manuscript. MD carried out the molecular genetic studies.

AW participated in the molecular genetic studies. AS participated on the identification of isolates. GC helped in drafting the manuscript. AIS helped on the collection and the identification of isolates in all sites. CB participated in the design and coordination of the study. AG-S designed the study, and wrote the manuscript. All authors read and approved the final manuscript.

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Bile Sensing: The Activation of *Vibrio parahaemolyticus* Virulence

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Bacteria must develop resistance to various inhospitable conditions in order to survive in the human gastrointestinal tract. Bile, which is secreted by the liver, and plays an important role in food digestion also has antimicrobial properties and is able to disrupt cellular homeostasis. Paradoxically, although bile is one of the guts defenses, many studies have reported that bacteria such as *Vibrio parahaemolyticus* can sense bile and use its presence as an environmental cue to upregulate virulence genes during infection. This article aims to discuss how bile is detected by *V. parahaemolyticus* and its role in regulating type III secretion system 2 leading to human infection. This bile–bacteria interaction pathway gives us a clearer understanding of the biochemical and structural analysis of the bacterial receptors involved in mediating a response to bile salts which appear to be a significant environmental cue during initiation of an infection.

Keywords: bacteria, human gastrointestinal tract, bile, *Vibrio parahaemolyticus*, type III secretion system 2

INTRODUCTION

Humans have a complex digestive system that not only aids in digestion of food but also has a role in self-defense against microorganisms in the body. Microorganisms such as bacteria have to tolerate various extreme environments in order to survive in the human gastrointestinal tract (Begley et al., 2005). Bile is an alkaline substance that is continuously secreted by liver and stored in the gall bladder in humans; the presence of bile plays an important role in the digestive system process. During the digestive process, the lipids are emulsified and solubilized by bile. In addition, bile has the capability to affect the cell membranes proteins and phospholipid structures and cause cellular homeostasis. Bile aids in the emulsification and solubilization of lipids in the gastrointestinal tract. In addition, it has the capability to affect the phospholipids and proteins of cell membranes and disrupt cellular homeostasis. Hence the ability to overcome the potentially lethal effects of bile is important for bacteria in order to survive and subsequently colonize the gastrointestinal tract (Begley et al., 2005; Hung and Mekalanos, 2005; Edwards and Slater, 2009). Recently, there has been increased evidence showing bile is been used as a signaling cue by enteric

bacteria to initiate virulence genes in host infection (Pope et al., 1995; Krukonis and DiRita, 2003; Prouty et al., 2004). Bile acids are a major component of crude bile that triggers the expression of bacterial virulence in the body. In this article, we aim to discuss how *Vibrio parahaemolyticus* senses bile in the human GI tract to regulate type III secretion system 2. This bile–bacteria interaction pathway gives us a clearer understanding of the biochemical and structural analysis of the bacterial receptors that takes action upon sensing the bile salts during an infection.

VIRULENCE FACTORS OF *Vibrio parahaemolyticus*

The bacterial protagonist in this story is *V. parahaemolyticus*, a Gram-negative, halophilic bacterium which naturally inhabits marine and estuarine environments worldwide (Zhang and Orth, 2013; Letchumanan et al., 2014). *V. parahaemolyticus* is recognized as the causative agent of foodborne gastroenteritis, a disease often associated with consumption of raw or undercooked seafood (Raghunath, 2015). Global climate change and rising ocean temperatures have led to the increase in the distribution of this pathogen worldwide (O'Boyle and Boyd, 2014). This is of concern as approximately half the reported foodborne cases in Asian countries are caused by *V. parahaemolyticus* (Alam et al., 2002; Bhuiyan et al., 2002). Frequent outbreaks of *V. parahaemolyticus* cases have also been reported in the United States and coastal countries of Europe such as Spain, Italy, and Norway (Caburlotto et al., 2008; Scallan et al., 2011; Ottaviani et al., 2013).

V. parahaemolyticus possess wide range of virulence factors that enables them to cause a gastrointestinal infection including adhesin (Liu and Chen, 2015), toxins, and secreted effectors (Zhang and Orth, 2013). These virulence factors play a vital role in the pathogenesis of the disease. During the initial host cell binding, adhesion is the first important step in bacterial pathogenesis (Liu and Chen, 2015). This factor is present on the surface of all *V. parahaemolyticus* to form a platform for them to attach onto host cell and secrete toxins during an infection (Broberg et al., 2011; Zhang and Orth, 2013; Letchumanan et al., 2014). The thermostable direct hemolysin (*tdh*) and TDH related hemolysin (*trh*) are the two major toxins found in *V. parahaemolyticus* (Honda et al., 1988; Nishibuchi et al., 1992; Okada et al., 2009). These two virulence toxins are believed to cause hemolysis and cytotoxic activity in a host cell (Broberg et al., 2011; Ceccarelli et al., 2013). The *tdh* is a pore-forming toxin which forms pores in the erythrocyte's membrane (Matsuda et al., 2010). The large pore size enables both water and ions to flow through the membrane (Honda and Iida, 1993). The subsequent alterations in ion flux in the intestine causes the diarrhea which is observed during an infection (Raghunath, 2015). Similar to the *tdh* gene, the *trh* gene also triggers cl^- channels resulting in altered ion flux during an infection (Takahashi et al., 2000). Both the *tdh* and *trh* are correlated with pathogenic *V. parahaemolyticus* strains, however, these genes do not completely account for the pathogenicity of *V. parahaemolyticus* (Lynch et al., 2005). There are several studies have reported that even in the absence of *tdh* and/or *trh* genes, *V. parahaemolyticus* strains remain virulent indicating the existence of other virulence factors (Jones et al., 2012; Pazhani et al., 2014). The thermolabile hemolysin (*tlh*), a type of phospholipase is another virulence toxin found in *V. parahaemlyticus* (DePaola et al., 2003; Zhang and Austin, 2005). Although the specific function of this gene in human infection remains unclear, *tlh* gene expression is upregulated under conditions mimic the intestinal environmental of human (Broberg et al., 2011; West et al., 2013). Hence, in the process of infection, *tlh* gene may be equally important as the *tdh* and *trh* genes.

The type III secretion system (T3SSs) is another important virulence factor of *V. parahaemolyticus* which is responsible for its pathogenicity (Broberg et al., 2011). This protein like structure has a secretion apparatus consisting of three main parts: the basal body that extends into the inner and outer membranes; a needle like structure that allows toxins to travel; and the translocon which is a pore injected into a target cell membrane (Izore et al., 2011). The T3SS1 and T3SS2 are the two main T3SSs encoded by *V. parahaemolyticus*. The cytotoxic T3SS1 is reported to be present in all *V. parahaemolyticus* and causes mouse lethality and possible initiation of autophagy (Park et al., 2004; Burdette et al., 2009; Hiyoshi et al., 2010). The enterotoxin T3SS2, on the other hand plays a vital part in determining the environmental fitness of strains (Hiyoshi et al., 2010; Matz et al., 2011). The T3SS2, *tdh* and *trh* are also known to be encoded on the pathogenicity island (Vp-PAI), signifying that *V. parahaemolyticus* acquires virulence determines through horizontal gene transfer (Okada et al., 2009; Matz et al., 2011). It is believed that the progression and severity of infection in humans are effected by the *V. parahaemolyticus* T3SS toxins (Ono et al., 2006). The strains that possess this needle-like T3SSs have the advantage of being able to secrete bacterial protein effectors directly into the host cell membrane and cytoplasm without facing the extracellular environment (Cornelis, 2006). In addition, the T3SS2 is suggested to be associated with *tdh*- and/or *trh*-positive *V. parahaemolyticus* strains (Raghunath, 2015). There are two distinct lineages of T3SS2 that have been described and associations were demonstrated of *tdh* with T3SSα and *trh* with T3SSβ (Park et al., 2004; Noriea et al., 2010). This could suggest that *V. parahaemolyticus* strains with the *tdh* and/or *trh* genes and T3SSs system have better ability to overcome host defenses in humans, conferring virulence that facilitates the development of infection.

Further analysis on the virulence properties has led to the discovery of type VI secretion systems encoded by T6SS1 and T6SS2 in *V. parahaemolyticus*. The T6SS1 is located on chromosome 1 where else, T6SS2 is located on chromosome 2 on *V. parahaemolyticus* RIMD 2210633 (Boyd et al., 2008; Izutsu et al., 2008). Salomon et al. (2013) proposed the role of T6SSs in *V. parahaemolyticus*. The T6SS1 is very active under warm marine-like conditions where else, T6SS2 is active under low salt conditions. It is also noted that surface sensing and quorum sensing differentially regulate both systems (Salomon et al., 2013). The T6SS2 and T3SS2 co-exist, suggesting the both systems may cooperate during an infection. T6SS2 takes the first step of

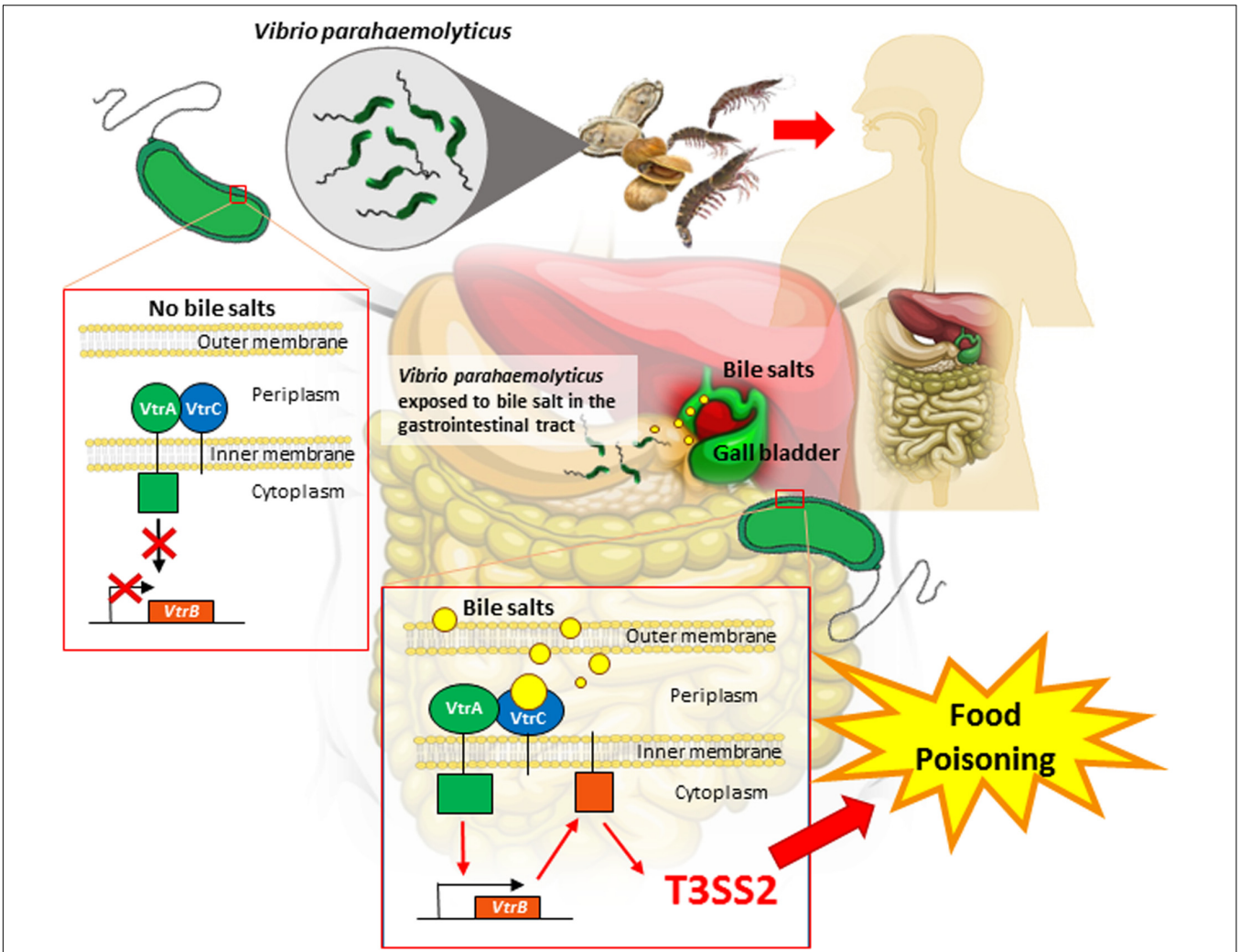

FIGURE 1 | Illustration on how *Vibrio parahaemolyticus* sense bile salts in the human gastrointestinal tract. Human ingest *V. parahaemolyticus* from contaminated seafood such as clams, shrimps and oysters. Upon ingestion, *V. parahaemolyticus* is exposed to bile salts in the gastrointestinal tract. VtrA and VtrC are two gene encoded proteins that is identified to be responsible for bile salt sensing. VtrA and VtrC will interact and form a protein complex on the surface of the membrane of the bacterial cell. This complex structure then binds to bile salts and triggers the cell to produce toxins. Upon binding of bile salts to the VtrA/VtrC complex, the cytoplasmic DNA binding domain of VtrA is activated which in turn induces VtrB to activate, resulting in the T3SS2 expression. T3SS2 virulence is secreted thus causing illness to human.

infection as a role of adhesion where else T3SS2 exports effectors by inducing enterocytotoxicity (Park et al., 2004; Yu et al., 2012).

THE SENSING OF BILE

V. parahaemolyticus with the virulence factors described are able to launch an attack on and cause illness to humans. Even with its arsenal of virulence factors, this bacterium still has to first survive the harsh conditions in the human gastrointestinal tract. It is suggested that exposure to harsh environmental conditions enables bacteria to be able to withstand the effects of bile in humans. The various pH conditions, temperatures and growth harden the bacteria toward the antimicrobial effects of bile in human. This will eventually increase their tolerance toward bile and the bacteria is able to survive in the human gastrointestinal tract. In addition, the bile levels in the human intestine are not constant and particularly in the presence of food, the bacteria would be less affected by the bile (Begley et al., 2005). Therefore, with these added advantages, it could be suggested that *V. parahaemolyticus* can indeed survive in the human gastrointestinal tract and regulate virulence during infections.

Bile is a bactericidal agent that are made up from various proteins, ions, pigments, cholesterol and bile salts. In an infection, the bile salts is believe to provide protection against bacteria (Merritt and Donaldson, 2009). When there is high amount of bile acids in the small intestine, the bacterial growth is inhibited (Inagaki et al., 2006). Where else, the growth of bacteria increases in the small intestine when bile is secreted in low amount, such seen in liver cirrhosis patients (Slocum et al., 1992). However,

enteric pathogens including *Vibrio* species have now developed a mechanism to resist the action off bile.

Gotoh et al. (2010) discovered the production of T3SS2 proteins are induced by bile under osmotic conditions similarly to the environments in gastrointestinal tract. They identified that the T3SS2 system is encoded in the pathogenicity island (Vp-PAI) and causes enterotoxicity effects to host cell. The VtrA and VtrB are the two transcriptional regulators that regulates encoded genes. Based on the study, *V. parahaemolyticus* initially recognizes its location in the human gastrointestinal tract by detecting bile acids. The transcription of Vp-PAI will be induced by bile acids via two main proteins, the VtrA and VtrB. The virulence genes then are regulated by the transduction of signals in the human intestinal tract (Gotoh et al., 2010). It was revealed that crude bile is a potent host derived inducer of *tdh* gene and T3SS2 under osmotic conditions corresponding to those in the intestinal tract.

Recently, Li et al. (2016) reported how *V. parahaemolyticus* has the ability to sense bile as an environmental cue to regulate its virulence mainly the T3SS2 during an infection. The study utilized bioinformatics tools to identify the proteins that are responsible for bile salt sensing and T3SS2 activation. **Figure 1** illustrates how the bacteria-bile sensing mechanism happens in the human body. VtrA and VtrC are two gene encoded proteins that is identified to be responsible for bile salt sensing. These two genes interact to form a protein complex on the surface of the membrane that surrounds the bacterial cell. The two proteins then create a barrel like structure that binds to bile salts and triggers the cell to produce toxins. Upon binding of bile salts to the hydrophobic chamber in the VtrA/VtrC complex, the cytoplasmic DNA binding domain of VtrA is activated which in turn induces VtrB to activate the T3SS2 virulence system. The VtrA/VtrC complex is described to be highly conserved in a group of diverse Vibrionaceae family (Li et al., 2016). Additionally, the study also found a family of monomeric lipid binding calycin domain proteins that has expanded to include an obligate heterodimer which binds to bile salts and can be utilized to transmit a signal. This increases the ability of *V. parahaemolyticus* to sense bile salt as an environmental cue to regulate virulence.

It is well understood that enteric bacteria including *V. parahaemolyticus* has the ability to sense bile which helps them identify their immediate environments and virulence factors can be expressed. *V. parahaemolyticus* releases toxins and type III secretion systems (T3SS2) in order to trigger virulence during an infection. This mode of mechanism ensures the survival of pathogenic *V. parahaemolyticus* in the environments and increase in the bacterial infections. However, this mechanism will cause more harm to we humans in future. Our own body defense fails to protect us against bacterial infections and on the other hand helps bacteria to release virulence. This situation will be worsened by the emergence of antimicrobial resistant strains in the environment which has become a major therapeutic challenge. As the effectiveness of treating bacterial infections declines, interest has been renewed toward using bacteriophages as a non-antibiotic approach to control the spreading of evolutionary *V. parahaemolyticus* strains worldwide (Wittebole et al., 2014; Letchumanan et al., 2016). Bacteriophage belonging to the *Siphoviridae* family is suitable in controlling *Vibrio* species (Letchumanan et al., 2016). This bacteriophage is highly specific to the bacterial host cell, do not affect or alter the gut microbiota (Hagens and Offerhaus, 2008), and safe to be consumed by humans. The phages are able to perform as a bio-control agent to control and inhibit virulence of pathogenic *Vibrio* species from clinical and environmental samples (Jassim and Limoges, 2014). In addition, the application of bacteriophage in the aquaculture industry can reduce the dependency of antibiotics and control the spreading of antimicrobial resistant bacteria in the environment (Letchumanan et al., 2016). The listed advantages make bacteriophage therapy a promising tool to control bacterial infections.

CONCLUSION AND FUTURE PERSPECTIVE

In summary, bile salts in human not only aid during digestion of food but possess antimicrobial activities as they have the ability to inhibit the survival of bacteria in the human gastrointestinal tract. However, certain conditions enable *V. parahaemolyticus* to develop resistance toward bile and eventually use bile as an environmental cue to regulate virulence. In order to treat infections, it is important to understand how *V. parahaemolyticus* senses bile salts and how this relates to their ability to regulate their virulence in the host during an infection. Given that there have been increasing numbers of multidrug resistant *Vibrio* strains from both clinical and environmental studies worldwide, drugs targeting suppression of bacterial virulence mechanisms should be designed instead of focusing on killing or inhibiting the growth of bacteria. Seen in this light, researchers will be able to design new drugs that may prevent the production of bacterial toxins and alleviate food poisoning symptoms. Future studies could focus on how other disease causing bacteria sense environmental cues to produce virulence during an infection.

AUTHOR CONTRIBUTIONS

VL performed the literature review and manuscript writing. KG-C, TK, SB, N-SAM, B-HG, and L-HL provided vital guidance and insight to the writing. The project was conceptualized by L-HL.

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REVIEW
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New Insights into Pathogenic Vibrios Affecting Bivalves in Hatcheries: Present and Future Prospects

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Hatcheries constitute nowadays the only viable solution to support the husbandry of bivalve molluscs due to the depletion and/or overexploitation of their natural beds. Hatchery activities include the broodstock conditioning and spawning, rearing larvae and spat, and the production of microalgae to feed all stages of the production cycle. However, outbreaks of disease continue to be the main bottleneck for successful larval and spat production, most of them caused by different representatives of the genus *Vibrio*. Therefore, attention must be paid on preventive and management measures that allow the control of such undesirable bacterial populations. The present review provides an updated picture of the recently characterized *Vibrio* species associated with disease of bivalve molluscs during early stages of development, including the controversial taxonomic affiliation of some of them and relevant advances in the knowledge of their virulence determinants. The problematic use of antibiotics, as well as its eco-friendly alternatives are also critically discussed.

Keywords: *Vibrio*, bivalve hatchery, pathogenesis, antibiosis, probiosis, phage therapy

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INTRODUCTION

According to latest SOFIA report (FAO, 2016), the worldwide food production must be increased considerably since global population will reach 9.7 billion people in 2050. In this context, marine products are an essential part of the human diet as one of the main resources of animal protein and its worldwide consumption *per capita* has been duplicated since 1960. Nowadays, more than half of these products come from aquaculture due to the overexploitation of the traditional fisheries and this proportion will exceed 65% in 2030. Bivalves are one of the most important food products for the aquaculture industry and the worldwide production (mainly oysters, mussels, clams and scallops) was close to 14 Mt with an economic value of more than $16 billion (FishStatJ, FAO).

Depletion and/or overexploitation of natural beds promoted that bivalve hatcheries gained importance in the shellfish aquaculture as the only viable solution to support the bivalve husbandry (Ojea et al., 2008; da Costa et al., 2013). Hatcheries generally provide spat of different bivalve species to the shellfish farmers (**Figure 1**). The term spat is applied to the early juvenile stage of bivalve development. It is commonly applied to juveniles in hatcheries and is related to bivalve larvae that have set and undergone metamorphosis. Then, spat is fattened in the natural environment until reach the commercial size (Helm and Bourne, 2004). These authors also described the term seed as the juvenile products supplied by hatcheries to shellfish farmers. In other cases, these facilities

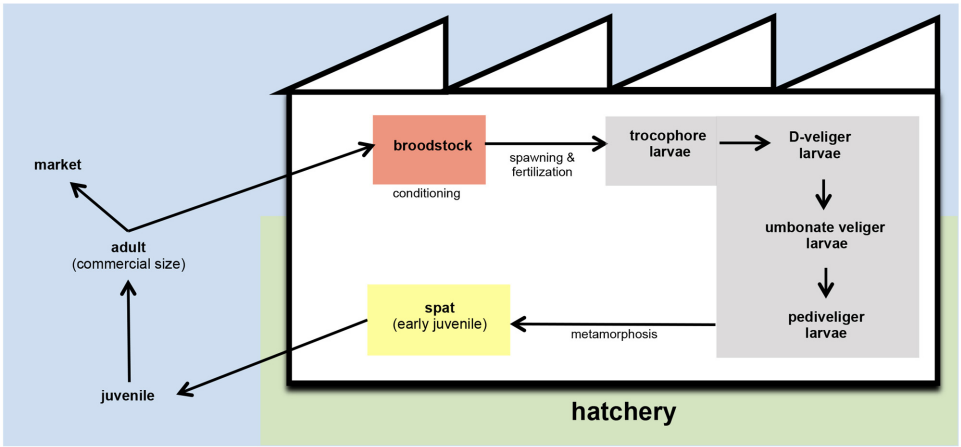

FIGURE 1 | Schematic representation of bivalve husbandry. In hatcheries, broodstock are kept in conditioning tanks (red) until spawning. After fertilization, embryos are transferred to larval tanks (gray) where larvae reach the different stages of development. From settlement and metamorphosis, spat is kept in spat tanks (yellow) and grow until the adequate size to carry on the culture out of the hatchery. In the field, juveniles are fattened with the nutrients content in natural environment until they reach the commercial size.

could also supply mature larvae to the farmers, as occurs with pediveliger Pacific oyster (*Crassostrea gigas*) larvae on the Pacific coast of North America (Helm and Bourne, 2004).

Hatchery activities include the broodstock conditioning and spawning, rearing and setting larvae, rearing spat to an acceptable size and the production of large quantities of microalgae to feed all stages of the production cycle (**Figures 1, 2**) (Prado et al., 2010). According to the **Figure 1**, the first step in hatchery culture is the broodstock conditioning which is performed in tanks where maturation of individuals is induced by artificial means until spawning. After fertilization, embryos are transferred to larval culture tanks where larvae reach the different stages of development. Bivalve cultures continue in spat tanks from settlement and metamorphosis, where spat is kept until they reach the adequate size to be transfer to the natural environment (Helm and Bourne, 2004; Dubert, 2015; Dubert et al., 2016a). All these hatchery activities are highly susceptible to bacterial contamination (e.g., seawater and broodstocks are introduced from the natural environment, phytoplankton cultures are not axenic, improper management of the cultures and seawater circuit. . .) or cross contamination as consequence of the bacterial feedback among compartments (vertical transmission from broodstocks to larvae, through the phytoplankton used as food. . .) (**Figure 2**) (Dubert, 2015; Dubert et al., 2015, 2016a). Special attention should be focused on seawater, the common nexus among different compartments, which is generally renewed in larval and spat tanks every 2 days after filtration and UV-sterilization. In addition, and due to their filter feeding nature, bivalves act as a bacterial reservoir, including vibrios, and can release them to the seawater even after every renewal (Prado et al., 2014a). Broodstock, phytoplankton or seawater are key players in the *Vibrio* dissemination within the hatchery, especially to larval and spat cultures (Beaz-Hidalgo et al., 2010; Prado et al., 2014a; Romalde et al., 2014; Dubert et al., 2015, 2016a; Holbach et al., 2015). Interestingly, microbiota reported in some of these studies included opportunistic pathogens harmless to broodstock or microalgae, but potentially harmful to larvae or spat. In summary, this bacterial feedback among the different hatchery compartments seems to be inevitable and its correct management plays an essential role in the successful working of the shellfish hatcheries (Dubert, 2015; **Figure 2**).

Clearly, microbiological aspects play an important role in the successful bivalve culture. Knowledge of *Vibrio* populations is essential since vibriosis caused by pathogenic species constitute the main bottleneck in the bivalve production process during the early stages of development, leading to high mortality rates and the rapid loss of production batches (Dubert et al., 2016b). Larval and spat mortalities associated to *Vibrio* spp. were described in hatcheries more than 50 years ago (Guillard, 1959) and still is awaiting a solution. Initially, Tubiash et al. (1965) proposed the term bacillary necrosis to describe a lethal disease of bivalve larvae and juveniles caused by bacteria classified as either *Aeromonas* sp. or *Vibrio* sp. Taxonomic affiliation of the etiological agents as *Vibrio* spp. was confirmed later (Tubiash et al., 1970). Subsequently, Elston (1999) suggested that the term bacillary necrosis should be replaced by a more descriptive name based on the type of infection. Hence, term vibriosis is generally extended to refer the bacterial disease in bivalve larvae and spat caused by pathogenic *Vibrio* species (Brown and Losee, 1978; Elston and Leibovitz, 1980; Elston, 1999). Different *Vibrio* species have been described as the etiological agent responsible of vibriosis promoting the larval and spat mortalities of different hatchery cultured bivalve species worldwide (Tubiash et al., 1965; Jeffries, 1982; Lodeiros et al., 1987; Nicolas et al., 1996; Estes et al., 2004; Gómez-León et al., 2005; Prado et al., 2005; Elston et al., 2008; Kesarcodi-Watson et al., 2009; Travers et al., 2014; Richards et al., 2014b; Rojas et al., 2015; Dubert et al., 2016d,e).

The aim of this review is to provide an overview on the vibriosis that affect bivalve larvae and spat in hatcheries due to its dramatic effects for the bivalve industry. We summarized the

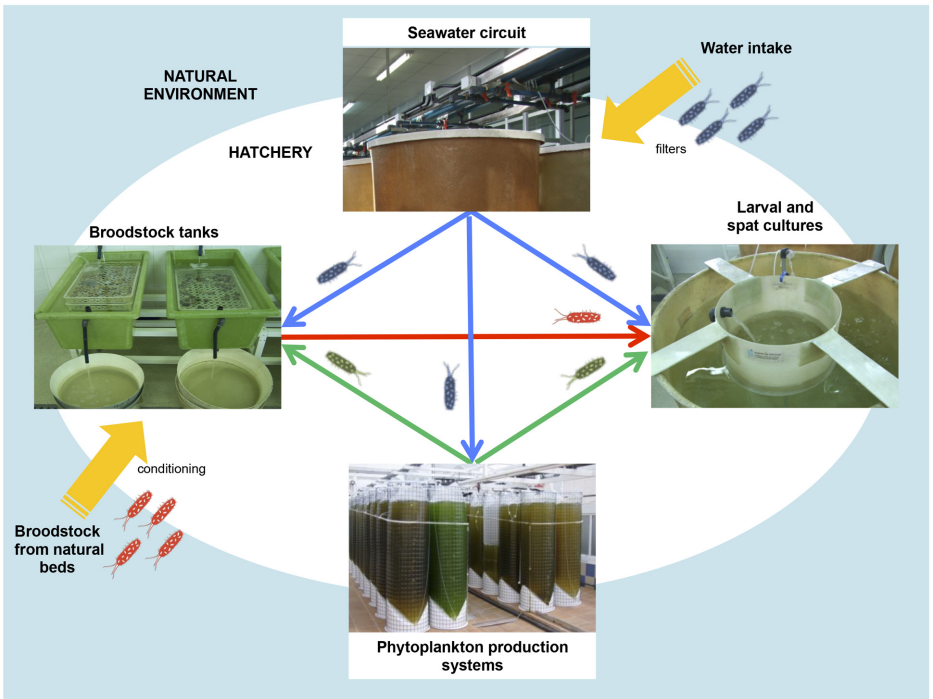

FIGURE 2 | Bacterial feedback among different hatchery compartments: broodstock conditioning tanks, phytoplankton production system, seawater circuit and larval and spat tank.

recent advances on this topic, with a focus on the aetiological agents, pathogenesis and preventive strategies described until now as well as the future prospects.

OVERVIEW OF THE PATHOGENIC VIBRIOS FOR BIVALVE LARVAE AND SPAT: SPECIES AND VIRULENCE FACTORS

One of the most important problems to define the current *Vibrio* species pathogenic to bivalve larvae and spat is related with their misleading taxonomic affiliation. In genomic era, techniques as multilocus sequence analysis (MLSA) or whole genome sequencing (WGS) are essential to provide a better understanding of the taxonomic position of the pathogenic *Vibrio* isolates and then to define them accurately (Sawabe et al., 2007, 2013; Urbanczyk et al., 2013). To avoid confusions, in the present review only the studies with *bona fide* identified strains were considered, but not those with presumptive *Vibrio* strains identified on the basis of phenotypic tests and not subjected to further molecular studies, i.e., studies by DiSalvo et al. (1978), Jeffries (1982), Lodeiros et al. (1987), Riquelme et al. (1995), Sainz et al. (1998), and among others.

In the next sections, the *Vibrio* species with importance for bivalve aquaculture due to the known pathogenicity for larvae and spat are summarized. We have also included the information available about their virulence factors. These species were clustered according with the *Vibrio* clades proposed by Sawabe et al. (2013) as easy way to stablish a good taxonomic approach (**Figure 3**).

Anguillarum clade

Vibrio aestuarianus

Vibrio aestuarianus is an important pathogenic species responsible of massive mortalities of spat, juveniles and adult Pacific oyster (*C. gigas*) in France (Saulnier et al., 2010; Madec et al., 2014; Barbosa-Solomieu et al., 2015; Green et al., 2016; Azéma et al., 2016). Taxonomic studies let to classify these pathogenic French isolates as a new subespecies, *V. aestuarianus* subsp. *francensis* (Garnier et al., 2008), distinguishable from the American strains isolated in absence of animal mortalities (*V. aestuarianus* subsp. *aestuarianus*) (Tison and Seidler, 1983).

In relation with virulence factors, Labreuche et al. (2010) characterized from the extracellular products (ECPs) a zinc metalloprotease (Vam) with lethal effects for the host. Recently, Goudenège et al. (2015) have demonstrated that *varS* gene, which codes for a signal transduction histidine-protein kinase, is a key regulator of virulence and the secretion of Vam metalloprotease.

Coralliilyticus clade

Vibrio coralliilyticus

This species was initially described as a coral pathogen responsible for coral bleaching (Ben-Haim et al., 2003a,b). Genard et al. (2013) described the physiological response of

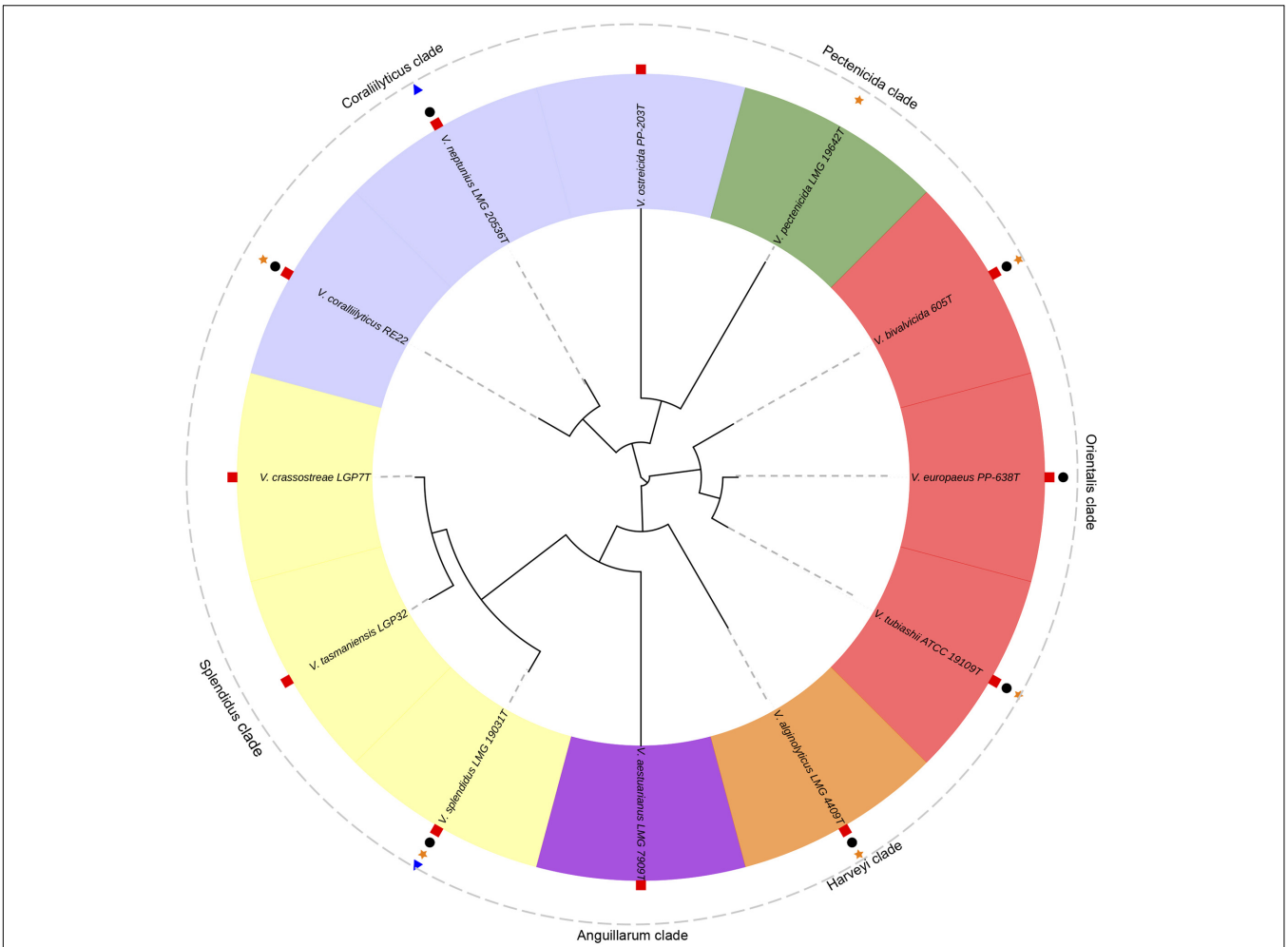

FIGURE 3 | Phylogenetic tree of the pathogenic *Vibrio* species responsible of outbreaks in bivalve hatcheries. *V. tasmaniensis* and *V. crassostreae,* well-known pathogenic species for spat and juveniles in the natural environment, were also included. Analyses were based on the concatenation of the partial sequences of five housekeeping genes (*gyr*B, *fts*Z, *pyr*H, *rec*A and *rpo*A; 3073 bp) after multiple data alignment with MUSCLE software using ML algorithm (GTR+G model). Symbols show the pathogenicity of these species for larvae and/or spat of: oysters (red squares), clams (black circles), scallops (orange stars) and mussels (blue triangles).

the *C. gigas* larvae during the bacterial infection of a strain of *V. coralliilyticus*, isolated from diseased oyster larvae and identified by 16S rRNA gene sequencing. Richards et al. (2014b) have demonstrated the virulence of the type strain ATCC BAA-450^T against Eastern (*Crassostrea virginica*) and Pacific (*C. gigas*) oysters (76.1–100% and 56.5–100% mortality, respectively). Recently, Mersni-Achour et al. (2015) demonstrated the high virulence of *V. coralliilyticus* against *C. gigas* larvae.

Sometimes, WGS is essential to elucidate the bacterial taxonomic position with accuracy. In fact, strains identified originally as *V. tubiashii* by partial sequencing of 16S rRNA gene (Estes et al., 2004; Elston et al., 2008) were further reclassified as *V. coralliilyticus* using such techniques (Wilson et al., 2013; Richards et al., 2014a). Hence, studies in which *V. coralliilyticus* is associated to outbreaks of vibriosis increased considerably (Tubiash et al., 1965; Estes et al., 2004; Elston et al., 2008; Gómez-León et al., 2008), extending its host range to other bivalve species as hard clam (*Mercenaria mercenaria*), flat oyster (*Ostrea edulis*), Atlantic bay scallop (*Argopecten irradians*) and naval shipworm (*Teredo navalis*). In summary, *V. coralliilyticus* constitutes serious threat for bivalve industry, being one of the most important emerging pathogens responsible of larval mortalities detected in shellfish hatcheries in France, New Zealand, and USA.

In relation with the virulence factors, several authors demonstrated that the high degree of virulence of some strains is associated to the production of high levels of extracellular metalloprotease (VtpA) and hemolysin (VthA) (Kothary et al., 2001; Delston et al., 2003; Hasegawa et al., 2008; Hasegawa and Häse, 2009a). Hasegawa and Häse (2009b) demonstrated that VtpA is a structural toxin to the host since this protein promotes significantly high toxicity to *C. gigas* larvae. Moreover, this metalloprotease appears to be the main secreted toxin in supernatants (Hasegawa et al., 2008; Hasegawa and Häse, 2009a). Later, Hasegawa et al. (2009) demonstrated that the

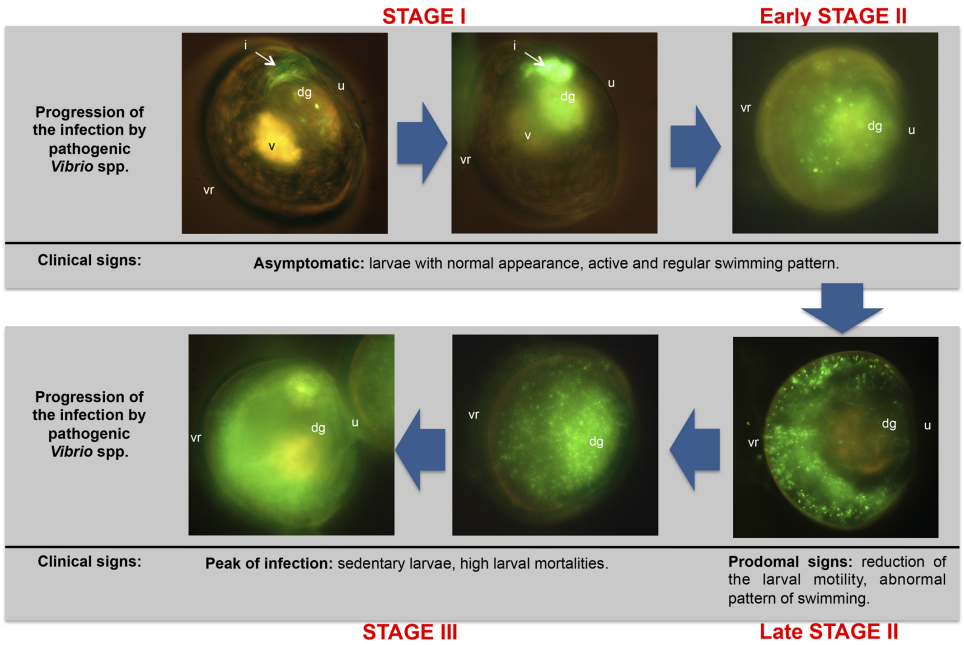

FIGURE 4 | Progression of the vibriosis infecting *R. philliphinarum* larvae with GFP-tagged pathogenic vibrios. dg: digestive gland; i, intestine; u: umbo; v: vellum; vr: ventral region. Adapted from Dubert et al. (2016b).

VtpR protein, that belongs to the TetR family of transcriptional regulators, play a key role as a global regulator of potential virulence factors. Indeed, this protein activates VtpA production and the expression of an additional metalloprotease (VtpB). In addition, Spinard et al. (2015) discovered two other putative extracellular metalloproteases, one with similarities to the Epp protease in *V. anguillarum* and the other containing a conserved domain in the M4 family of metalloproteases. They also found different putative hemolysin/cytolysin genes, including a phospholipase/hemolysin with similarity to Plp in *V. anguillarum* and a hemolysin hlyA. Moreover, they identified a putative MARTX toxin operon encoding three transport proteins of the type I secretion system (T1SS). On the other hand, Weynberg et al. (2015) demonstrated by *in silico* comparative genomic analysis that bacteriophage genomes encoding toxin genes are integrated in *V. coralliilyticus* genomes, suggesting that virulence is driven by prophages and other horizontally acquired elements.

Vibrio neptunius

The first description of this species as a bivalve pathogen was reported by Prado et al. (2005) in a study with diseased flat oyster larvae. The strains, identified by 16S rRNA sequencing, showed a high degree of virulence against *O. edulis* and Manila clam (*Ruditapes philliphinarum*) larvae, with mortalities higher than 98% at 48 h (Prado et al., 2005; Dubert et al., 2016b). Kesarcodi-Watson et al. (2009) reported high mortality rates in Greenshell mussel larvae (*Perna canaliculus*) (100% in 2–3 days) during *in vivo* assays with other strain identified as *V. coralliilyticus/neptunius-like*. Later, these authors included Pacific oysters as susceptible host (Kesarcodi-Watson et al., 2012). However, the high degree of relatedness with *V. coralliilyticus* (Thompson et al., 2003, 2005) and the lack of genomic information hinder the accurate taxonomic affiliation of these strains as *V. neptunius*. Hence, comparative studies based on WGS should be done between *V. neptunius* and *V. coralliilyticus*.

Vibrio ostreicida

This species was described by Prado et al. (2014b). Type strain PP-203^T was obtained from inner surfaces of nursery containers with continuous mortalities of young *O. edulis* spat (Prado et al., 2005). Another two similar strains, PP-200 and PP-204, were obtained from different bins during the same outbreak and included in the taxonomic description reported by Prado et al. (2014b). These authors suggested the possibility that these strains were able to survive the water changes forming biofilms on the inner tank surfaces. In virulence assays, the type strain caused 86.4–98.5% mortality of *O. edulis* larvae after 24–48 h. No further reports of this species associated to bivalve mortalities or about virulence have been published. Further analyses based on WGS are needed to elucidate its taxonomic position within the clade.

Harveyi clade

Vibrio alginolyticus

The first description of *V. alginolyticus* as a bivalve pathogen was reported by Tubiash et al. (1965). Indeed, they demonstrated its virulence for larvae of *M. mercenaria*, *O. edulis*, *A. irradians* and *T. navalis*. Luna-González et al. (2002) reported that scallops (*Argopecten ventricosus* and *Nodipecten subnodosus*) were more susceptible to the pathogen than other species tested, including *Atrina maura* and *C. gigas*, whereas Gómez-León et al. (2005) demonstrated the virulence of *V. alginolyticus* for carpet shell clam (*Ruditapes decussatus*) larvae and spat. On the other hand,

Estes et al. (2004) associated the degree of virulence, at least for *C. gigas*, with an increase in the water temperature. In any case, isolation of this species has been not common in bivalve hatcheries in comparison with bivalve adults (Wang et al., 2016).

Recently, Castillo et al. (2015) identified putative virulence factors involved in adhesion and destruction of tissues (collagenases, arylsulfatases, proteases, and hemolysin), ABC-type transport systems (spermidine, putrescine, iron), and toxins (RTX, YafQ) from the draft genomes of two *V. alginolyticus* strains.

Orientalis clade

Vibrio bivalvicida

Vibrio bivalvicida was described by Dubert et al. (2016d) who isolated three strains obtained from cultures of carpet shell clam in a Spanish hatchery. These strains led to high mortality rates (>96%) at 72 h in all experimental challenges including larvae of different clam species, such as *R. decussatus*, *R. philippinarum*, or *Donax trunculus*, as well as flat oyster. Recently, Rojas et al. (2016) reported the first isolation of *V. tubiashii* in Chile, demonstrating its pathogenic activity on the Chilean scallop larvae (*A. purpuratus*). However, the strain studied was really a representative of *V. bivalvicida* (unpusblished results) by means of WGS comparisons. Hence, it represents the first isolation of this species out of Europe. Overall, these results support the pathogenic potential of *V. bivalvicida* to kill the larvae of a broad range of bivalve species in the Atlantic and the Pacific oceans.

Interestingly, Dubert et al. (2016d) identified from the genome sequencing of the type strain three putative extracellular proteins characterized in other pathogenic *Vibrio* spp. A phospholipase/hemolysin and a HlyA hemolysin showing similarity with Plp and Vah1 of *V. anguillarum*, respectively, and a metalloprotease that shows similarity to VtpA of *V. coralliilyticus*. In addition, they also identified other five hemolysins, two phospholipases and type III (T3SS) and VI (T6SS) secretion systems involved in the extracellular secretion of effectors into a host cell.

Vibrio europaeus

Vibrio europaeus is the pathogen of bivalve larvae most recently described (Dubert et al., 2016e). Initially, this taxon was described as a subespecies of *V. tubiashii*, *V. tubiashii* subsp. *europaeus* by Prado et al. (2015). However, this study showed some taxonomic incongruities. Later, studies carried out by Dubert et al. (2016e) employing a polyphasic approach that included the WGS analysis, as well as phenotypic tests and chemotaxonomic techniques, supported their elevation to the rank of species. Strains described in the initial study were isolated from *O. edulis* and *R. philippinarum* during disease outbreaks in different Spanish hatcheries (Prado et al., 2005, 2015). Interestingly, Dubert et al. (2016e) included in the description of the new taxa a French isolate initially identified as *V. tubiashii*, and highly pathogenic for *C. gigas* larvae and spat (Mersni-Achour et al., 2014, 2015; Travers et al., 2014). Recently, Dubert et al. (2017) have reported a mortality event in larvae of other clam species, namely *R. decussatus*, involving this bacterial species. In summary, *V. europaeus* is an emergent bivalve pathogen responsible of severe losses that affected Spanish and French hatcheries (Travers et al., 2014; Prado et al., 2015; Dubert et al., 2016e, 2017)

In relation to the virulence factors, Mersni-Achour et al. (2014) identified an extracellular zinc metalloprotease belonging to the thermolysin family, close to predicted extracellular zinc metalloproteases of *V. tubiashii*. Later, Mersni-Achour et al. (2015) studied the two major fractions (F1 and F2) from ECPs by GP-HPLC and found differences in their toxicity to larvae (43% mortality for F1 fraction, 70% in the presence of the F2 fraction and 100% mixing both fractions). MS-MS analysis revealed a diversity of outer-membrane proteins in F1 (porin-like protein H precursor, outer-membrane channel protein, long-chain fatty acid transport protein, outer-membrane protein N and hypothetical proteins), whereas F2 showed a unique extracellular zinc metalloprotease. Recently, Spinard et al. (2016) found in the genome of the type strain PP-638^T two putative metalloproteases with 75% and 71% similarities respect to VtpA of *V. coralliilyticus* and to Epp of *V anguillarum*, respectively. Moreover, they detected three putative hemolysins and phospholipases encoded in the genome. Finally, T3SS and T6SS genes were also found. Interestingly, T6SS structural components are encoded on the p251-like megaplasmid, whereas the protein VgrG responsible for forming the puncturing tip of the T6SS appears to be encoded by two genes located in chromosomes 1 (Chr1) and 2 (Chr2) respectively.

Vibrio tubiashii

Strains ATCC 19109^T (=Mildford 74 J) and ATCC 10106 (=Mildford 27 O) were originally isolated in a North American hatchery from diseased juvenile clams (*M. mercenaria*) and oyster (*C. virginica*) larvae, respectively (Tubiash et al., 1965). Later, Hada et al. (1984) would describe the species formally. Pathogenicity of these strains was demonstrated in that report using larvae of clam (*M. mercenaria*), oyster (*O. edulis*), scallop (*A. irradians*) and shipworm (*T. navalis*). Takahashi et al. (2000) demonstrated a high degree of virulence degree of the strain ATCC 10106 using larvae of *C. gigas* in *in vivo* assays. Contradictory results were obtained by Richards et al. (2014b), since they did not detect significant mortalities for *C. gigas* larvae, but certain virulence (55.6–70.7% mortality) for *C. virginica* larvae. Scarcity of studies related with the virulence factors for *bona fide V. tubiashii* is due to the recently reclassification of some strains as *V. coralliilyticus*.

Pectenecida clade

Vibrio pectenecida

First, Nicolas et al. (1996) isolated different strains associated to bacterial problems in hatchery cultures of great scallop (*Pecten maximus*) affected by recurrent larval mortalities. Among isolates tested, strain A-365 promoted considerable mortalities in *P. maximus* larvae, reaching 100% after 4 days. However, this strain did not cause significant losses in *C. gigas* challenges (>30% at 6 days). Later, Lambert et al. (1998) described the species taxonomically, proposing this isolate as type strain. This species has the particularity that does not grow on TCBS medium.

Pathogenicity of the strain *V. pectenicida* A496 for *P. maximus* was demonstrated by Sandlund et al. (2006).

In relation to virulence factors, Lambert et al. (2001) demonstrated the toxic activity of *V. pectenicida* cytoplasmic extract on *P. maximus* hemocytes due to the vibrio hemocyte-killer toxin (VHKT).

Splendidus clade

Vibrio crassostreae

This species was described as pathogenic for the spat of Pacific oyster in French farming areas, although no hatchery outbreaks have been reported yet (Faury et al., 2004; Gay et al., 2004a). Later, Lemire et al. (2014) demonstrated that *V. crassostreae* strains encode a putative outer membrane protein that is necessary for virulence. Recently, Bruto et al. (2016) demonstrated that this species is a non-virulent oyster colonizer that subsequently turns into a pathogen by acquisition of a virulence plasmid, essential for killing.

Vibrio splendidus

The high homogeneity of 16S rRNA gene within the Splendidus clade has led to difficulties in the taxonomy and identification of the strains and many authors have described closely related bivalve pathogens as *V. splendidus*-related strains. This group of strains has been commonly isolated from disease outbreaks. Indeed, pathogenicity of *V. splendidus*-related strains has been demonstrated for larvae of mussels (*Mytilus edulis* and *Perna canaliculus*), clams (*R. decussatus*), scallops (*P. maximus*), as well as for oysters (*C. gigas*) spat (Nicolas et al., 1996; Gatesoupe et al., 1999; Gay et al., 2004b; Gómez-León et al., 2005; Torkildsen et al., 2005; Sandlund et al., 2006; Kesarcodi-Watson et al., 2009; Rojas et al., 2015; Ben Cheikh et al., 2016; De Rijcke et al., 2016). Recently, Pérez-Cataluña et al. (2016) suggested a synonymy among *V. splendidus* and the later described species *V. hemicentroti*.

Knowledge of the virulence factors is essential to distinguish the virulent and non-virulent strains, since this species is commonly detected even in the absence of disease. A lot of information has been published on the virulence factors of *V. splendidus* strain LGP32 (see below). However, this strain was re-classified as *V. tasmaniensis* (Sawabe et al., 2013). Regarding virulence factors of *V. splendidus*-related strains, Macpherson et al. (2012) identified from the pathogenic strain DMC-1 a new haemolysin, termed vibrioaerolysin, with homology to aerolysin produced by several *Aeromonas* spp. Expression of the vibrioaerolysin is controlled by a ToxR-like gene located close to vibrioaerolysin gene, since the transposon insertion into the ORF of ToxR-like gene rendered mutants unable to produce haemolysin. Well known virulence factors, as Vsm and OmpU, have been also identified in the scallop pathogen *V. splendidus*-related strain JZ6 (Liu et al., 2013). For this strain hemolysis was temperature-dependent with highest hemolytic level at 10 °C and decreasing with the increase of temperature. Recently, Liu et al. (2016) carried out a comparative transcriptome analysis of *V. splendidus* JZ6, which shows highest virulence at 10°C. They identified 10 pivotal genes related to the virulence at 10°C involved in adhesion, protein secretion and virulence of *V. splendidus*: two genes (*secE* and *ftsY*) in Sec dependent pathway, two genes (flhG and VS_2437) for Flp pilus assembly and six genes (*toxS*, *cqsA*, *cqsS*, *rpoS*, *hapR*, and *vsm*) in "Vibrio Cholerae pathogenic cycle". Moreover, a novel mono-ADP-ribosyltransferase (MART) toxin, named Vis toxin, produced by the oyster pathogenic *V. splendidus* 12B01 was characterized by Ravulapalli et al. (2015).

Vibrio tasmaniensis

Strain LGP32 (Gay et al., 2004a,b), formerly designed as *V. splendidus*, was taxonomically reclassified as *V. tasmaniensis* (Sawabe et al., 2013). This strain is a well-known pathogen for spat of *C. gigas* oyster in French farming areas (Gay et al., 2004a,b; Green et al., 2016). However, as for *V. crassostreae*, any outbreak of vibriosis due to this species has been reported in a bivalve hatchery until present.

Virulence factors of strain LGP32 have been widely studied. First, Vsm metalloprotease was identified due to a role in ECP toxicity for oysters. However, expression of *vsm* gene is not necessary for bacterial virulence in the oyster infection model when bacteria are injected (Le Roux et al., 2007). By means of comparative genomics, Binesse et al. (2008) demonstrated that Vsm was the major factor in the toxicity of the ECPs and that non-virulent strains lacked this marker. Complete genome sequence of strain LGP32 revealed homologues of genes usually associated with virulence, e.g., haemolysins, siderophore transport and utilization and adhesins (Le Roux et al., 2009). Interestingly, they found in Chr2 a haemolysin-co-regulated protein gene (*hcp*) and the *vas* operon, which encodes a type VI secretion system. Further studies revealed the importance of an outer membrane protein, OmpU porin, as the major determinant of *V. tasmaniensis* LGP32 pathogenicity in oyster experimental infections, contributing to resistance to antimicrobial peptides/proteins (AMPs) which are involved in *C. gigas* immunity, and confering adhesive properties (Duperthuy et al., 2010). These authors also elucidate the role of OmpU, as an adhesin/invasin required for β-integrin recognition and to attach and invade oyster hemocytes (Duperthuy et al., 2011), and defined this species as a facultative intracellular pathogen that manipulates host defense mechanisms to enter and survive in host immune cells.

RECENT ADVANCES ON THE PATHOGENESIS OF VIBRIOSIS

In most reports is not clear if pathogenic vibrios are the primary causative agent, secondary opportunistic colonizers or commensals, due to the limitation of the experimental procedures used. Interestingly, the vast majority virulence assays in larvae were carried out by immersion in *Vibrio*-inoculated seawater being a good reflect of the natural route of infection (Tubiash et al., 1965; Lodeiros et al., 1987; Gómez-León et al., 2005; Prado et al., 2005, 2015; Kesarcodi-Watson et al., 2009; Richards et al., 2014b; Rojas et al., 2015, 2016; Dubert et al., 2016d,e). Obviously, time-course of mortalities depends on the degree of virulence of the pathogenic strain and the bacterial concentration

inoculated. In contrast, spat and adults have shown reliance on infection via injection discarding the immersion for these assays (Duperthuy et al., 2011). However, in both cases, researchers generally inoculate a single *Vibrio* strain, whereas in the natural environment hosts are colonized by an assemblage of diverse vibrios (Gay et al., 2004a; Wendling et al., 2014; Le Roux et al., 2016). Thus, some studies have recently investigated the fact that the functional unit of pathogenesis is a bacterial clone, a population or a consortium. Interestingly, adaptive responses of the host to sympatric isolates have to be taken into account during the infection, in contrast to the virulence of the allopatric strains. For instance, cross-infection experiments in larvae demonstrated that *C. gigas* larvae showed lower mortalities with sympatric *Vibrio* combinations, demonstrating the adaptive potential of the host (Moehler et al., 2011; Wendling and Wegner, 2015).

Different reports have studied the mechanism of bivalve host response during vibriosis outbreaks by means of transcriptomic approach (Genard et al., 2013; Bassim et al., 2014). However, studies related with the onset and advance of the pathogenic bacteria are scarce in bivalves. Hence, we have described the new insights on this topic focused on the following of the infection process in bivalve larvae and spat from a bacterial point view.

Pathogenesis in Bivalve Larvae

Clinical signs of vibriosis are well known in bivalve larvae in comparison with the processes of bacterial colonization and infection. In any case, the order of appearance of the clinical signs is the same regardless of the pathogenic *Vibrio* species: prodromal signs are constituted by reduction of larval motility, abnormal circular pattern of swimming and tendency to the quiescence by the inability to swim. At the peak of infection, dead and moribund larvae exhibit bacteria swarming on the margins and inside, disruption and/or extension of the velum, detachment of portions of the velum with cilliary action after all other soft tissues are destroyed. A phenomenon called 'spotting' is regularly observed, consisting in the accumulation and agglutination of moribund and dead larvae at the bottom of the tank (Tubiash et al., 1965; Elston, 1999; Estes et al., 2004; Prado et al., 2005, 2015; Torkildsen et al., 2005; Elston et al., 2008; Gómez-León et al., 2008; Kesarcodi-Watson et al., 2009; Beaz-Hidalgo et al., 2010; Mersni-Achour et al., 2015; Rojas et al., 2015, 2016; Dubert et al., 2016a,b).

First references about colonization routes for different bivalve species were exclusively described by means of histological observations (Tubiash et al., 1965; Elston and Leibovitz, 1980). However, microscopy techniques used in both studies did not let to distinguish the primary pathogen from the regular microbiota in the different larval tissues. Dubert et al. (2016b) described for the first time in bivalve larvae the complete colonization process by means of fluorescent tagging of pathogenic *Vibrio* species (*V. europaeus*, *V. bivalvicida* and *V. neptunius*), demonstrating that a bacterial clone is the most accurate approach for these bacterial species. Hence, they demonstrated the onset and advance of the vibriosis and accurately described the route of infection. Mortality and morbidity was studied in detail in that work using *R. philliphinarum* larvae as animal model and defined three infection stages (I, II and III) (**Figure 4**). In the Stage I, pathogenic *Vibrio* were filtered by the bivalve larvae through the vellum and entered the digestive system through the oesophagus and stomach, colonizing the digestive gland and quickly proliferating in the intestine during the first hours of infection. Stage II was characterized by the rapid expansion of the GFP-tagged *Vibrio* spp. to the surrounding organs in the body cavity from the dorsal to ventral region. From late Stage II, typical prodomal signs were observed. In the Stage III, pathogenic *Vibrio* spp. colonized completely the larvae at the peak of infection and the clinical signs corresponding to advanced infection were then observed. Interestingly, these authors demonstrated that the vibriosis is asymptomatic in the bivalve larvae during the early infection stages. This fact conditions the preventive treatments since once the pathogen is inside the larvae the infection process cannot be stopped.

Pathogenesis in Bivalve Spat

Bivalve larvae are more susceptible to vibriosis than adults since the resistance to bacterial infection significantly increases with age of the bivalves (Gómez-León et al., 2008). Some authors have proposed different bacterial strategies to infect the bivalve host. Thus, the study of diseases using specific-pathogen-free (SPF) oysters has enabled the assessment of the infection process under natural conditions (Le Roux et al., 2016). Lemire et al. (2014) demonstrated that ecological populations often represent the functional unit of pathogenesis in which the presence of non-virulent strains increased the virulence of *V. crassostreae*. They suggested that non-pathogenic population could promote a high bacterial load necessary to either overcome host defenses or to induce expression of virulence factors via *quorum sensing* (QS). More recently, Bruto et al. (2016) identified abundant number of strains belonging to this species in oyster tissues which are nearly absent in the surrounding water. They identified virulent and non-virulent *V. crassostreae* strains and proposed the dynamic colonization of the host in which the non-virulent strains turn into a pathogen by introgression of a virulence plasmid. These authors hypothesized that the acquisition of the virulent plasmid is favored by the elevated host density in farming areas. On the other hand, Saulnier et al. (2010) demonstrated the synergistic effect over the virulence by means of co-infection of *C. gigas* spat with *V. tasmaniensis* and *V. aestuarianus*. Moreover, Goudenège et al. (2015) proposed that the functional unit of *V. aestuarianus* pathogenesis are clones. They clustered the virulent strains into two lineages and demonstrated that a regulatory gene *VarS* is essential for the infection.

PREVENTIVE TREATMENTS: PRESENT AND FUTURE PROSPECTS

The classical treatments were directed toward the complete elimination of bacteria from seawater, which constitutes an unfeasible and undesirable objective, because the cultures are not axenic and some bacteria even enhance larval development. At present, the efforts should be devoted to the microbiological improvement through the proper management of seawater circuit, the phytoplankton production systems, or the broodstock

conditioning to maintain the necessary balance in the microbiota that allow the successful development of molluscan larvae.

Water Treatments

In hatcheries, the water is subjected to treatments, including filtration, pasteurization, ozone, and UV radiation, with the aim to reduce the associated bacterial population. Generally, the first step is the decantation of the water pumped from the sea and, once most of the solid particles are eliminated, the water is treated. Filtration is an expensive treatment and only larval cultures and small-scale phytoplankton cultures receive maximum filtration water. Filtration is an advisable practice to reduce contents of bacteria as well as organic matter, and its results are better than the obtained by other systems like pasteurization, as showed by Lewis et al. (1988) in a hatchery of the Pacific oyster, *C. gigas*. The disinfection with chlorine or ozonization are alternatives also employed. Use of chlorine has shown some problems, including the interference in the larval mechanism of pumping (Vasconcelos and Lee, 1972) or the reactivity with organic nitrogen in the water that can produce toxic residues for marine organisms (Jorquera et al., 2002). On the other hand, application of ozonization can be complex and costly to disinfect aquaculture systems (Summerfelt, 2003) and can led to the appearance of oxidants toxic to aquaculture species (Richardson et al., 1982; Summerfelt, 2003). Although radiation of seawater with ultraviolet light has an unquestionable lethal power on bacteria, there are disagreements about the true effects when the treatment is used on water culture in hatcheries. Some authors have pointed out the advantages of this procedure, like the decrease of different bacterial populations including bivalve pathogens (Vasconcelos and Lee, 1972; Lodeiros et al., 1987), while other authors found important variations in effectiveness among samples and/or bacterial pathogens (Murchelano et al., 1975; Brown, 1981). Thus, the effects of UV-radiation treatment are variable and this variability may rise with factors such as the dose and the individual efficiency of the radiation unit, the water flow, or the presence of organic matter in the water (Brown and Russo, 1979; Liltved et al., 1995; Liltved and Cripps, 1999), existing the risk of a change from bactericide to only bacteriostatic effects. Therefore, the selection of undesirable populations resistant to the treatment, the high economic cost and the impossibility for the treatment of big volumes of water are the main disadvantages of this method.

In order to stabilize and increase larval survival, the development of new technologies and they integration into commercial hatcheries must be encouraged. The technology used in bivalve hatcheries has not progressed very much since the birth of this industry and still essentially relies on the static water methods developed in the 1960s for *C. virginica* and *O. edulis* (Loosanoff and Davis, 1963; Walne, 1974), which have been adapted to most cultured bivalves though without any great changes (Helm and Bourne, 2004). Nevertheless, about fifteen years ago, flow-through systems were developed for rearing bivalve larvae (Magnesen et al., 2006; Rico-Villa et al., 2008, 2009), some of them coupled with monitoring of different seawater parameters such as temperature, oxygen, pH, turbidity and ammonia, which allow the reduction in labor necessary for larval rearing and the increase of the larval density maintaining the same growth and survival rate but with high needs of water and energy.

Using recirculation systems (RAS) where water is treated and re-used, the seawater and energy needed can be reduced considerably. In addition, such systems would provide better water quality control, since water will be taken in only once per larval rearing cycle being not subject to fluctuations in the quality of the natural water supply. Furthermore, well-managed RAS has a stable microclimate that is hardly influenced by the low water intake. In finfish culture, this type of system has contributed to increased survival of the larvae in hatcheries and during later grow-out stages. Although at present there are no recirculation systems for bivalve larvae in commercial use, a number of studies have been performed on marine bivalve species (Widman, 1998; Pfeiffer and Rusch, 2000; Suantika et al., 2000; Xiongfei et al., 2005; Zohar et al., 2005) with promising results.

Impact of the Antibiotic Use in Hatchery Environment

The use of antibiotics is one of the most widespread strategies for the control and prevention of vibrios in hatcheries. In hatcheries, antimicrobial agents have routinely been applied to water to treat and prevent disease, particularly during the first stages of bivalve development (Prado et al., 2014a; Dubert et al., 2016c). Therefore, antimicrobial agents as florfenicol, erythromycin, oxolinic acid and specially chloramphenicol are used in bivalve hatcheries to prevent the vibriosis and to improve the survival rates of larvae and juveniles (Lodeiros et al., 1987; Nicolas et al., 1996; Uriarte et al., 2001; Torkildsen et al., 2005; Campa-Córdova et al., 2006; Miranda et al., 2014; Dubert et al., 2016c). The use of chloramphenicol in Europe is currently banned in animals raised for human consumption, including aquaculture, because it has been associated with aplastic anemia and it is difficult to establish a safe level of human exposure (Schwarz et al., 2004). However, numerous studies have linked its use to higher larval survival rates and its efficacy in controlling the *Vibrio* populations and despite its prohibition, some authors justify that the brief use of chloramphenicol during larval development does not pose a risk to the consumer since the larvae are subsequently fattened in the sea for at least 1 or 2 years (Uriarte et al., 2001; Helm and Bourne, 2004; Torkildsen et al., 2005; Campa-Córdova et al., 2006).

The main risk associated with the extensive use of antibiotics is the development of resistant bacteria, which can transmit quickly resistance genes in the hatchery environment by horizontal transfer mechanisms (Zanetti et al., 2001; Kümmerer, 2004; Kitiyodom et al., 2010; Cabello et al., 2013; Miranda et al., 2013). In this way the use of antibiotics would have a detrimental effect in selecting resistant bacterial populations, including those with pathogenic potential. Indeed, Dubert et al. (2016c) suggested that these treatments limit the bacterial diversity and competition, favoring the proliferation of resistant vibrios in the hatchery environment and increasing the risk of bacterial contamination to the cultures. These authors demonstrated that the continued use of antibiotic (chloramphenicol) in a shellfish hatchery, far from optimizing and favoring the success of larval cultures,

promoted the rapid development and persistence of resistant vibrios, most of them with pathogenic potential, that carry different R plasmids. Moreover, they demonstrated the transfer of R-plasmids from these resistant vibrios to other bacteria, including bivalve and human pathogens. This fact, constitutes a serious risk to the aquatic environment and public health. Interestingly, the persistence of these resistant populations in the hatchery environment could be promoted by subinhibitory and even residual concentrations of antibiotics (Beaber et al., 2004; Hastings et al., 2004; Buschmann et al., 2012; Davies and Davies, 2010; Andersson and Hughes, 2014).

Some authors have found the use of certain antibiotic in shellfish hatcheries promotes the co-selection of resistance to antibiotics (Dang et al., 2006; Dubert et al., 2016c). Hence, occurrence of multiple resistant genes in the same R-plasmid should be taken into account due to the mechanisms of co-resistance and cross- resistance (Courvalin and Trieu-Cuot, 2001).

The use of antibiotics in shellfish hatcheries is highly unadvisable since these facilities constitute a potential source of antibiotic residues and resistant bacteria to the aquatic environment. Even bivalve larvae and spat could act as delivery vehicles of resistant bacteria, including pathogenic vibrios, in different geographical locations and aquatic environments due to aquaculture exports. The exposure to these risks for aquatic environment and public health has to be taken into account.

Eco-friendly Alternatives: Probiosis, Quorum Quenching (QQ), and Phage-therapy

In recent years, the use of probiotics become an interesting alternative to the utilization of antibiotics, although most of the studies about probiotics in aquaculture were focused on fish and crustaceans being scarce those focussed on molluscs. According to Verschuere et al. (2000), up to now the best adapted definition to application to larval cultures of bivalves, a probiotic would be a live microbial additive with a beneficial effect on the host, modifying the microbiota associated with the host or the environment, ensuring an optimal use of the feed or improving its nutritional value, improving the host response against the disease, or getting a better quality of its environment. In this sense, several studies have demonstrated high survival ratios when bivalve larvae are treated with probiotics prior to experimental infection with vibrios (Lodeiros et al., 1987; Douillet and Langdon, 1993, 1994; Gibson et al., 1998; Riquelme et al., 2000; Kesarcodi-Watson et al., 2012; Karim et al., 2013; Sohn et al., 2016a,b; Zhao et al., 2016). Among the antibiotic-producing marine bacteria used by these authors there are representatives of different bacterial taxa including, *Pseudoalteromonas haloplanktis* (formerly *Alteromonas haloplanktis*), *Aeromonas media*, *Alteromonas macleodii*, *Neptunomonas* sp., *Pseudoalteromonas* sp., *Pseudomonas* sp., *Vibrio* sp. or *Bacillus* sp.

In the last years, different isolates of *Phaeobacter gallaeciensis* (formerly *Roseobacter gallaeciensis*) and *P. inhibens* have received special attention by different research groups, due to their great spectrum of *in vitro* inhibition against pathogenic bacteria from aquaculture systems (Ruiz-Ponte et al., 1999; Prado et al., 2009, 2010; Kesarcodi-Watson et al., 2012; Sohn et al., 2016a,b; Zhao et al., 2016). Prado et al. (2009) in experiments performed in marine water, with phytoplankton cultures and with larvae of flat oyster (*O. edulis*) and clam (*R. philippinarum*) cultures confirmed its potential use as control method in mollusc hatcheries, if its action is allowed before the pathogens reach high concentrations in the system. Similar results were obtained by Kesarcodi-Watson et al. (2012) in challenge experiments of *P. maximus*, *O. edulis* and *C. gigas* larvae with different pathogenic vibrios including *V. coraliilyticus* and *V. splendidus*. On the other hand, Sohn et al. (2016a,b) and Zhao et al. (2016) demonstrated that probiotic of *P. inhibens* involves contributions from biofilm formation and antibiotic production and that, as for *P. gallaeciensis*, colonization in the system prior the introduction of the pathogens is needed for probiotic activity. Our research group has also obtained promising results on the improvement of larval survival and growth using a mixture of marine bacteria with probiotic activity in a scallop (*P. maximus*) hatchery (unpublished results).

More in depth works on probiosis in bivalve larval cultures are needed, to clarify the interaction among bacteria and the other live organisms, to establish the ability of probiotics to remain in the systems and to determine the appropriate dosage to achieve the highest effectiveness. In addition, methods to improve the conservation, storage and manipulation of probiotic in hatcheries are also needed.

One of the most promising alternatives to control pathogens in bivalve hatcheries is based on the inhibition of the expression of virulence genes, regulated in many aquaculture pathogens by bacterial cell-to-cell signaling, known as QS (de Kievit and Iglewski, 2000; Deep et al., 2011). QS is the regulation of gene expression in response to fluctuations in cell population density, which correlates with signaling molecule (autoinducer) concentration (Miller and Bassler, 2001; González and Marketon, 2003). The most thoroughly characterized Gram-negative, bacterial intraspecific autoinducers are *N*-acylhomoserine lactones (AHLs), which have been reported to accumulate in the culture medium, and bind to an AHL-receptor protein belonging to the LuxR family of transcriptional regulators. The activated LuxR/AHL complex then binds specific DNA sequences, resulting in the activation or repression of target genes, including in many cases the activation of important virulence phenotypes (Eberhard et al., 1991; Fuqua et al., 1994; Natrah et al., 2011). Some aquatic organisms, including micro-algae, macroalgae, invertebrates and also other bacteria, have the potential to disrupt QS by means of various different mechanisms (Natrah et al., 2011). A mechanism involves the production of compounds known as quorum sensing inhibitors (QSIs), that interfere with the detection of signal molecules (Givskov et al., 1996; Rasch et al., 2004; Teasdale et al., 2009). Such compounds were first described in the red marine algae *Delisea pulchra*, which synthesizes halogenated furanones with protective effect of both fish and shrimp from vibriosis (Givskov et al., 1996; Rasch et al., 2004). A second mechanism is the quorum quenching (QQ), which can be defined as the enzymatic inactivation of AHLs by the production of acylases or lactonases (Tait et al., 2005; Defoirdt

et al., 2007; Romero et al., 2010, 2011). The use of AHL-degrading bacteria has been successful in increasing the survival of turbot (*Scophthalmus maximus*) and freshwater prawn have (*Macrobrachium rosenbergii*) larvae (Tinh et al., 2008; Nhan et al., 2010). Torres et al. (2013) detected and isolated AHL-degrading bacteria from a bivalve hatchery, including representatives of genera *Alteromonas* and *Thalassomonas* (further reclassified as belonging to genus *Thalassotalea*) (Deering et al., 2016), pointing out their potential to be employed to attenuate the production of virulence factors by bivalve pathogens. Since new agents for controlling bacterial diseases can be considered only when their efficacy is demonstrated using different challenge tests, further research is needed in order to elucidate the *in vivo* interactions of quorum-quenching microorganisms with aquaculture pathogens and animals.

Phage therapy also represents a promising alternative strategy for prevention of disease outbreaks (Housby and Mann, 2009). In natural environments, phages and their bacterial hosts maintain equilibrium. The "kill the winner" theory (Thingstad, 2000), and its further development "Cost of Resistance" (Våge et al., 2013), hypothesize that populations of bacteria that bloom are often controlled by phage infection, which subsequently reduces their numbers. Therefore, in natural ecosystems, wherever bacteria can be isolated, a specific phage can also generally be found (Chibani-Chennoufi et al., 2004; Stenholm et al., 2008).

Phages have been used for decades to effectively treat bacterial infectious diseases, including wound and gastrointestinal infections (Sulakvelidze et al., 2001; Sulakvelidze and Morris, 2001; Sulakvelidze and Kutter, 2005; Sulakvelidze, 2011) as well as to reduce food borne illnesses, including those caused by *V. parahaemolyticus* or *Salmonella* (Tan et al., 2014; Letchumanan et al., 2016; Pereira et al., 2016a,b). Commercial phages are now available for treating bacterial diseases in humans, animals (including aquaculture) and agricultural crops (Housby and Mann, 2009; Ly-Chatain, 2014). Thus, one Israeli company, Phage Biotech Ltd., has developed a phage treatment for *V. harveyi* in shrimp (Hodgson, 2013), the Australian biotechnology company Biologix is developing phage therapy for *Vibrio* sp. associated with mortalities in aquaculture (Letchumanan et al., 2016), and, in Baltimore, MD, Intralytix Inc. is also developing a phage treatment against *V. tubiashii* and related pathogens in larval oyster and clam hatcheries (Richards, 2014).

Phages typically are highly specific in terms of the bacterial species that they will infect and, indeed, commonly will only infect certain strains of any species (Housby and Mann, 2009). This host specificity of phages can be considered as both an advantage or disadvantage for phage therapy. Thus, although phages have a specific effect on the target bacterium and do not alter the normal microbiota of the culture system, a precise identification of the target bacterium is required before an appropriate phage can be selected for therapy (Housby and Mann, 2009). Another advantage of the use of phages is that they have the ability to disrupt bacterial biofilms (Azeredo and Sutherland, 2008). Thus, Luo et al. (2015) demonstrated that phages belonging to the *Siphoviridae* family successfully reduced *Vibrio* biofilms in an abalone farm. The potential emergence of phage-resistant bacteria or the role of some phages in the transfer of virulence genes are possible drawbacks of phage therapy.

More research would be needed in a near future to understand how the interaction of environmental factors, including pH, temperature, salinity and organic matter content, influences the efficiency of phage therapy in aquaculture systems. In this sense, Silva et al. (2014) demonstrated that salinity and organic matter although did not affect the survival of the bacteriophages, had a clear influence in the efficacy of the treatment. Therefore, to establish the best conditions to improve the efficiency of phage therapy and, in addition, to adapt its use in closed recirculated systems will be also key areas of research.

CONCLUDING REMARKS

As considered along this review, the molluscan hatcheries contitute a singular and complex environment, i.e., "a sea of bacteria (mainly vibrios) in a world of larvae", subjected to rapid changes that can lead to an imbalance in such ecosystem causing the culture failure and the death of the animals.

Despite the years passed since the first recognition of the role of the vibrios in as agents of larvae and spat diseases, the situation has not been solved yet. Hence, it is necessary in the coming years to focus the research on several aspects: (i) the *Vibrio* species implicated in the outbreaks. With the new genomic tools, *bona fide* identification of pathogens is feasible. New tools are necessary for early detection of these pathogens in the aquaculture systems. PCR procedures are available for some pathogens but procedures for use in the field are highly advisable; (ii) the virulence genes implicated specifically in the pathogenicity for bivalves should be determined in comparison with those already recognized for fish; (iii) knowledge on the disease onset. Are populations or clones the units of vibrio pathogenesis in bivalves?; and (iv) advances in the management of microbiological aspects of the water circulation system are devised. RAS systems specific for these invertebrates should be improved considering also the integration of preventive measures such as probiotic, QQ bacteria or bacteriophages, taking into account their desirable withstand period in these systems to successfully control the *Vibrio* populations.

AUTHOR CONTRIBUTIONS

JD, JB, and JR conceived and wrote, edited and approved the manuscript.

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A Comprehensive Epidemiological Research for Clinical *Vibrio parahaemolyticus* in Shanghai

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Vibrio parahaemolyticus is one of the most important pathogen for seafood-borne gastroenteritis in Shanghai and the rest of the world. A total of 42 *V. parahaemolyticus* strains were isolated from 1900 fecal specimens collected from patients in Shanghai hospital presenting from January 2014 to December 2015. All isolates were evaluated for potential virulence factors [*tdh*, *trh*, and type three secretion system (T3SS) genes], typed using multilocus sequence typing (MLST) and screened for antimicrobial resistance phenotype and genotype. And for the first time, the relationship between virulence, genetic diversity and antimicrobial resistance of these isolates were identified. The results showed that 37 isolates carried the *tdh* gene (88.1%) and only seven isolates were positive for the *trh* gene. The T3SS1 and T3SS2 genes were detected in all strains and only *trh*-positive isolates are also containing the T3SS2β genes. MLST analysis of the 42 Shanghai isolates identified 20 sequence types (STs) with 16 novel STs and that these clinical *V. parahaemolyticus* strains showed high degrees of genetic diversity. All isolates expressed high levels of resistance against Ampicillin (100.0%), Streptomycin (100.0%), Cephazolin (92.9%), Kanamycin (92.8%) and Amikacin (90.5%), and eight out of 38 resistance genes (*SHV*, *tet*(B), *str*A, *qnr*A, *gry*A, *qnr*B, *sul*I, *sul*II) were detected in at least two isolates. This study confirms that antimicrobial resistance of clinical *V. parahaemolyticus* isolates is greater than those of environmental isolates. Furthermore, no clear correlation between antimicrobial resistance and virulence or genetic diversity was found in this study. These results add to epidemiological data of clinical *V. parahaemolyticus* isolates in Shanghai and highlight the need for additional mechanistic studies, especially antimicrobial resistance, to reduce the burden of disease caused by this pathogen in China.

Keywords: *Vibrio parahaemolyticus*, diarrhoea samples, virulence, genetic diversity, antimicrobial susceptibility

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INTRODUCTION

Illnesses caused by foodborne pathogens are an increasingly critical public health concern (Velusamy et al., 2010). *Vibrio parahaemolyticus* is recognized as a major foodborne pathogen for causing gastroenteritis worldwide, especially in coastal countries and regions (McLaughlin et al., 2005; Su and Liu, 2007; Letchumanan et al., 2014; Hubbard et al., 2016). Clinical symptom of

V. parahaemolyticus infections include diarrhea, abdominal cramps, vomiting and fever, which also progresses to septicemia can sometimes lead to death in patients (Scallan et al., 2011; Lopatek et al., 2015). In 1950, this pathogen was first discovered in Japan, which resulted 272 illnesses with 20 deaths (Fujino et al., 1953). From 1997 to 2000, 84 food poisoning outbreaks caused by *V. parahaemolyticus* were recorded in Spain (Fournier and Ogata, 2005). From 2000 to 2008, it was reported around 35,000 *V. parahaemolyticus* infections annually in US (Haendiges et al., 2015). In China, during 2003 to 2008, this microorganism has caused 9041 illnesses and 3948 hospitalizations (Wu et al., 2014). It is essential to gather the epidemiological data of *V. parahaemolyticus* to reduce the burden of disease caused by this pathogen in China.

Virulence of *V. parahaemolyticus* is primarily attributed to the production of a thermostable direct haemolysin (TDH), TDH-related haemolysin (TRH) and two type III secretion systems, T3SS (Makino et al., 2003; Ritchie et al., 2012; Zhang et al., 2013). TDH and TRH are encoded by the *tdh* gene and *trh* gene, respectively (Letchumanan et al., 2014). Although the specific actions of these genes in human infection remain unknown, the relevance between pathogenicity of *V. parahaemolyticus* and the presence of *tdh* and *trh* is well recognized (Broberg et al., 2011; Ceccarelli et al., 2013). Contamination of foods with *tdh*- and/or *trh*-positive *V. parahaemolyticus* strains is considered a public health risk (Pazhani et al., 2014). The two T3SS systems in *V. parahaemolyticus* are known as T3SS1 and T3SS2 (Wang et al., 2015). T3SS1 is encoded by the first pathogenicity island on chromosome I and is involved in cytotoxicity (Paranjpye et al., 2012). T3SS2 is located on chromosome II and is also encoded by a pathogenicity island. As a newly identified type of secretion system, T3SS2 appears to be associated with enterotoxicity and cytotoxicity, in experiments conducted in vitro and in intestinal cell lines (Ritchie et al., 2012). Currently, it is possible to detect the presence of *tdh*, *trh* and T3SS genes in *V. parahaemolyticus* isolates by PCR-based methods (West et al., 2013).

Vibrio parahaemolyticus strains exhibit high genetic diversity due to high rates of recombination and mutation, which caused potential infection risk for human health (Ludeke et al., 2015). A number of molecular typing methods have been used to determine the molecular epidemiology of *V. parahaemolyticus*, and these methods include multilocus sequence typing (MLST), serotyping, and pulsed-field gel electrophoresis (PFGE) (Banerjee et al., 2014; Xu et al., 2015). MLST has proven to be powerful tool for investigating the prevalence and diversity of *V. parahaemolyticus* strains in recent years (Wu et al., 2016). MLST is based on the sequencing of seven housekeeping genes and can be analyzed directly via the internet (Gonzalez-Escalona et al., 2008). MLST is commonly used for identifying the relationship between isolates in public database and has proven to be an important method for investigation of the evolution and epidemiology of *V. parahaemolyticus* (Banerjee et al., 2014).

Antimicrobials are used in the treatment of infectious diseases and improper or enhanced application of antimicrobials leads to development of antimicrobial resistant (AMR) bacteria (Yano et al., 2014). The Economic Forum for Global Risks indicates that the problem of AMR is projected to be one of the greatest threats to human health in the future (Koser et al., 2014; Blair et al., 2015). The critical factors for the emergence of AMR are antimicrobial resistance genes (ARGs) which can be transferred by the horizontal gene transfer (Thomas and Nielsen, 2005). ARGs are emerging contaminants posing a potential worldwide human health risk (Allen et al., 2010; Lou et al., 2016). It is vital to monitor the AMR and ARGs of *V. parahaemolyticus* strains, which can be used for disease management and reducing the burden of disease caused by this pathogen.

Shanghai is one of the largest prosperous cities in China with high annual consumption of seafood and many cases of *V. parahaemolyticus* infections (Zhang and Orth, 2013; Qi et al., 2016), which have become a potential threat for human health. The researches for *V. parahaemolyticus* strains isolated from aquatic products are widely reported (Wang et al., 2011; Guo et al., 2013; He et al., 2015, 2016; Lou et al., 2016; Hu and Chen, 2016; Yu et al., 2016; Zhang et al., 2017), while studies on clinical isolates has been poorly documented (Zhang and Orth, 2013; Qi et al., 2016).

The main objectives of this study are to monitor the virulence, genetic diversity and antimicrobial susceptibility of clinical *V. parahaemolyticus* isolates from Shanghai. We hope to provide reliable information, for assessing the genetic traits and the antimicrobial resistance risk of *V. parahaemolyticus* strains, and for better management of foodborne infections in Shanghai.

MATERIALS AND METHODS

Specimen Collection and Bacteria Isolation

A total of 1900 fecal specimens were collected by Shanghai hospital from patients who presented with acute diarrhea to gastroenteritis outpatient clinics during the period from January 2014 through to December 2015. These fecal samples were placed in sterile sealed plastic bags and stored at 4°C prior to further analysis. Confirmation of *V. parahaemolyticus* samples were performed using standard culture methods (ISO, 2007).

Briefly, 25 g of each fecal specimen was homogenized for 2 min in a stomacher 400 with 225 mL of alkaline peptone water (APW; Beijing Land Bridge Technology Company Ltd., Beijing, China) containing 3% NaCl, and incubated at 37°C for 16-18 h. After incubation, a loop from the top 1 cm was streaked onto thiosulfate-citrate-bile salts-sucrose (TCBS; Beijing Land Bridge Technology Company Ltd., Beijing, China) agar plates and incubated at 37°C for 18–24 h. Presumptive individual bacterial colony (green or blue green colony, 2–3 mm in diameter) were grown in 10 ml tryptic soy broth (TSB; Beijing Land Bridge Technology Company Ltd., Beijing, China) supplemented with 3.0% NaCl and incubated at 37°C for 18–24 h. After cultivation, the bacterial liquid and 50% glycerol in the proportion of 1:1 were placed in a glycerol tube and stored at –80°C for further analysis.

DNA Extraction

DNA extraction of all presumptive *V. parahaemolyticus* isolates was performed using the TIANamp Bacteria DNA Kit (Tiangen

Biotech Beijing Co., Ltd, Beijing, China), in accordance to the manufacturer's recommended protocols and then stored it at –20°C prior to PCR analysis.

Identification of *Vibrio parahaemolyticus*

The presumptive *V. parahaemolyticus* isolates were tested for the presence of the species specific gene *tlh* by using polymerase chain reaction (PCR). Detection of *tlh* gene was carried out using the primer *tlh*-F (5- AAA GCG GAT TAT GCA GAA GCA CTG -3) and *tlh*-R (5- GCT ACT TTC TAG CAT TTT CTC TGC -3) as specified in (Food and Drug Administration [FDA], 2004). The reaction mixture for this PCR assay was performed in 25 μL, containing 1 μL of DNA template, 12.5 μL of PCR Mix (Sangon Biotech, Shanghai, China), 9.5 μL of dd H_2O and 1 μL of each primer. The thermal-cycling program is as follows: initial denaturation at 94°C for 3 min, 25 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min, and a final extension at 72°C for 3 min. Finally, PCR products were analyzed by agarose gel electrophoresis.

We also chose the API 20E system (BioMerieux, Inc., Durham, NC, United States) and DBI-08 (Beijing Land Bridge Technology Company Ltd., Beijing, China) to analyze and identify the *V. parahaemolyticus* isolates, according to the procedure described by the manufacturer and using *V. parahaemolyticus* ATCC 33847 as the reference strain (Croci et al., 2007; Li et al., 2016; Xie et al., 2016).

Detection of Virulence-Associated Genes

Detection of the *V. parahaemolyticus* virulence genes *tdh* (West et al., 2013) and *trh* (Nilsson and Turner, 2016) were also performed by PCR. We designed a primer for detection of the *ureR* gene to study the variation of the *trh* gene as outlined in Nilsson and Turner, 2016. The *ureR* gene encodes for the transcriptional activator of the urease gene cluster located immediately upstream from *trh* and is widely reported to be genetically linked to *trh* (Nilsson and Turner, 2016). *V. parahaemolyticus* virulence associated genes of type III secretion system-1 (T3SS1) genes (VP1670 [*vscP*], VP1686 [*putative*], VP1689 [*vscK*] and VP1694 [*vscF*]), T3SS2α genes (VP1362 [*vopB2*], VP1339 [*vscC2*], VP1335 [*vscS2*] and VP1327 [*vopT*]) and the T3SS2β genes (*vscC2*, *vopB2*, *vopC*, *vscS2*) were tested by conventional PCR (Jones et al., 2012). In our study, the oligonucleotide primers were synthesized by Sangon Biotech (Sangon Biotech, Shanghai, China). Particularly worth mentioning is that the *V. parahaemolyticus* ATCC17802 (*trh*+) and ATCC33847(*tdh*+) were used as the reference strains, and distilled water was used as the negative control.

Multilocus Sequence Typing

Seven housekeeping genes, *dnaE*, *gyrB*, *recA*, *dtdS*, *pntA*, *pyrC*, and *tnaA* (Supplementary Table S1), were used for *V. parahaemolyticus* characterization under the MLST scheme, PCR fragments were sequenced by Sangon Biotech (Sangon Biotech, Shanghai, China) and alignments of these sequences were determined using DNAMAN. The sequences were analyzed online[1] to assign allele numbers and define sequence types (STs). New sequences for alleles and new ST profiles were submitted to the *V. parahaemolyticus* MLST database. Based on the relatedness of the STs, all of the isolates were subdivided into clonal complexes (CCs) or groups by eBURST program. Nucleotide sequence analyses were evaluated by MEGA5.1 program. In this study, the primary founder of a CC, a single locus variants (SLVs), double locus variants (DLVs), and

[1]http://pubmlst.org/v.parahaemolyticus/

TABLE 1 | Information of 42 *Vibrio parahaemolyticus* clinical isolates.

Name	Gender	Age	*tlh*	*tdh*	*trh*	*ureR*
VPC1	Female	57	+	+	–	–
VPC2	Female	53	+	+	–	–
VPC15	Female	30	+	+	–	–
VPC16	Female	31	+	+	–	–
VPC17	Male	56	+	+	–	–
VPC18	Female	29	+	–	+	+
VPC19	Male	16	+	+	–	–
VPC20	Male	13	+	+	–	–
VPC21	Female	54	+	+	–	–
VPC22	Male	17	+	+	–	–
VPC25	Female	35	+	+	–	–
VPC26	Female	36	+	+	–	–
VPC27	Male	22	+	+	–	–
VPC28	Male	48	+	+	–	–
VPC29	Male	26	+	+	–	–
VPC32	Male	57	+	+	–	–
VPC33	Male	38	+	+	–	–
VPC34	Male	37	+	+	–	–
VPC35	Male	56	+	+	–	–
VPC36	Female	47	+	–	+	+
VPC37	Male	63	+	+	–	–
VPC38	Female	56	+	+	–	–
VPC40	Female	58	+	+	–	–
VPC41	Female	79	+	+	–	–
VPC42	Male	55	+	+	–	–
VPC43	Male	56	+	+	+	+
VPC44	Male	33	+	–	–	–
VPC45	Female	54	+	+	–	–
VPC46	Male	26	+	+	–	–
VPC47	Female	86	+	+	–	–
VPC48	Female	45	+	+	–	–
VPC49	Female	56	+	+	+	+
VPC50	Male	46	+	+	–	–
VPC51	Male	24	+	+	–	–
VPC54	Female	39	+	+	+	+
VPC55	Male	22	+	+	–	–
VPC85	Female	58	+	–	+	+
VPC89	Female	46	+	+	–	–
VPC90	Male	37	+	+	–	–
VPC94	Male	28	+	–	+	+
VPC97	Female	26	+	+	–	–
VPC100	Male	56	+	+	–	–

singletons were defined as described previously (Han et al., 2015).

Antimicrobial Susceptibility Testing

The antibiotic susceptibilities of the 42 isolates were assessed using the disk diffusion method on Mueller Hinton agar (MHA) (OXOID Limited, China) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015; Lou et al., 2016). Briefly, Muller–Hinton agar and a panel of 18 antibiotics disks were selected for resistance tests. The 18 common antimicrobials belonging to 6 classes used in this study were: β-lactam (ampicillin: AMP, amoxicillin-clavulanic: AMC, piperacillin: PRL, cefotaxime: CTX, ceftazidime: CAZ, cefoxitin: FOX, cephazolin: KZ, imipenem: IPM, meropenem: MEM), aminoglycoside (amikacin: AK, gentamicin: CN, kanamycin: K, streptomycin: S), tetracycline (tetracycline: TET), quinolone (ciprofloxacin: CIP, levofloxacin: LEV), sulfonamides (trimethoprim-sulfamethoxazole: SXT), chloramphenicol (chloramphenicol: C). The results were expressed as sensitive (S), intermediate (I), or resistant (R) according to the methods of the CLSI (CLSI, 2015). *Escherichia coli* ATCC 25922 was used as the quality control organism for the antimicrobial susceptibility testing.

Evaluation of Antibiotic Resistance-Encoding Genes

The 38 antibiotic resistance genes (Supplementary Table S2) of six classes of antibiotics were identified by PCR, as previously described (Lou et al., 2016). All obtained PCR products were purified and sequenced by Sangon Biotech (Sangon Biotech, Shanghai, China). The acquired sequences were aligned and analyzed with the BLAST program[2].

TABLE 2 | Distribution of T3SS genes among 42 clinical *V. parahaemolyticus* isolates.

Gene	No. of strains (n = 42)			
	tdh+ *trh*− (n = 34)	*tdh*+ *trh*+ (n = 3)	*tdh*− *trh*+ (n = 4)	*tdh*− *trh*− (n = 1)
T3SS1				
VP1670 (*vscP*)	34	3	4	1
VP1686 (*putative*)	34	3	4	1
VP1689 (*vscK*)	34	3	4	1
VP1694 (*vscF*)	34	3	4	1
All 4 genes present	34	3	4	1
T3SS2α				
VP1362 (*vopB2*)	34	2	3	1
VP1339 (*vscC2*)	34	1	3	1
VP1335 (*vscS2*)	34	1	2	1
VP1327 (*vopT*)	34	1	2	1
All 4 genes present	34	1	2	1
T3SS2β				
vscC2	0	3	4	0
vopB2	1	3	4	0
vopC	1	2	4	0
vscS2	1	3	4	0
All 4 genes present	0	2	4	0

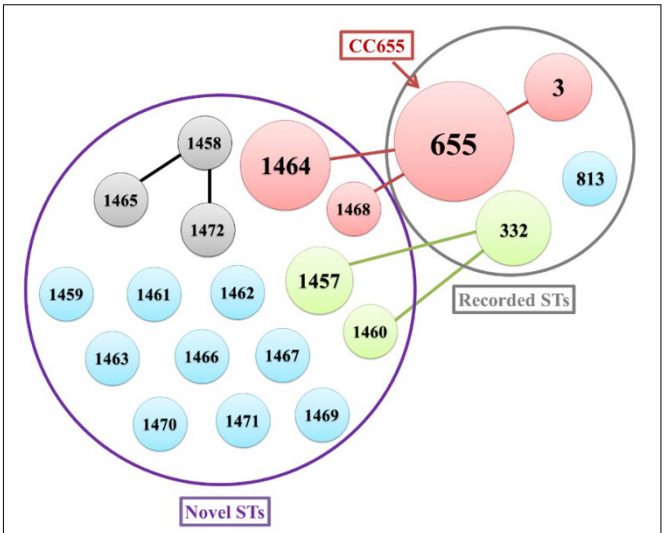

FIGURE 1 | Multilocus sequence typing (MLST) of 42 *V. parahaemolyticus* clinical isolates.

RESULTS

Prevalence of *V. parahaemolyticus*

A total of 42 presumptive *V. parahaemolyticus* strains were isolated from 1900 fecal specimens (2.2%) collected from patients presenting in Shanghai hospital during January, 2014 to December, 2015. The PCR results showed all isolates were positive for the presence of the *tlh* gene (**Table 1**), which indicated that these 42 isolates were *V. parahaemolyticus* strains. And the results of API 20E and DBI-08 provided further evidence to the veracity of PCR outcomes, with all 42 isolates being identified with 99% confidence as *V. parahaemolyticus*. The demographic characteristics of patients about 42 *V. parahaemolyticus* isolates are also presented in **Table 1**. The 42 individuals enrolled in this research included 20 females and 22 males. The male patients' ages ranged from 13 to 57, while for the female patients, the age distribution was more uniform in women whose median ages above 30 years-old.

Distribution of Virulence-Associated Genes

From our study, the hemolysin gene *tdh* was detected in most of the isolates (88.1%, 37/42), whereas the *trh* gene was present in only 7 strains (16.7%, 7/42). We further determine the distribution of the *trh* gene by the *ureR* gene. The *ureR* gene and the variable *trh* gene were observed in the same seven *V. parahaemolyticus* isolates. Of these, 3 of 42 (7.1%) clinical isolates were positive for both *tdh* and *trh*. Only one strains

[2]http://www.ncbi.nlm.nih.gov/BLAST

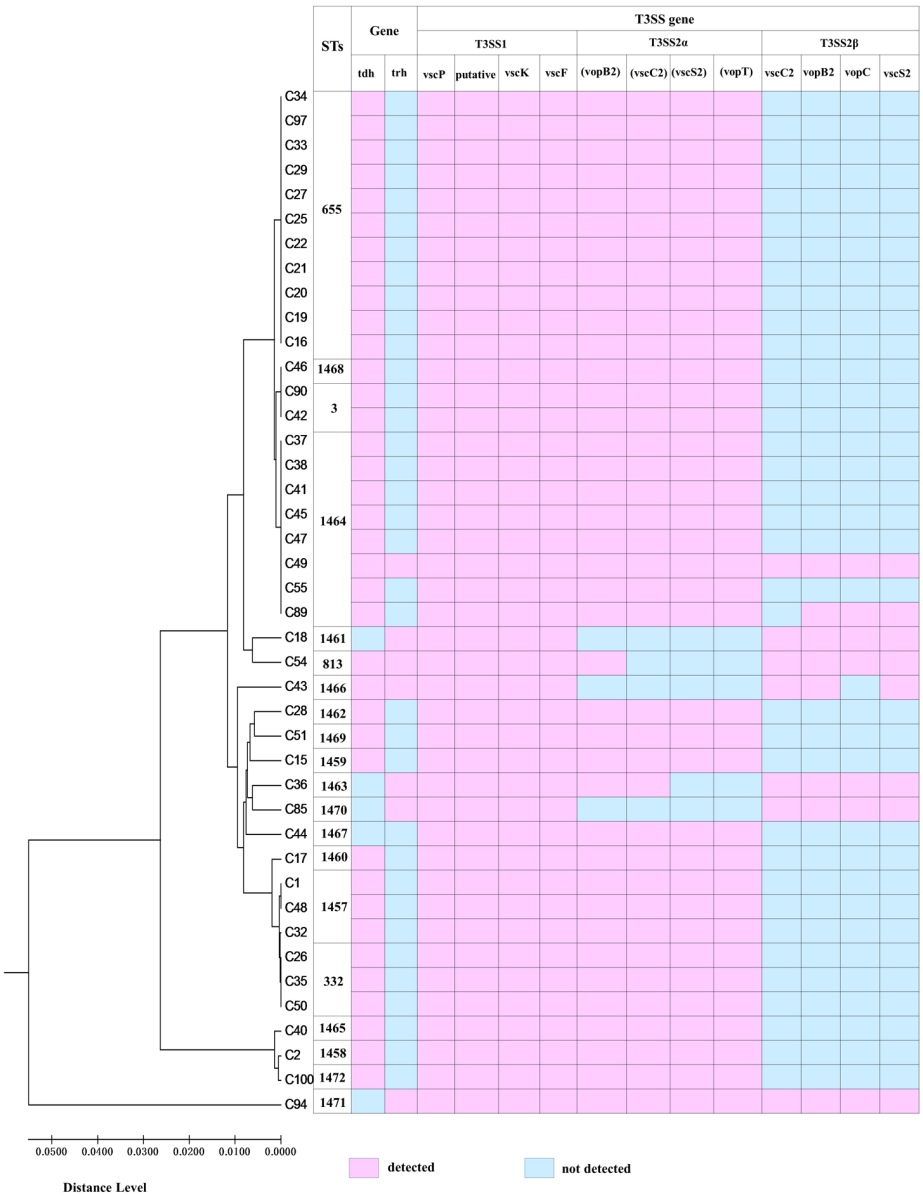

FIGURE 2 | The relationship between genetic diversity and virulence-associated genes characterization of isolates. ST, sequence type.

(2.4%, 1/42) from the diarrheal patients contained neither the *tdh* nor the *trh* gene.

The two types of T3SS complexes are features in the virulence mechanism of *V. parahaemolyticus* and the distribution of T3SS genes is presented in **Table 2**. T3SS1 genes were identified in all of the *V. parahaemolyticus* isolates and these samples contained all four T3SS1 genes (100%). All thirty-four of the *tdh*+/*trh*- and one of the *tdh*-/*trh*- clinical isolates contained all four genes of the T3SS2α genes. However, the isolates of *tdh*+/*trh*+ and *tdh*-/*trh*+ the detected percentage of all T3SS2α genes was only 33.3% and 50.0%, respectively. Additionally, four of the *tdh*-/*trh*+ isolates were amplified all four genes of the T3SS2β genes, followed by the isolates of *tdh*+/*trh*+ the detected percentage of all T3SS2β genes was 66.7%. Only one remaining *tdh*+/*trh*- isolate (VPC89) amplified all four T3SS2β genes but *vscC2*. As expected, one of the *tdh*-/*trh*- clinical isolate was evaluated in negative of all four T3SS2β genes. Overall, the T3SS2α-associated genes were most prevalent in *tdh*+ isolates (93.5%, 35/37), and the T3SS2β genes were detected prevalent in the *trh*+ clinical isolates (85.7%, 6/7).

Multilocus Sequence Typing Analysis

The genetic characteristic of the *V. parahaemolyticus* isolates was analyzed by MLST. MLST classified the 42 *V. parahaemolyticus* isolates into 20 different STs (**Figure 1**), of which 16 ST were novel (ST1457, ST1458, ST1459, ST1460, ST1461, ST1462, ST1463,

ST1464, ST1465, ST1466, ST1467, ST1468, ST1469, ST1470, ST1471, and ST1472). The go eBURST algorithm used in our study categorized 20 different STs into 10 singletons, one clone complexes (CC655) and Two groups (**Figure 2**). Among these, CC655 was the most prevalent clone complexes, including 22 isolates with ST655(50%), ST1464(36.4%), ST3(9.1%) and ST1468(4.5%). In addition, ST655 was the most frequent sequence type, which including 11 isolates (VPC16, VPC19 VPC20, VPC21, VPC22, VPC25, VPC27, VPC29, VPC33, VPC34, VPC97).

Antimicrobial Resistance Profile

As shown in the **Table 3**, the isolates of *V. parahaemolyticus* were detected for different levels of antibiotic resistance. One concern, all *V. parahaemolyticus* strains were resistant to ampicillin (100%) and streptomycin (100%), followed by Cephazolin (92.9%), Kanamycin (92.9%) and Amikacin (90.5%). Isolates were also commonly resistant to Gentamicin (71.4%), Piperacillin (54.8%), and Cefoxitin (50%). In addition, all the clinical isolates showed susceptibility to six antibiotics, including Chloramphenicol, Cefotaxime, Imipenem, Ceftazidime, Trimethoprim-sulfamethoxazole and Meropenem. And only one *V. parahaemolyticus* strains (VPC2) were not found in multidrug resistance (MDR, defined as resistance to 3 or more different antimicrobials). So the multidrug resistance rate reaches to 97.6% of all 42 clinical *V. parahaemolyticus* isolates. Of these, there were 92.9% multidrug-resistant isolates showing resistance to more than five antibiotics. Strikingly, we found that four isolates showed resistance to ten antibiotics.

TABLE 3 | Antimicrobial resistance profiles of 42 clinical *V. parahaemolyticus* isolates.

Classify	Antimicrobial agent	*Vibrio parahaemolyticus* ($n = 42$)		
		No. (%) of R	No. (%) of I	No. (%) of S
β- lactam	Ampicillin	42 (100.0)	0 (0.0)	0 (0.0)
	Amoxicillin-Clavulanic	0 (0.0)	8 (19.0)	34 (81.0)
	Piperacillin	7 (16.7)	16 (38.1)	19 (45.2)
	Cefotaxime	0 (0.0)	0 (0.0)	42 (100.0)
	Ceftazidime	0 (0.0)	0 (0.0)	42 (100.0)
	Cefoxitin	0 (0.0)	21 (50.0)	21 (50.0)
	Cephazolin	16 (38.1)	23 (54.8)	3 (7.1)
	Imipenem	0 (0.0)	0 (0.0)	42 (100.0)
	Meropenem	0 (0.0)	0 (0.0)	42 (100.0)
Tetracyclines	Tetracycline	0 (0.0)	2 (4.8)	40 (95.2)
Aminoglycosides	Amikacin	2 (4.8)	36 (85.7)	4 (9.5)
	Gentamicin	3 (7.1)	27 (64.3)	12 (28.6)
	Kanamycin	3 (7.1)	36 (85.7)	3 (7.1)
	Streptomyein	30 (71.4)	12 (28.6)	0 (0.0)
Quinolones	Ciprofloxacin	0 (0.0)	11 (26.2)	31 (73.8)
	Levofloxacin	0 (0.0)	2 (4.8)	40 (95.2)
Chloramphenicol	Chloramphenicol	0 (0.0)	0 (0.0)	42 (100.0)
Sulfonamides	Trimethoprim -sulfamethoxazole	0 (0.0)	0 (0.0)	42 (100.0)

R, resistant; I, intermediate; S, sensitive.

Antimicrobial Resistance Genotypes of *V. parahaemolyticus*

The 38 antibiotic resistance genes of 6 classes of antibiotics searched in 42 pathogenic *V. parahaemolyticus* isolates are shown in Supplementary Table S3. Eight out of 38 resistance genes (*SHV*, *tet*(B), *str*A, *qnr*A, *gry*A, *qnr*B, *sul*I, *sul*II) were detected in at least one isolates. Notably, all of the clinical isolates carried two or more ARGs evaluated. Among them, *tet(*B) was the most prevalent gene, with the detection frequencies of 100%, followed by *str*A, *sul*I, *SHV*, *qnr*A, *qnr*B *gry*A, and *sul*II the detected percentage of them was 92.9, 90.5, 28.6, 28.6, 26.2,19.0, and 4.8%, respectively.

Correlation among Virulence genes, STs, Resistance Phenotype, and Genotype

A minimum spanning tree (MST) of the sequence types (STs) that was constructed based on subtyping information, including sequence type and virulence-associated genes, is shown in **Figure 2**. As shown in **Figure 2**, We can see that the most prevalent clonal complexes were CC655, all of them were positive for virulence-related *tdh* (100%), T3SS1(100%) genes and T3SS2α (100%) genes and the majority of them were negative for the *trh* (95.5%) and T3SS2β (95.5%) genes. Notably, two distinct lineages of the T3SS2 have been described with a correlation between the presence of *tdh* with T3SS2α and *trh* with T3SS2β. From this we can observe that the virulence-related gene of *trh* and T3SS2β are co-occurrences and disappearance simultaneously. Specifically, the *tdh* (100%), T3SS1(100%) genes and T3SS2α (100%) genes were detected in all ST655 isolates.

We conclude that correlation between virulence-related genes, AMR phenotypes and genotypes was absent. Likewise, as shown in **Figure 3**, genetic diversity is not apparently associated with the AMR phenotypes and genotypes.

DISCUSSION

In this study, we analyzed 1900 fecal specimens collected from Shanghai hospital from January, 2014 to December, 2015 and isolated 42 *V. parahaemolyticus* strains, with an isolation rate of 2.2%. Compared to other studies in China, this rate is significantly less than the 6.0% reported for southern coastal China in 2007–2012 (Li et al., 2014) and the 8.1% observed in southeastern China in 2009–2013 (Chen et al., 2016). Compared to other developing countries, our result is also less than the clinical *V. parahaemolyticus* isolation rate of 5.1% detected in Northwestern Mexico from 2004 to 2010 (Velazquez-Roman et al., 2012). That indicates the current status of *V. parahaemolyticus* infection in Shanghai is better than other regions.

The thermostable direct haemolysin (TDH), the TDH-related haemolysin (TRH) and the two type III secretion systems (T3SS1 and T3SS2) are recognized as major virulence factors in *V. parahaemolyticus* (Ceccarelli et al., 2013; Letchumanan et al.,

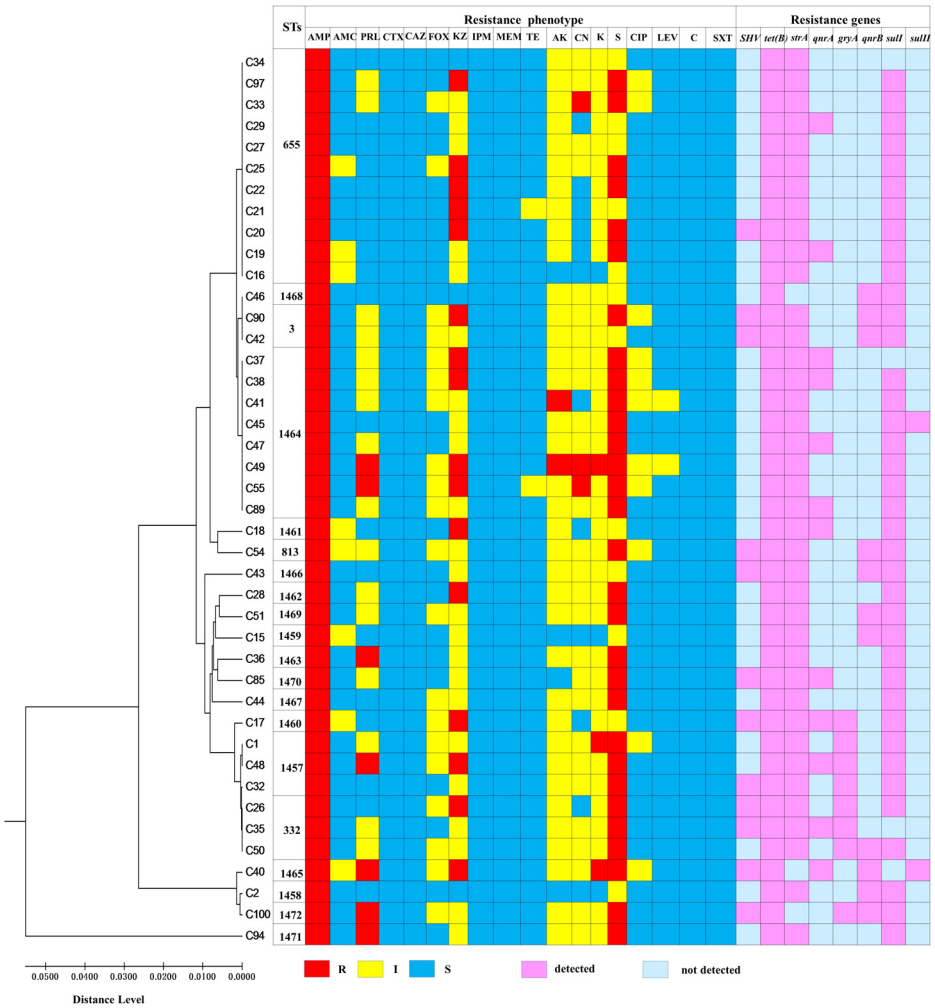

FIGURE 3 | The relationship of genetic diversity, resistance phenotypic and genotypic characterization of 42 clinical *V. parahaemolyticus* isolates. ST, sequence type; R, resistant; I, intermediate; S, sensitive.

2014; Raghunath, 2015). In our study, the hemolysin gene *tdh* was detected in 88.1% *V. parahaemolyticus* isolates, whereas the *trh* gene was present in only 7 strains. And we found that one of the clinical isolates is co-negative for *tdh* and *trh* gene. This finding is consistent with prior studies that not all clinical strains harbor these genes (Okuda et al., 1997). All *V. parahaemolyticus* strains in this study contains the T3SS1 component genes, which is also consistent with a previous study (Jones et al., 2012). The T3SS2 contains two gene clusters, T3SS2α and T3SS2β (Ritchie et al., 2012; Wang et al., 2015), which is closely related to *tdh*-positive and *trh*-positive *V. parahaemolyticus*, respectively (Jones et al., 2012). However, in this study, T3SS2α genes didn't appear with *tdh*+ genes simultaneously, which indicates that there is high genetic heterogeneity in *V. parahaemolyticus* T3SSs.

The genetic diversity of *V. parahaemolyticus* was also investigated using MLST. Compared to other molecular methods, such as the identification of known virulence genes, phylogenetic analysis of housekeeping genes, microarray, and PFGE, MLST give a better understanding of the genetic relationships among *V. parahaemolyticus* isolates (Perez-Losada et al., 2013; Hazen et al., 2015; Ludeke et al., 2015). In this study, 42 *V. parahaemolyticus* isolates were classified into 20 sequence types (STs) with 16 novel STs. The high proportion of novel STs indicated a high genetic diversity of *V. parahaemolyticus* strains, and shows that the information content in the MLST database on this strain is still evolving. Thus, more MLST surveillances should be performed in China and the rest of world, to contributed to better understanding the genetic diversity of *V. parahaemolyticus*. Furthermore, ST655 was the most frequent hypotype in our study, which was clustered into the major clonal complex, CC655. Previous studies have been reported that ST3 belonged to the most prevalent clonal complex CC3, which is widely distributed and plays an important role in *V. parahaemolyticus* infections in multiple countries (Fuenzalida et al., 2006; Martinez-Urtaza et al., 2013; Turner et al., 2013; Haendiges et al., 2015; Han et al., 2016). There is only one locus difference between ST3

and ST655, which indicates that these STs are closely related. We recommend further research on the pandemic clonal complexes CC655 containing ST655 for management of *V. parahaemolyticus* infections.

Antibiotic treatment is necessary for controlling *V. parahaemolyticus* infections, but overuse of antibiotics has led to the generation and distribution of antimicrobial-resistant bacteria, which is becoming a major concern for human health (Ji et al., 2011; Shaw et al., 2014; Blair et al., 2015). This study also investigated the antimicrobial resistance phenotype and genotype of the 42 clinical *V. parahaemolyticus* strains. All isolates showed a high level of resistance against Ampicillin (100.0%), Streptomycin (100.0%), Cephazolin (92.9%), Kanamycin (92.8%), and Amikacin (90.5%), and eight out of 38 resistance genes (*SHV*, *tet*(B), *str*A, *qnr*A, *gry*A, *qnr*B, *sul*I, *sul*II) were detected in at least two isolates. According to this study and some previous researches (Chen et al., 2016; Xie et al., 2017), the antimicrobial resistance of clinical *V. parahaemolyticus* was significantly higher than that of environmental strains which were isolated from water (Shaw et al., 2014), aquatic products (Letchumanan et al., 2015; Lou et al., 2016; Yu et al., 2016; Xie et al., 2017) or ready-to-eat foods (Xie et al., 2016). As the human gastrointestinal tract is a conducive environment for promoting horizontal ARGs transfer (Hu et al., 2013; Theethakaew et al., 2013), we speculate that the complex gastrointestinal environment may accelerate the acquisition of antimicrobial resistance in *V. parahaemolytiucs*. The microevolution mechanisms for the different rates of acquisition of antibiotic resistance between clinical and environmental pathogens should be studied further.

CONCLUSION

This study is the first comprehensive research describing the virulence, genetic diversity, antibiotic resistance phenotype, and genotype of *V. parahaemolyticus* from diarrhea patients in Shanghai. The study reveals that *tdh*, *trh* and T3SS genes are of equal importance as virulence associated factors. MLST analysis showed that the novel loci and STs points to high genetic diversity of *V. parahaemolyticus* strains isolated in Shanghai. ST655 was the most prevalent STs and this ST could have evolved from the global pandemic ST3. The antimicrobial resistance profiles indicated that the multidrug-resistant isolates were also widespread and measures to contain or slowdown the emergence of drug-resistant strains should be a top priority in China. These results add to the epidemiological data of clinical *V. parahaemolyticus* isolates in Shanghai and highlight the need for more AMR type research for managing the burden of disease caused by this pathogen in China.

AUTHOR CONTRIBUTIONS

HL, RT, and YL contributed equally to carrying out the experiments and writing the draft manuscript. YZ (Corresponding Author), YP, PM, and ZZ provided support for experimental design and editing of final manuscript. ZC, WC, QH assisted in completing the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01043/full#supplementary-material

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Vibrio cholerae O1 with Reduced Susceptibility to Ciprofloxacin and Azithromycin Isolated from a Rural Coastal Area of Bangladesh

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Cholera outbreaks occur each year in the remote coastal areas of Bangladesh and epidemiological surveillance and routine monitoring of cholera in these areas is challenging. In this study, a total of 97 *Vibrio cholerae* O1 isolates from Mathbaria, Bangladesh, collected during 2010 and 2014 were analyzed for phenotypic and genotypic traits, including antimicrobial susceptibility. Of the 97 isolates, 95 possessed CTX-phage mediated genes, *ctxA*, *ace*, and *zot*, and two lacked the cholera toxin gene, *ctxA*. Also both CTX^+ and CTX^- *V. cholerae* O1 isolated in this study carried *rtxC*, $tcpA^{ET}$, and *hlyA*. The classical cholera toxin gene, *ctxB1,* was detected in 87 isolates, while eight had *ctxB7.* Of 95 CTX^+ *V. cholerae* O1, 90 contained $rstR^{ET}$ and 5 had $rstR^{CL}$. All isolates, except two, contained SXT related integrase *intSXT*. Resistance to penicillin, streptomycin, nalidixic acid, sulfamethoxazole-trimethoprim, erythromycin, and tetracycline varied between the years of study period. Most importantly, 93% of the *V. cholerae* O1 were multidrug resistant. Six different resistance profiles were observed, with resistance to streptomycin, nalidixic acid, tetracycline, and sulfamethoxazole-trimethoprim predominant every year. Ciprofloxacin and azithromycin MIC were 0.003–0.75 and 0.19–2.00 μg/ml, respectively, indicating reduced susceptibility to these antibiotics. Sixteen of the *V. cholerae* O1 isolates showed higher MIC for azithromycin (≥0.5 μg/ml) and were further examined for 10 macrolide resistance genes, *erm*(A), *erm*(B), *erm*(C), *ere*(A), *ere*(B), *mph*(A), *mph*(B), *mph*(D), *mef*(A), and *msr*(A) with none testing positive for the macrolide resistance genes.

Keywords: *Vibrio cholerae*, El Tor, antibiotic resistance, reduced susceptibility, ciprofloxacin, azithromycin

INTRODUCTION

Vibrio cholerae, the causative agent of cholera, is autochthonous to the estuarine and marine environment worldwide. Of more than 200 O-antigen serogroups identified in *V. cholerae*, only toxigenic O1 and O139 are primarily associated with epidemics and pandemics (Sack et al., 2004). Cholera, an ancient diarrheal disease, continues to be a serious threat in countries of Asia,

Africa, and South America. Even though cholera is underreported in many countries, 3–5 million cases are recorded annually in different parts of the world, with a significant number of deaths (Ali et al., 2012). The case fatality rate of cholera has been reduced over the past few decades, mainly because patients are treated with oral and/or intravenous rehydration therapy together with appropriate dosage of antibiotics. Effective antibiotic treatment shortens the duration of diarrhea and limits the loss of body fluids by ca. 50% (Sack et al., 2004). However, antibiotic resistant enteropathogens, including *V. cholerae,* are emerging rapidly due to the selective pressure of antibiotics existing in the environment and from excessive use (Laxminarayan et al., 2013; Andersson and Hughes, 2014). *V. cholerae,* both O1 and O139, have developed resistance to several antimicrobial drugs, including tetracycline (TE), chloramphenicol (C), furazolidone, ampicillin (AM), and trimethoprim-cotrimoxazole, used successfully to treat cholera over the years (Garg et al., 2001; Kitaoka et al., 2011). As a consequence, multidrug resistant (MDR) *V. cholerae* has been on the rise, causing clinicians to face a serious challenge when deciding a drug of choice and regimen for treating cholera patients.

Two biotypes of *V. cholerae* O1, Classical (CL) and El Tor (ET) are universally recognized, with each possessing distinct phenotypic and genetic traits, including major virulence genes, i.e., toxin coregulated pilus (*tcpA*) and B-subunit of cholera toxin (*ctxB;* Kaper et al., 1995; Safa et al., 2010). Of the two biotypes, CL is associated with the sixth and presumably the earlier pandemics of cholera that occurred between 1817 and 1923 (Kaper et al., 1995; Devault et al., 2014), while ET is reported to have initiated the ongoing seventh pandemic in the early 1960s, gradually displacing the CL biotype (Kaper et al., 1995; Kim et al., 2015). Over the past two decades, variants of ET with only a few CL attributes (phage-encoded repressor $rstR^{CL}$ and B-subunit of cholera toxin $ctxB^{CL}$) have emerged in Asia and Africa. These variants are collectively known as atypical ET (Safa et al., 2010; Kim et al., 2015). Moreover, based on amino acid substitutions in CtxB, 12 different *ctxB* genotypes have been identified in *V. cholerae* (Kim et al., 2015). In 1992, *V. cholerae* O139 carrying the SXT/R391 family integrative conjugative element (ICE) appeared transiently as the major cause of cholera in Bangladesh and India (Albert et al., 1993; Ramamurthy et al., 1993; Waldor et al., 1996). SXT/R391 ICE was the first MDR marker detected in *V. cholerae*, conferring resistance to streptomycin (S), sulfamethoxazole, and trimethoprim (Waldor et al., 1996; Hochhut et al., 2001). SXT/R391 ICE also found to provide a selective advantage to *V. cholerae* O1 ET, a strain that has been tracked globally in three overlapping waves during the seventh pandemic (Mutreja et al., 2011).

Interestingly, outbreaks of cholera that occur in the coastal areas are seasonal each year in Bangladesh. For example, in Mathbaria, cholera occurs predominantly during the spring, months of March through May, with inhabitants lacking safe drinking water are most susceptible (Emch et al., 2008; Akanda et al., 2013). Several antibiotics are used to treat cholera, including doxycycline, ciprofloxacin (CIP), and azithromycin (AZ), all greatly influenced by the drug sensitivity pattern of the bacterium reported in the contemporary literature (Harris et al., 2012). In Bangladesh, a single dose of AZ or CIP currently is used for prophylactic treatment. Not surprising, *V. cholerae* O1 is now reported to have reduced susceptibility to CIP in Bangladesh, India, Vietnam, Haiti, Zimbabwe, and Western Africa (Islam et al., 2009; Quilici et al., 2010; Sjölund-Karlsson et al., 2011; Tran et al., 2012; Kumar et al., 2014; Khan et al., 2015). MDR *V. cholerae* O1 resistant to TE, AM, S, sulfonamides, norfloxacin, gentamicin, furazolidone, kanamycin (K), sulfamethoxazole-trimethoprim (SXT), and erythromycin (E), is currently circulating in cholera endemic countries of Asia and Africa (Finch et al., 1988; Faruque et al., 2007; Jain et al., 2011; Rashed et al., 2012; Dixit et al., 2014). Furthermore, genes conferring resistance to CIP and AZ have been shown to be transferred to *V. cholerae* via plasmids, gene cassettes, and mobile genetic elements with horizontal gene transfer mechanisms in environmental reservoir implicated in transforming sensitive bacteria to resistant (Kitaoka et al., 2011). Considering these phenotypic and genetic modifications that are reported, a study of 97 *V. cholerae* O1 isolates was undertaken to determine the antibiotic resistance/susceptibility status of *V. cholerae* O1 isolated from environmental samples and cholera cases in cholera endemic Mathbaria, Bangladesh.

MATERIALS AND METHODS

Bacterial Strains

In this study, a total of 97 *V. cholerae* O1 isolated from rectal swabs and surface water samples collected in the coastal villages of Mathbaria, Bangladesh, between June, 2010 and December, 2014, as a part of epidemiological surveillance conducted by the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B) were analyzed for antibiotic susceptibility and genotypic traits. Mathbaria is geographically adjacent to the Bay of Bengal, located ∼165 km south-west of Dhaka city. Clinical isolates ($n = 52$) were obtained from rectal swabs of suspected cholera patients seeking treatment at the local health center during the cholera peak and off-peak season. Environmental isolates ($n = 45$) were obtained from water and plankton samples collected periodically at six different ponds and a river in the same area where the clinical samples were collected. The clinical and environmental samples were collected, transported, and subjected to bacteriological analysis for *V. cholerae,* following standard procedures (Alam et al., 2006a; Huq et al., 2012). Isolation and identification were performed according to standard methods (Alam et al., 2006a,b; Huq et al., 2012). All samples were collected according to protocols approved by institutional review boards at the Johns Hopkins University, University of Maryland (College Park, MD, USA), and ICDDR,B. Informed consent was obtained from the patients, and parents or legal guardians of the children who participated in this study. Genomic DNA was prepared from the presumptively identified *V. cholerae* isolates using the boiling lysis method of Park et al. (2013) and *V. cholerae* species-specific *ompW* PCR was done to confirm identity of the isolates (Nandi et al., 2000).

Serogrouping

The serogroups of the *V. cholerae* isolates were confirmed by a slide agglutination test using specific polyvalent antisera for *V. cholerae* O1 and O139. Isolates showing positive agglutination reaction with O1 antisera were tested further using a serotype-specific monoclonal antibody, i.e., Inaba and Ogawa (Alam et al., 2006b). The serogroups of these isolates were reconfirmed by multiplex PCR, targeting O1-(*wbe*) and O139-(*wbf*) specific O biosynthetic genes, together with the cholera toxin gene (*ctxA;* Hoshino et al., 1998).

Antimicrobial Susceptibility

Susceptibility to antimicrobials was determined by standard disc diffusion on Muller-Hinton agar (BD, USA) according to Clinical and Laboratory Standards Institute guidelines for *V. cholerae* (CLSI, 2010a) and *Enterobacteriaceae* (CLSI, 2010b). *Escherichia coli* ATCC 25922 was used as a control for antimicrobial susceptibility. All strains of *V. cholerae* were tested for resistance to AM (10 μg), CIP (5 μg), C (30 μg), E (15 μg), K (30 μg), S (10 μg), TE (30 μg), nalidixic acid (NA, 30 μg), penicillin (P, 10 μg), and SXT (23.75 and 1.25 μg, respectively) using commercially available discs (BD BBL Sensi-Disc). Minimum inhibitory concentrations (MIC) of CIP and AZ were determined using *E*-test strips (bioMérieux-USA), according to the manufacturer's instructions. Cut-off levels for assessing resistance were determined following the CLSI document M45 guidelines (CLSI, 2010b).

PCR Assay

PCR assays were carried out to detect genes encoding accessory cholera enterotoxin (*ace*), zonula occludens toxin (*zot*), hemolysin (*hlyA;* Rivera et al., 2001), SXT-related integrase (*int*SXT; Hochhut et al., 2001) and biotype-specific (ET and CL) toxin coregulated pilus (*tcpA;* Rivera et al., 2001), phage-encoded repressor (*rstR;* Kimsey et al., 1998), and repeat in toxin (*rtxC;* Chow et al., 2001) using primers and conditions described previously. Double mismatch amplification mutation assay (DMAMA)-PCR was performed to identify three genotypes of the cholera toxin gene, i.e., *ctxB1*, *ctxB3*, and *ctxB7*, based on nucleotide substitution at position 58, 115, and 203 (Naha et al., 2012). *V. cholerae* O1 strains O395 (CL), N16961 (ET), and 2010EL-1786 were used as control for the PCR analysis. *V. cholerae* O1 isolates showing MIC for AZ $\geq$ 0.5 μg/mL were analyzed further for the macrolide resistance genes: *erm*(A), *erm*(B), and *erm*(C), that encode methylase; *ere*(A) or *ere*(B) encoding esterases; *mph*(A), *mph*(B), and *mph*(D) encoding phosphotransferases; and *mef*(A) and *msr*(A) encoding efflux pumps (Phuc Nguyen et al., 2009).

RESULTS

Phenotypic and Geneotypic Characteristics

All 97 isolates produced colonies typical of *V. cholerae* on both taurocholate tellurite gelatin agar (TTGA) and thiosulfate citrate bile-salts sucrose (TCBS) agar. These isolates gave biochemical reactions characteristic of *V. cholerae* and reacted to polyvalent antibody specific for *V. cholerae* serogroup O1. Of 97 isolates, 89 gave positive agglutination with monovalent Ogawa antisera, while the remaining eight reacted positively with monovalent Inaba antisera. All isolates were serologically identified as *V. cholerae* O1. Notably, the serotype of 89 strains was determined to be Ogawa and eight to Inaba (**Table 1**).

Genomic DNA of all isolates ($n = 97$) amplified *V. cholerae* species-specific genes, namely *ompW* and O-antigen biosynthetic-*wbe* (O1) confirming identification as *V. cholerae* O1. None amplified the O-antigen biosynthetic-*wbf* (O139). As shown in **Table 1**, except for two isolates, all amplified the CTX-phage mediated genes *ctxA*, *ace*, and *zot*, suggesting 95 of the isolates were toxigenic *V. cholerae* O1. The PCR assay results also showed *hlyA* gene present in all isolates (**Table 1**). Of 97 *V. cholerae* O1, *intSXT* was identified in 95 isolates, while two lacked *intSXT*. All *V. cholerae* O1 isolates contained the ET biotype specific *tcpA* and *rtxC,* reflecting ET attributes. Among the 95 toxigenic *V. cholerae* O1 isolates, 90 possessed *rstR* of the ET biotype ($rstR^{ET}$), while the remaining five revealed CL biotype specific $rstR^{CL}$. Unlike hybrid ET strains, none of the toxigenic isolates contained both $rstR^{ET}$ and $rstR^{CL}$. DMAMA-PCR detected cholera toxin gene of CL biotype (*ctxB*1) in 87 *V. cholerae* O1 isolates, while 8 had Orissa variant or Haiti variant cholera toxin (*ctxB7*; **Table 1**). Overall, the PCR results confirmed that 90 of the *V. cholerae* O1 isolates were atypical ET, possessing the $rstR^{ET}$ and either *ctxB1* or *ctxB7* gene. Five toxigenic *V. cholerae* O1 possessing $rstR^{CL}$ and *ctxB1* are designated as variant ET and their genetic attributes were similar to the Matlab variant (MJ1236) isolated in 1994 in Matlab, Bangladesh. As shown in **Table 1**, *V. cholerae* O1 variant ET was isolated from both clinical and environmental sources in Mathbaria, Bangladesh only in 2012. *V. cholerae* O1 atypical ET was associated with cholera cases that occurred during June, 2010, and December, 2014, in Mathbaria, Bangladesh and these strains were also isolated frequently from environmental sources (**Table 1**).

As shown in **Figure 1**, the CL type cholera toxin genotype, *ctxB1,* was predominant, having been detected in 73, 80, 95, 100, and 91% *V. cholerae* O1 isolates in 2010, 2011, 2012, 2013, and 2014, respectively. In contrast, Orissa, or Haiti variant cholera toxin genotype *ctxB7* was found in 27, 20, and 5% isolates in 2010, 2011, and 2012, respectively. Remarkably, *ctxB7* was not detected in *V. cholerae* O1 isolated in 2013 and thereafter (**Table 1**). Although, 9% of the *V. cholerae* O1 were non-toxigenic in 2014, *ctxB1* was the only genotype prevailed among toxigenic isolates in 2013 and 2014.

Antimicrobial Susceptibility

Antimicrobial susceptibility tests, using ten different antibiotics revealed that 93% of the total set of *V. cholerae* O1 isolates were MDR, i.e., resistant to at least three different antibiotics drugs (**Table 1**). As shown in **Figure 2A**, six different resistance profiles were observed, with a range of resistance to one to five antibiotics during 2010 and 2014. *V. cholerae* O1 showing resistance to S, NA, TE, and SXT was the dominant pattern (53–91%) each year between 2010 and 2014 (**Figure 2A**). Interestingly, resistance of *V. cholerae* O1 to P, S, NA, SXT, E, and TE varied during

TABLE 1 | Genetic characteristics and drug resistance of *V. cholerae* O1 isolated in Bangladesh.

Year of isolation	Number of strains	Source	Serotype	*wbeO1*	*ctxA*	*ace*	*zot*	*tcpA*	*rtxC*	*ctxB* type	*rstR*	*hlyA*	int_{SXT}	Drug resistance profile
2010	7	Env	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	S, NA, TE, SXT
	3	Env	OGET	+	+	+	+	ET	+	*B7*	ET	+	+	S, NA, SXT
	1	Clinical	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	S, NA, TE, SXT
2011	3	Env	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	S, NA, TE, SXT
	1	Env	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	S, NA, SXT
	11	Clinical	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	S, NA, TE, SXT
	4	Clinical	OGET	+	+	+	+	ET	+	*B7*	ET	+	+	S, NA, SXT
	1	Clinical	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	S, NA, TE, E, SXT
2012	5	Env	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	S, NA, TE, SXT
	3	Env	INET	+	+	+	+	ET	+	*B1*	CL	+	+	S, SXT
	1	Env	INET	+	+	+	+	ET	+	*B1*	ET	+	+	S, NA, SXT
	4	Clinical	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	S, NA, TE, SXT
	2	Clinical	INET	+	+	+	+	ET	+	*B1*	CL	+	+	S, SXT
	1	Clinical	INET	+	+	+	+	ET	+	*B1*	ET	+	+	S, NA, TE, SXT
	1	Clinical	INET	+	+	+	+	ET	+	*B1*	ET	+	+	S, NA, SXT
	1	Clinical	OGET	+	+	+	+	ET	+	*B7*	ET	+	+	S, NA, SXT
	1	Clinical	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	P, S, NA, TE, SXT
2013	10	Env	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	S, NA, TE, SXT
	2	Env	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	P, S, NA, TE, SXT
	10	Clinical	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	S, NA, TE, SXT
	1	Clinical	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	P, S, NA, TE, SXT
	1	Clinical	OGET	+	+	+	+	ET	+	*B1*	ET	+	–	NA
2014	9	Env	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	S, NA, TE, SXT
	1	Env	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	P, S, NA, TE, SXT
	10	Clinical	OGET	+	+	+	+	ET	+	*B1*	ET	+	+	S, NA, TE, SXT
	2	Clinical	OGET	+	–	–	–	ET	+	–	–	+	+	S, NA, TE, SXT
	1	Clinical	OGET	+	+	+	+	ET	+	*B1*	ET	+	–	NA

Env, environmental; OGET, Ogawa El Tor; INET, Inaba El Tor; ET, El Tor; CL, classical.

the years of the study period. As shown in **Figure 2B**, 100% of the *V. cholerae* O1 showed resistance to S and SXT in 2010, 2011, and 2012. However, S and SXT resistance fell to 96% the following 2 years, 2013 and 2014. The SXT-related integrase (*intSXT*) was detected in all isolates resistant to S and SXT, suggesting the SXT/R391 ICE mediated the resistance to S and SXT. Except for five of the *V. cholerae* O1 variant ET isolated in 2012, all were resistant to NA (**Figure 2B**), an indicator of reduced susceptibility to CIP. TE resistant *V. cholerae* O1 comprised 73, 75, 58, 96, and 96% in 2010, 2011, 2012, 2013, and 2014, respectively (**Figure 2B**). Of 97 *V. cholerae* O1, five showed resistance to P during 2012 and 2014, while only one isolate showed resistance to E in 2011. Notably, all 97 *V. cholerae* O1 isolates were uniformly sensitivity to AM, CIP, C, and K.

MIC of Ciprofloxacin

The MIC of CIP of all 97 *V. cholerae* O1 isolates was determined to be 0.003–0.75 μg/ml during 2010 and 2014. As shown in **Table 2**, MIC_{50} and MIC_{90} of CIP was 0.5 μg/ml in 2010. However, the MIC_{50} and MIC_{90} were 0.38 μg/ml in 2011, maintained consistently over the following years until 2014. Five of the *V. cholerae* O1 variant ET isolated in 2012 had an MIC for CIP of 0.003 μg/ml. Only 1% of the total set of strains had the highest MIC, 0.75 μg/ml, while 77% had an MIC of 0.38 μg/ml (**Figure 3A**).

MIC of Azithromycin

The MIC of AZ for 97 of the *V. cholerae* O1 isolates was 0.19–2.00 μg/ml. As shown in **Table 2**, except for the 2012 isolates, MIC_{50} was consistently 0.25 μg/ml. Year-wise data revealed that the lowest MIC_{90} was 0.38 μg/ml in 2010 and 2013 and increased to 0.5 and 0.75 μg/ml in 2011 and 2012, respectively (**Table 2**). As shown in **Figure 3B**, 52 and 27% of the total isolates had MIC 0.25 and 0.38 μg/ml, respectively. The highest MIC of 2.00 μg/ml occurred in 1% of the *V. cholerae* O1 isolates (**Figure 3B**). Sixteen (16%) *V. cholerae* O1 with an MIC of ≥0.5 μg/ml were analyzed further for 10 macrolide resistance genes. PCR assay results revealed that none of the isolates

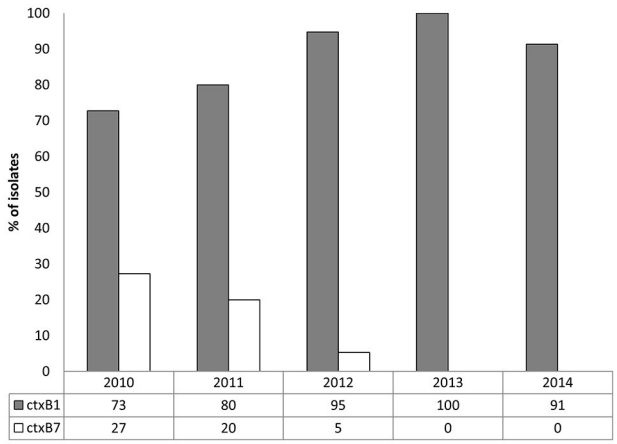

FIGURE 1 | Distribution of *ctxB* genotypes in 97 *V. cholerae* O1 from Mathbaria, Bangladesh isolated during 2010 and 2014. In both the clinical and environmental isolates, *ctxB1* (gray bars) was predominant in each year. Although *ctxB7* in the *V. cholerae* O1 isolates (white bars) was detected in relatively low percentage in 2010, 2011, and 2012, it was not detected in 2013 and 2014.

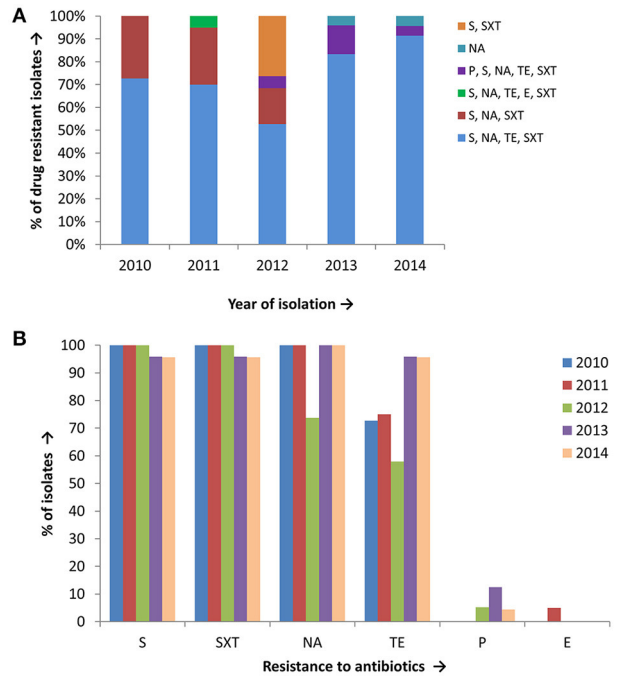

FIGURE 2 | (A) Drug resistance profile of 97 *V. cholerae* O1 isolates from Mathbaria, Bangladesh. Six different resistance profiles were observed for *V. cholerae* O1 isolated during 2010 and 2014, of which four profiles were multidrug resistance: resistance to S, NA, TE, and SXT was most abundant and predominant in *V. cholerae* O1 **(B)** *V. cholerae* O1 isolated during 2010 and 2014 showing resistance to six different drugs. The majority of the isolates were resistant to S, SXT, TE, and NA, while only a few were resistant to P and E.

contained the following macrolide resistance genes: *erm*(A); *erm*(B); *erm*(C); *ere*(A); *ere*(B); *mph*(A); *mph*(B); *mph*(D); *mef*(A); and *msr*(A).

DISCUSSION

Endemic cholera occurs in many geographic locations of Bangladesh each year, with two distinct seasonal peaks, one in the spring (March–May) and the other in the fall (September–November; Emch et al., 2008; Akanda et al., 2013). The Ganges delta region of the Bay of Bengal is a well-known reservoir of *V. cholerae* where it has established residence for centuries (Sack et al., 2004). Historically, this part of Asia has been affected severely by both CL and ET cholera during the seventh pandemic up to 1991, prior to the disappearance of CL strains from Bangladesh (Siddique and Cash, 2014). Epidemiological data suggest that most of the recorded epidemics struck the coastal populations first (Jutla et al., 2010), a pattern typical of recent epidemics in Bangladesh as well before reaching inland (Akanda et al., 2013). Since the beginning of the ongoing seventh pandemic, *V. cholerae* O1 strains have undergone multiple genetic changes, with the evolution of new clones and also atypical ET strains (Chun et al., 2009; Safa et al., 2010; Kim et al., 2014). Results of this study show association of *V. cholerae* O1 atypical ET with cholera occurring in the coastal areas of Mathbaria, Bangladesh during 2010 and 2014. Since 2001, atypical ET emerged as the major cause of cholera in Bangladesh superseding prototype ET, although isolated a decade earlier in the 1990s in Matlab, Bangladesh (Nair et al., 2006; Safa et al., 2006). This transition was considered remarkable for cholera epidemiology, mainly because atypical ET strains possessing CL cholera toxin (*ctxB1*) cause a more severe cholera than prototype ET (Siddique et al., 2010). In recent years, several non-synonymous mutations have been detected in the *ctxB* gene, although, correlation of these mutations with clinical outcomes of the disease remains to be clarified (Kim et al., 2015). The *ctxB7* genotypes have an amino acid substitution at position 20 [histidine (H)→asparagine (N)] first reported in a cholera outbreak in Orissa, India (Kumar et al., 2009). Later, *V. cholerae* O1 carrying *ctxB7* was determined to be associated with cholera in Haiti, Zimbabwe, India, Bangladesh, Nepal, Nigeria, and Cameroon (Quilici et al., 2010; Chin et al., 2011; Hasan et al., 2012; Rashed et al., 2012; Marin et al., 2013; Dixit et al., 2014). In Mathbaria, Bangladesh, *ctxB1* was consistently dominant to *ctxB7* during 2010 and 2012, whereas *ctxB7* was not detected thereafter. The alternating dominance of *ctxB1* and *ctxB7*, i.e., one genotype disappearing transiently for 2 or 3 years and reappearing in the following years with remarkable dominance, was previously observed in *V. cholerae* O1 causing cholera in Kolkata, India, and Dhaka, Bangladesh (Rashed et al., 2012; Mukhopadhyay et al., 2014; Rashid et al., 2016).

Bacterial resistance to antimicrobial drugs is a serious public health concern worldwide and antibiotic therapy constitutes a major component of the clinical management of cholera. An antimicrobial drug considered to be a successful therapeutic agent may not be successful in the future and notably so if *V. cholerae* acquires resistance to drugs of choice. Resistance can arise from single or multiple mutations in target genes or by acquisition of resistance genes carried by mobile genetic elements, such as plasmids, transposons, integrons, and ICEs (Kitaoka et al., 2011). Prior to the use of macrolides and

TABLE 2 | Minimum inhibitory concentration of ciprofloxacin and azithromycin for 97 *V. cholerae* O1 isolates.

Year of isolation	No. of strains	Ciprofloxacin			Azithromycin		
		MIC Range (μg/ml)	MIC_{50}	MIC_{90}	MIC Range (μg/ml)	MIC_{50}	MIC_{90}
2010	11	0.38–0.75	0.5	0.5	0.19–0.75	0.25	0.38
2011	20	0.25–0.5	0.38	0.38	0.25–1.5	0.25	0.5
2012	19	0.003–0.38	0.38	0.38	0.25–0.75	0.38	0.75
2013	24	0.25–0.38	0.38	0.38	0.25–1	0.25	0.38
2014	23	0.25–0.38	0.38	0.38	0.19–2	0.25	0.5

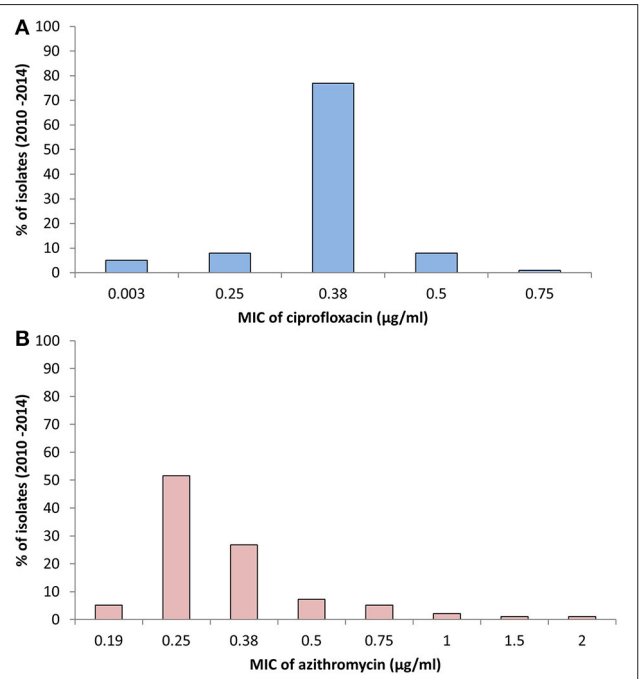

FIGURE 3 | (A) MIC of CIP for 97 *V. cholerae* O1 isolates from Mathbaria, Bangladesh. The MIC range for CIP was 0.003–0.75 μg/ml. The majority of the isolates showed MIC 0.38 μg/ml, while a low percentage had MIC of 0.5 and 0.75 μg/ml. **(B)** MIC of AZ for 97 *V. cholerae* O1 isolates from Mathbaria, Bangladesh. The range of MIC for AZ was 0.19–2 μg/ml. A relatively low percent of the isolates had a MIC of 2 μg/ml, the sensitivity-borderline for *V. cholerae.*

fluoroquinolone drugs for treatment of cholera, TE was the drug of choice, except for young children and pregnant women (Greenough et al., 1964; Sack et al., 2004). However, tetracycline was limited as a drug of choice because of the emergence of resistant *V. cholerae* O1 to AMP, KN, S, and SXT, as well as TE, in Asia and Africa (Mhalu et al., 1979; Glass et al., 1980). In this study, 93% of the *V. cholerae* O1 strains tested proved to be multidrug resistant, mostly resistant to S, NA, TE, and SXT. Despite having spatio-temporal variation in the resistance profile, the multidrug resistant *V. cholerae* O1 was consistently identified as the etiological agent of cholera epidemics in Asia and Africa, and most recently in Haiti (Mhalu et al., 1979; Glass et al., 1980; Dalsgaard et al., 2001; Jain et al., 2011; Sjölund-Karlsson et al., 2011; Rashed et al., 2012; Tran et al., 2012). A recent study from China reported that the prevalence of multidrug resistant *V. cholerae* O1 strains has been increased rapidly since 1993, showing resistance to AMP, NA, TE, and SXT (Wang et al., 2012). The same study also revealed relatively low number of *V. cholerae* O1 has reduced susceptibility to azithromycin in China that were isolated only in 1965, 1998, and 2006 (Wang et al., 2012). Drug resistant markers, such as SXT ICE, class 1 integrons, and low molecular weight plasmids are commonly found in multidrug resistant *V. cholerae* O1 (Kitaoka et al., 2011). Interestingly, a recent study reported the presence of a transmissible multidrug resistant plasmid (IncA/C) in Haitian *V. cholerae* isolates possessing several multidrug resistance determinants, i.e., *aac(3)-IIa*, bla_{CMY-2}, $bla_{CTX-M-2}$, bla-$_{TEM-1}$, *dfrA15*, *mphA*, *sul1*, *tetA*, *floR*, *strAB*, and *sul2* (Folster et al., 2014).

Among the fluoroquinolone antibiotics, only CIP has been recommended by the Pan American Health Organization and International Centre for Diarrhoeal Disease Research, Bangladesh for treatment of cholera. Although, *V. cholerae* O1 has not shown complete resistance to CIP, current epidemiological data confirm a gradual increase in the MIC of CIP has been occurring (Khan et al., 2015). *V. cholerae* O1 with reduced susceptibility to CIP has been reported in different parts of the world and appears to be disseminating globally (Islam et al., 2009; Quilici et al., 2010; Sjölund-Karlsson et al., 2011; Tran et al., 2012; Khan et al., 2015). A recent study showed that the MIC of CIP for *V. cholerae* O1 has increased 45-fold in a 19 year time-span in Bangladesh. That is, the MIC was 0.010 μg/ml in 1994 and has increased dramatically to 0.475 μg/ml in 2012 (Khan et al., 2015). In our study, 95% of the *V. cholerae* O1 isolated in Mathbaria, Bangladesh, showed reduced susceptibility to CIP during 2010 and 2014. Notably, the CIP MIC_{50} and MIC_{90} did not show rapid change in the 5 year of our study period and the MIC remained below the susceptibility breakpoint ($\leq$1 μg/ml) according to CLSI guidelines (CLSI, 2010a). It is important to note that all *V. cholerae* O1 atypical ET isolates were resistant to NA, another drug in the quinolone group. *V. cholerae* O1 showing resistance to NA is an indicator of reduced susceptibility to CIP (Khan et al., 2015). The genetic basis of quinolone drug resistance in *V. cholerae* is the accumulation of mutations in *gyrA* ($83_{Ser \rightarrow Ile}$) and *parC* ($85_{Ser \rightarrow Leu}$) (Kitaoka et al., 2011). These point mutations have been detected in currently circulating *V. cholerae* O1 associated with cholera epidemics in Bangladesh, India, Nepal, Nigeria, Cameroon, and Haiti (Quilici et al., 2010; Sjölund-Karlsson et al., 2011; Hasan et al., 2012; Dixit et al., 2014).

Frequent use of a specific group of antibiotics for treatment of cholera over a prolonged period will increase the likelihood of bacterial resistance. Global dissemination of *V. cholerae* O1 with reduced CIP sensitivity raises a serious concern for clinical management of cholera in countries where the disease is endemic. Results of a recent study showed that single-dose CIP used to treat cholera was not as effective as it was in the past because of the emergence of *V. cholerae* O1 less susceptible to CIP and NA (Khan et al., 2015). Single dose AZ has been introduced as an alternative treatment for cholera in India and Bangladesh. However, the sensitivity breakpoint guidelines for the AZ disc diffusion assay has not yet been published by the CLSI for *V. cholerae* (CLSI, 2010a). In this study, all *V. cholerae* O1 isolates showed a reduced susceptibility to AZ and the MIC for 1% of the isolates was at the sensitivity breakpoint borderline ($\leq$2 μg/ml). Interestingly, none of the *V. cholerae* O1 (AZ MIC $\geq$ 0.5 μg/ml) possessed macrolide resistance genes that have been reported for the *Enterobacteriaceae* (Phuc Nguyen et al., 2009). Although at a relatively low incidence, E and AZ resistant *V. cholerae* O1 have been reported in Bangladesh and India (Faruque et al., 2007; Bhattacharya et al., 2012).

Reduced susceptibility to CIP and AZ is alarming for cholera-endemic countries of Asia and Africa. Environmental factors trigger seasonal cholera in endemic countries including Bangladesh, but cholera cases have occurred in other countries immediately after a devastating natural calamity, e.g., floods, earthquakes, typhoons, and cyclones. The morbidity and mortality rates of cholera, which were under control for several decades, can be expected to increase if *V. cholerae* O1 acquires full resistance to currently used drugs. Considering the global burden of cholera, it is important that the appropriate antibiotic and appropriate concentration be used to treat cholera. Indiscriminate use of antibiotics, for example in agriculture and animal husbandry for disease management should be controlled to assure continued success of antibiotic for the treatment of disease in humans, including cholera. Therefore, global monitoring of antimicrobial sensitivity of *V. cholerae* O1 is essential to assess clinical efficacy of drugs worldwide.

AUTHOR CONTRIBUTIONS

SR and AH contributed to the design of the study. SR also performed all research works in the laboratory, analyzed data, and wrote the manuscript. AS and MS collected clinical and environmental samples from the field area and processed all samples at ICDDR,B. NH, MA, MH, RS, RC, and AH contributed to revising the manuscript critically for important intellectual content. All authors discussed, read, and approved the final manuscript.

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frontiers in Microbiology

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Antimicrobial Susceptibility among Urban Wastewater and Wild Shellfish Isolates of Non-O1/Non-O139 *Vibrio cholerae* from La Rance Estuary (Brittany, France)

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The early 2000s marked the end of the Golden age of the antibiotics and the beginning of the awareness on the potential threat to human health due to the dissemination of antimicrobial resistance. As a base-line study, we investigated the antimicrobial susceptibility of 99 strains of non-O1/non-O139 *Vibrio cholerae* isolated from wastewater and shellfish in 2000/2001 within La Rance estuary (Brittany, France). All isolates were susceptible to amoxicillin-clavulanic acid, cefotaxime, imipenem, chloramphenicol, nalidixic acid, ciprofloxacin, norfloxacin, amikacin, gentamicin, tetracycline, doxycycline, trimethoprim-sulfamethoxazole, and erythromycin. The only resistances were to streptomycin, sulfonamides and ampicillin: 54.6% of the isolates had acquired resistance to at least one antimicrobial agent among them and only six isolates from cockles were multidrug resistant. On the basis of the distribution of a limited selection of resistance associated genes, our study shows that *V. cholerae* can constitute an environmental reservoir for these genes. However, none of our isolates harbored integron. This result casts doubt on the capacity of non-O1/non-O139 *V. cholerae* to acquire resistance-associated genes in such context, and on its potential role of indicator of the dissemination of antimicrobial resistance in the aquatic environment.

Keywords: *Vibrio cholerae* non-O1/non-O139, wastewater, wild shellfish, antimicrobial resistance, estuary

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INTRODUCTION

The early 2000s marked the end of a 50 year time referred as the "Golden age of antibiotics" and the beginning of the awareness of the public health importance of the dissemination of antimicrobial resistance. Recent studies highlighted the important role of the aquatic environment in the dissemination of antimicrobial resistance (Baquero et al., 2008) and particularly wastewater which is considered as a hot spot for horizontal genes transfer (Bouki et al., 2013; Rizzo et al., 2013).

Vibrio cholerae is known to be an autochthonous inhabitant of riverine and estuarine aquatic environments and also a waterborne bacterial pathogen. The agent of cholera, a major public health threat for developing countries, belongs to serogroups O1 or O139 and produces the cholera toxin.

The other serogroups are collectively referred as non-O1/non-O139 *V. cholerae*. Majority of strains isolated from the environment do not produce the cholera toxin and belong to non-O1/non-O139 serogroups.

In Europe, the increase of the frequency of infections due to non-O1/non-O139 *V. cholerae* in connection with the aquatic environment is reported by numerous authors (Dalsgaard et al., 2000; Ninin et al., 2000; Andersson and Ekdahl, 2006; Ottaviani et al., 2011; Hirk et al., 2016). This increase may be partially related to global warming. Due to the impact of climate change, a rise in the occurrence of *V. vulnificus* and *V. cholerae* is predicted for European waters (Baker-Austin et al., 2017).

Vibrio cholerae may act as an environmental reservoir for antibiotic resistance genes (Ceccarelli et al., 2016). It has been established that *V. cholerae*, irrespective of serogroup, has a plastic genome and a long history of successful association with plasmids (Coppo et al., 1995; Carraro et al., 2016). In other words, *V. cholerae*, due to its genetic characteristics, is possibly capable of acquiring and of exchanging genes through either integrons or integrative and conjugative elements (ICE) such as the SXT element (Waldor et al., 1996; Hochhut et al., 2001).

Within La Rance estuary, a survey on the presence of non-O1/non-O139 *V. cholerae* was performed during 2000/2001 in a field of wild shellfish—a point of exposure for humans—and upstream in the discharge of wastewaters from an agglomeration. This estuary opens into The English Channel and is located in Brittany (France). It is a typical environmental aquatic composite, characterized by high anthropogenic pressure. The water body is subject (i) to an artificial tide controlled by tidal power plant, which also causes important sedimentation in the upper part of the estuary, and (ii) to heavy inputs from human activity, mainly diffuse pollution and wastewater discharge from the treatment plant of the Dinan urban area (11,000 inhabitants). It is also an area of intensive recreational activity (sailing, fishing, bathing, collection of shellfish from the shore), and aquaculture (algae cultivation, oyster farming). The estuary is therefore simultaneously an environment receiving discharges from human activities, a potential reactor for genetic exchanges between bacteria subjected to antibiotic pressure and a source of diverse human exposures.

The aim of this study therefore was to investigate the antibiotic susceptibility of isolates of non-O1/non-O139 *V. cholerae* collected in 2000/2001 in cockles and in treated wastewater, in order to be used as base-line study of antimicrobial resistance.

MATERIALS AND METHODS

Sampling Site and Study Period

Two sites located in La Rance estuary (North Brittany, France) were sampled in 2000 and 2001. A wild shellfish harvesting area located in Mordreuc (48°30′43 N, 1°58′37 W) on the shore was sampled seven times between June and October 2000 and twice in September 2001.

Treated wastewater was collected upstream at the outlet of the Dinan biological aerobic treatment plant (BTP) with trickling filters and activated sludge (48°27′48 N, 02°1′40 W). Eleven samples were collected, one every two weeks between June and October 2000 and two more in 2001 (in August and in September). The two sampling sites are 8.5 km apart following the bed of the river (4.8 km between the discharge of wastewater upstream and the lock on the river and 3.7 km between the lock and the wild cockle shellfish harvesting area downstream) (**Figure 1**).

Environmental Data

For each wastewater sample, salinity, pH and temperature were recorded *in situ*.

The enumeration of *E. coli* and intestinal *Enterococci* was carried out using the 96-wells microplate (BioRad, France) most probable number (MPN) methods ISO 9308-2 and ISO 7899-1, respectively. The detection of *E. coli* in the 96-wells microplate is based on the expression of β-D-glucuronidase enzyme, while β-glucosidase is the target for intestinal *Enterococci*.

Sample Processing

Harvested cockles and water samples were transported to the laboratory in coolers and examined within 3 h of collection.

Upon arrival, wild cockles (*Cerastoderma edule*) were scrubbed under running tap water and opened aseptically, using a sterile knife. 250 g of flesh plus intra-valve liquid were homogenized with a blender in Phosphate-Buffered Saline (1/3 w/w) for two times 30 s. Volumes of 10 mL and 1 mL of homogenate of cockles, and also volumes of 1 mL of tenfold dilutions were mixed with Alkaline Peptone Saline Water (ASPW; composition for 1 liter: 10 g peptone, 20 g NaCl and 5 g yeast extract; post-autoclave pH: 8.6 ± 0.2), in 100 and 10 mL volumes for the raw homogenate, and in 10 mL volumes for the tenfold dilutions.

Sample volumes of 1 L and 100 mL of treated wastewater were filtered (Diaphragm pump N035.3 AN.18 KNF Neuberger, Village-Neuf, France) successively with glass microfiber filters GF/D (grade D, 2.7 μm; Whatman, Maidstone, UK), glass microfiber filters GF/C (grade C, 1.2 μm; Whatman, Maidstone, UK), 0.45 μm cellulose ester membranes (Millipore, Watford, UK) and 0.22 μm cellulose ester membranes (Millipore, Watford, UK). The various filter sizes guaranteed the isolation of both fixed-form and free-living bacteria. For volumes of 1 L and 100 mL, the filters were placed in 250 mL of sterile ASPW. The volumes of 10 mL were incorporated directly in 100 mL of ASPW, and sample volumes of 1 mL to 0.001 mL were incorporated in 10 mL of ASPW.

Isolation of *V. cholerae*

After incubation at 41 ± 1°C for 16 to 18 h, 0.1 mL of tenfold dilutions of the enrichment were spread over TCBS Agar (Difco) (Muic, 1990) and incubated at 37°C for 36 h. From each enrichment, up to 32 yellow colonies isolated on TCBS Agar were transferred with sterile toothpicks onto Nutrient Agar without NaCl (NA_0, Difco) to test for growth at 37°C, and then submitted for an oxidase test (Bactident oxidase strips, Merck, Darmstadt, Germany). Positive isolates were considered to be presumptive *V. cholerae* (Baron et al., 2007). Identification of presumptive *V. cholerae* was confirmed by PCR (Nandi et al., 2000). Confirmed isolates ($n = 216$) were maintained at −80°C in

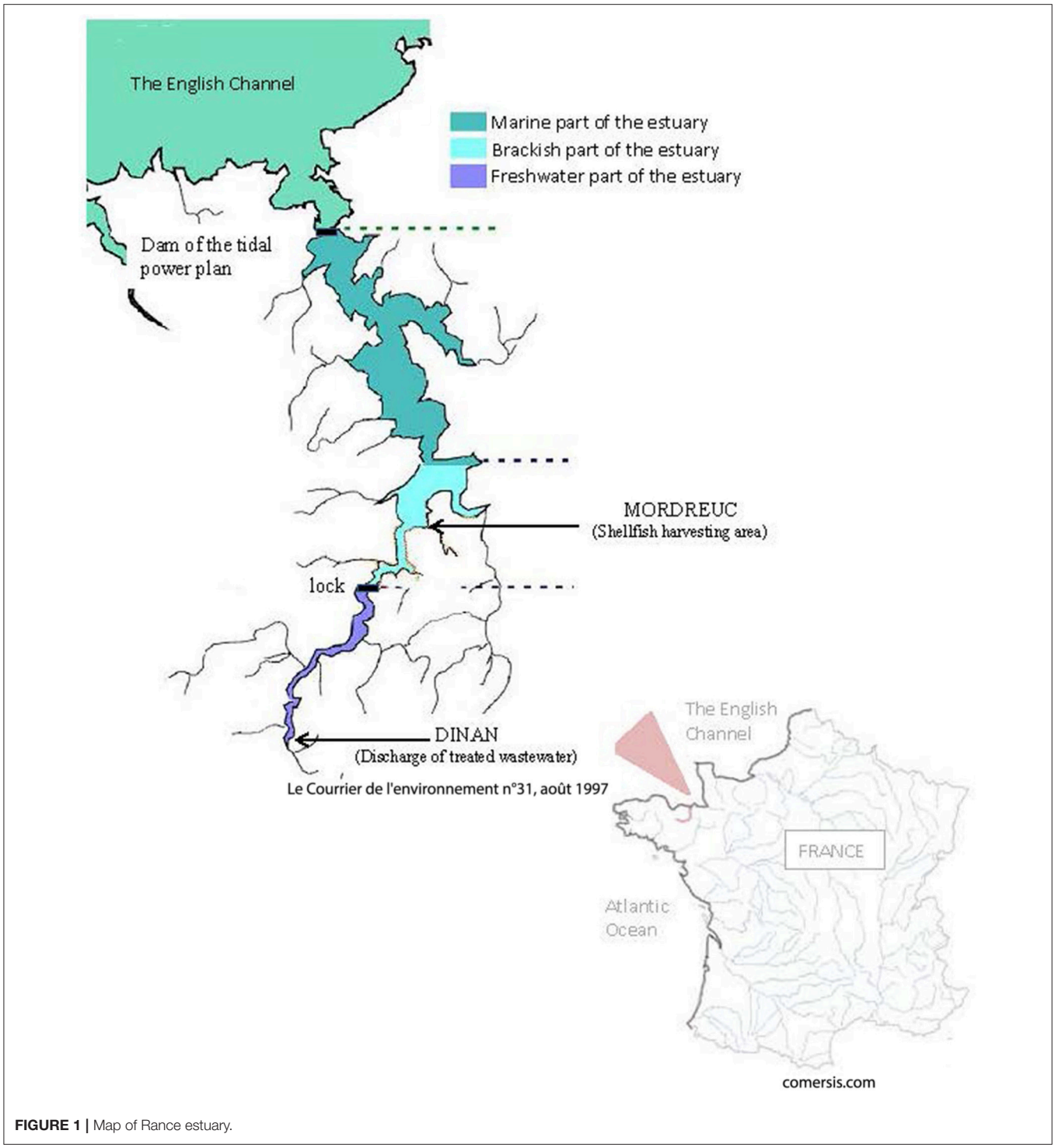

FIGURE 1 | Map of Rance estuary.

brain heart infusion broth (Bio-Rad) containing glycerol (10%). Only one positive isolate per enrichment was conserved for further study (characterization and antibiotic susceptibility).

Characterization of *V. cholerae*

Vibrio cholerae isolates were examined to determine whether they were members of the O1 serogroup via slide agglutination using a polyclonal antibody specific for the O1 surface antigen (Bio-Rad). A saline solution was used as a control to identify self-agglutinating isolates. The genes coding for the O1 and O139 surface antigens (*rfb*) were assessed with PCR using O1-and O139-specific primers (Hoshino et al., 1998).

The virulence associated genes *tcpA*, *ctxA* and *hlyA* were detected by PCR according to Rivera et al. (2001) and Fields

et al. (1992) in the whole collection of confirmed *V. cholerae* non-O1/non-O139. *Vibrio cholerae* O1, Classical and Inaba obtained from Centre National de Référence des Vibrions et du Choléra, Institut Pasteur, Paris (CNRVC 940147), and *Vibrio cholerae* O1, El Tor and Inaba (ATCC 39315) obtained from the collection of the Pasteur Institute (Paris) were included as positive and negative controls where appropriate. All primer pairs, annealing temperatures, and amplicon sizes corresponding to target genes are listed in **Table 1**.

Enumeration of *V. cholerae*

For samples collected in 2000, enumeration of *V. cholerae* was done by the MPN method (5 tubes, 5 dilutions) based on the number of positive enrichments by serial fractions of volume for a sample (Beliaeff and Mary, 1993). The results were expressed per 100 g of flesh plus intra-valve liquid for cockles and per liter for wastewater. The number of dilutions and tested volumes were adapted according to the expected abundances. In 2001 no enumeration was done, the objective being only to collect isolates.

Antimicrobial Susceptibility Testing

The susceptibility of 99 isolates collected from cockles ($n = 30$ in 2000 and $n = 21$ in 2001) and from wastewater ($n = 31$ in 2000 and $n = 17$ in 2001) was studied using the disk diffusion method) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2015). The 16 antimicrobial agents were chosen

TABLE 1 | The list of primer pairs, annealing temperatures, and amplicon sizes corresponding to target genes for PCR.

Targeted gene	Primer name	Primer sequence (5′ - 3′)	T°C[a]	Amplicon size (bp)	References
ermA	ermA1	TAACATCAGTACGGATATTG	54	139	Di Cesare et al., 2013
	ermA2	AGTCTACACTTGGCTTAGG			
ermB	ermB1	CCGAACACTAGGGTTGCTC		200	
	ermB2	ATCTGGAACATCTGTGGTATG			
mef	mef1	AGTATCATTAATCACTAGTGC		348	
	mef2	TTCTTCTGGTACTAAAAGTGG			
sul1	sul1-1	CGCACCGGAAACATCGCTGCAC	65	162	Pei et al., 2006
	sul1-2	TGAAGTTCCGCCGCAAGGCTCG			
sul2	sul2-1	TCCGGTGGAGGCCGGTATCTGG	57.5	190	
	sul2-2	CGGGAATGCCATCTGCCTTGAG			
O139 *rfb*	O139-1	AGCCTCTTTATTACGGGTGG	55	449	Hoshino et al., 1998
	O139-2	GTCAAACCCGATCGTAAAGG			
O1 *rfb*	O1-1	GTTTCACTGAACAGATGGG		192	
	O1-2	GGTCATCTGTAAGTACAAC			
ctxA	ctx Ats	CTCAGACGGGATTTGTTAGGCACG	64	301	Nandi et al., 2000
	ctx A	TCTATCTCTGTAGCCCCTATTACG			
ompW	ompW ts	CACCAAGAAGGTGACTTTATTGTG		588	
	ompW ta	GAACTTATAACCACCCGCG			
strA	strA-F	GAGAGCGTGACCGCCTCATT	57	862	Popowska et al., 2012
	strA-R	TCTGCTTCATCTGGCGCTGC			
strB	strB-F	GCTCGGTCGTGAGAACAATC	54	859	
	strB-R	AGAATGCGTCCGCCATCTGT			
ctxA	94F	AGAATGCGTAAGCCATCTGT	60	564	Fields et al., 1992
	614R	GAATGCGTAAGCCATCTGTTT			
tcpA Classical	72F	TCTGCTTCATCTGGCGCTGC	60	620	Rivera et al., 2001
	647R	TCTGCTTCATCTGGCGCTGC			
tcpA El Tor	72F	TCTGCTTCATCTGGCGCTGC		451	
	477R	TAACTGCTTCATCTGGCGCTGC			
hlyA Classical	744F	CACAAGGTGACTTTATTGTG		727	
	1184R	CACCAAGAAGGTGACTTTA			
hlyA El Tor	489F	TTGTTACCCTTTAATTGGCCC		738/727	
	1184R	CACCAAGAAGGTGACTTTA			

[a] *Annealing temperature.*

TABLE 2 | Phenotypic and genotypic profile of susceptibility in the 99 isolates of non-O1/non-O139 *V. cholerae*.

N°	Sample	Date	*hlyA*[a]	Phenotypic antimicrobial suceptibility	Molecular determinants[c]						
				Resistance profile[b] (CLSI M45 3rd edition)	*sul*1	*sul*2	*str*A	*str*B	*erm*A	*mef*A	Integron
812	Wastewater	06/19/00	ET	*pansusceptible*	+	+	−	+	+	−	−
816	Wastewater	06/19/00	ET	SSS	+	−	+	+	+	−	−
822	Wastewater	06/19/00	ET	*pansusceptible*	+	−	−	−	+	−	−
823	Wastewater	06/19/00	ET	STR	+	+	−	−	+	−	−
844	Wastewater	06/19/00	–	SSS	−	−	−	−	+	−	−
846	Wastewater	06/19/00	ET	SSS	+	−	−	−	+	−	−
1508	Wastewater	07/03/00	ET	SSS	+	+	+	−	+	−	−
1514	Wastewater	07/03/00	ET	SSS	+	−	−	−	+	−	−
1518	Wastewater	07/03/00	ET	STR	+	−	−	−	+	−	−
1530	Wastewater	07/03/00	CI	STR	+	−	−	−	+	−	−
1534	Wastewater	07/03/00	ET	*pansusceptible*	+	−	−	−	+	−	−
1538	Wastewater	07/03/00	ET	SSS	+	+	−	−	+	−	−
2602	Wastewater	07/17/00	CI	SSS	−	−	−	−	+	−	−
2606	Wastewater	07/17/00	ET	SSS	+	−	−	−	+	−	−
2610	Wastewater	07/17/00	ET	SSS	+	−	−	−	+	−	−
2622	Wastewater	07/17/00	ET	SSS	+	−	−	−	+	−	−
2626	Wastewater	07/17/00	CI	SSS	−	−	−	−	+	−	−
5109	Wastewater	08/14/00	ET	SSS-STR	−	+	+	+	+	−	−
5113	Wastewater	08/14/00	ET	SSS-STR	+	−	−	−	+	−	−
5117	Wastewater	08/14/00	–	STR	+	+	+	−	+	−	−
5125	Wastewater	08/14/00	ET	SSS-STR	−	−	−	−	+	−	−
5129	Wastewater	08/14/00	ET	*pansusceptible*	+	−	−	−	+	−	−
5141	Wastewater	08/14/00	ET	*pansusceptible*	+	−	−	−	+	−	−
5145	Wastewater	08/14/00	CI	*pansusceptible*	+	−	−	−	+	−	−
6525	Cockles	08/21/00	–	*pansusceptible*	+	+	−	+	+	−	−
6720	Cockles	08/24/00	CI	SSS	+	−	−	−	+	−	−
6724	Cockles	08/24/00	–	SSS-STR-AMP	+	+	+	−	+	−	−
6728	Cockles	08/24/00	CI	SSS-STR	+	−	−	−	+	−	−
6732	Cockles	08/24/00	–	*pansusceptible*	+	+	−	+	+	−	−
7101	Wastewater	08/28/00	CI	SSS	+	−	−	−	+	−	−
7105	Wastewater	08/28/00	ET	*pansusceptible*	+	+	−	+	−	−	−
7113	Wastewater	08/28/00	ET	SSS	+	−	−	−	+	−	−
7133	Wastewater	08/28/00	–	SSS	+	+	−	+	−	−	−
7165	Wastewater	08/28/00	–	*pansusceptible*	+	−	−	−	+	−	−
7169	Wastewater	08/28/00	CI	*pansusceptible*	−	−	−	−	+	−	−
7173	Wastewater	08/28/00	ET	SSS	−	−	−	−	+	−	−
8621	Cockles	09/04/00	–	SSS-STR-AMP	−	+	−	+	+	−	−
8625	Cockles	09/04/00	ET	SSS	+	−	−	−	+	−	−
8637	Cockles	09/04/00	–	SSS-STR-AMP	+	+	−	+	+	−	−
8645	Cockles	09/04/00	CI	SSS-STR	+	−	−	+	+	−	−
8660	Cockles	09/04/00	–	SSS	+	−	−	−	+	−	−
9050	Cockles	09/10/00	ET	SSS-STR-AMP	+	−	−	−	+	−	−
9067	Cockles	09/10/00	CI	*pansusceptible*	+	−	−	+	+	−	−
9083	Cockles	09/10/00	–	AMP	+	−	−	−	+	−	−
9092	Cockles	09/10/00	–	*pansusceptible*	+	−	−	−	+	−	−
9112	Cockles	09/10/00	ET	SSS	+	−	−	+	+	−	−
9114	Cockles	09/10/00	–	SSS	+	+	−	+	+	−	−
10205	Cockles	09/18/00	ET	SSS-AMP	+	−	−	−	+	−	−
10225	Cockles	09/18/00	ET	SSS	+	−	−	−	+	−	−
10229	Cockles	09/18/00	–	SSS-STR-AMP	+	−	−	−	+	−	−
10257	Cockles	09/18/00	ET	SSS-STR	+	+	+	+	+	−	−

(Continued)

TABLE 2 | Continued

N°	Sample	Date	*hlyA*[a]	Phenotypic antimicrobial suceptibility	Molecular determinants[c]						
				Resistance profile[b] (CLSI M45 3rd edition)	*sul*1	*sul*2	*str*A	*str*B	*erm*A	*mef*A	Integron
10285	Cockles	09/18/00	–	SSS	+	–	–	–	+	–	–
11013	Cockles	09/24/00	ET	SSS-STR-AMP	–	–	–	–	+	–	–
11017	Cockles	09/24/00	–	SSS	–	–	–	–	+	–	–
11021	Cockles	09/24/00	–	SSS	+	–	–	–	+	–	–
11037	Cockles	09/24/00	ET	SSS	+	–	–	–	+	–	–
11045	Cockles	09/24/00	–	SSS	+	–	–	–	+	–	–
11053	Cockles	09/24/00	Cl	SSS	+	–	–	–	+	–	–
11057	Cockles	09/24/00	ET	STR	+	–	–	–	+	–	–
11063	Cockles	09/24/00	–	*pansusceptible*	+	–	–	–	+	–	–
11109	Cockles	09/24/00	–	SSS-AMP	+	+	–	+	+	–	–
21850	Wastewater	08/06/01	Cl	*pansusceptible*	+	+	–	–	+	–	–
21852	Wastewater	08/06/01	–	*pansusceptible*	–	–	–	–	+	–	–
21853	Wastewater	08/06/01	–	*pansusceptible*	+	–	–	–	+	–	–
21854	Wastewater	08/06/01	–	*pansusceptible*	+	+	–	–	+	+	–
21855	Wastewater	08/06/01	–	STR	+	–	–	–	+	–	–
21856	Wastewater	08/06/01	ET	*pansusceptible*	+	+	–	–	+	+	–
21857	Wastewater	08/06/01	–	*pansusceptible*	+	+	–	–	+	–	–
21858	Wastewater	08/06/01	–	*pansusceptible*	+	+	–	–	+	–	–
21859	Wastewater	08/06/01	–	*pansusceptible*	+	–	–	–	+	–	–
21860	Wastewater	08/06/01	–	*pansusceptible*	+	+	–	–	+	–	–
21861	Wastewater	09/18/01	–	STR	+	+	–	–	+	+	–
21863	Wastewater	08/06/01	–	*pansusceptible*	+	+	–	–	+	+	–
21864	Wastewater	08/06/01	ET	SSS	+	–	–	–	+	–	–
21865	Wastewater	08/06/01	Cl	*pansusceptible*	+	+	–	–	+	+	–
22410	Wastewater	08/20/01	–	*pansusceptible*	+	+	–	–	+	–	–
22415	Wastewater	08/20/01	ET	SSS	+	+	–	–	+	–	–
22416	Wastewater	08/20/01	–	*pansusceptible*	+	+	–	–	+	–	–
28202	Cockles	09/18/01	Cl	*pansusceptible*	+	+	–	–	+	+	–
28203	Cockles	09/18/01	Cl	STR	+	+	–	–	+	+	–
28204	Cockles	09/18/01	–	*pansusceptible*	+	+	–	–	+	–	–
28205	Cockles	09/18/01	–	*pansusceptible*	+	+	–	–	+	+	–
28206	Cockles	09/18/01	Cl	*pansusceptible*	+	+	–	–	+	–	–
28207	Cockles	09/18/01	–	*pansusceptible*	+	+	–	–	+	–	–
28208	Cockles	09/18/01	–	*pansusceptible*	+	+	–	–	+	+	–
28209	Cockles	09/18/01	ET	*pansusceptible*	+	+	–	–	+	–	–
28210	Cockles	09/18/01	–	*pansusceptible*	+	+	–	–	+	+	–
28211	Cockles	09/18/01	Cl	STR	+	+	–	–	+	+	–
28219	Cockles	09/18/01	ET	*pansusceptible*	+	+	–	–	+	–	–
28220	Cockles	09/18/01	ET	*pansusceptible*	+	+	–	–	+	–	–
28222	Cockles	09/18/01	–	*pansusceptible*	–	+	–	–	+	–	–
28223	Cockles	09/18/01	–	*pansusceptible*	+	+	–	–	+	–	–
28224	Cockles	09/18/01	Cl	STR	–	+	–	–	+	+	–
28229	Cockles	09/18/01	–	*pansusceptible*	+	+	–	–	+	–	–
28237	Cockles	09/18/01	Cl	*pansusceptible*	+	+	–	–	+	+	–
28238	Cockles	09/18/01	Cl	*pansusceptible*	+	+	–	–	+	+	–
28242	Cockles	09/18/01	–	*pansusceptible*	+	+	–	–	+	–	–
28253	Cockles	09/18/01	Cl	*pansusceptible*	+	+	–	–	+	–	–
28254	Cockles	09/18/01	Cl	*pansusceptible*	+	+	–	–	+	–	–

[a] *ET, hemolysin El Tor; Cl, hemolysin Classical; –, no hemolysin detected.*

[b] *Resistance profiles were defined using with CLSI breakpoint when available in other cases referred to Table 1 in Supplementary Material.*

[c] *+, gene or integron was detected by PCR; –, gene or integron was not detected by PCR.*

Pansusceptible, profile susceptible to all the tested antimicrobial agents.

SSS, sulfonamide; STR, streptomycin; AMP, ampicillin.

in order to represent the main antimicrobial families used in France: ampicillin, amoxicillin-clavulanic acid, cefotaxime, imipenem, chloramphenicol, nalidixic acid, ciprofloxacin, norfloxacin, amikacin, gentamicin, streptomycin, tetracycline, doxycycline, sulfonamides, trimethoprim-sulfamethoxazole association, and erythromycin (Table 1 in Supplementary Material). The CLSI interpretative criteria for disk diffusion susceptibility testing of *Vibrio* spp. (CLSI, 2015) published in the M45 3rd edition were used for ampicillin, amoxicillin-clavulanic acid, cefotaxime, imipenem, chloramphenicol, ciprofloxacin, amikacin, gentamicin, tetracycline, sulfonamides, trimethoprim-sulfamethoxazole association (**Table 2**). For nalidixic acid, norfloxacin, amikacin and streptomycin, the interpretative criteria for *Enterobacteriaceae* were used (CLSI, 2016) (Table 1 in Supplementary Material). No criterion was available for erythromycin or doxycycline; therefore, the distribution of the inhibition diameters was recorded and interpretation was based on obtained distribution plots. The separation between wild-type (microorganisms without acquired resistance mechanisms) and non-wild-type populations (microorganisms with acquired resistance mechanisms) was determined by visual inspection of the diameter distribution (Hombach et al., 2014). The intermediate results were considered as resistant for this study. Wild-type populations were considered as susceptible populations and non-wild-type as resistant populations.

Antibiotic Resistance Associated Genes

Screening for resistance-associated genes in our collection of isolates was done particularly to provide information on the reservoir of resistance genes in the autochthonous aquatic non-O1/non-O139 *V. cholerae* populations under study. As, in the early 2000s CTX-M enzymes have become the most prevalent extended-spectrum β-lactamases in Europe (Cantón and Coque, 2006), the bla_{CTX-M} was detected using PCR (Woodford et al., 2005). Genes associated with resistance to streptomycin (*strA* and *strB*), to sulfonamides (*sul1* and *sul2*)—which may be associated with the presence of *V. cholerae* SXT element—and to erythromycin (*ermA*, *ermB* and *mef*) were detected by PCR (Pei et al., 2006; Popowska et al., 2012; Di Cesare et al., 2013). Class 1, 2, and 3 integrons were screened using qPCR (Barraud et al., 2010). All primer pairs, annealing temperatures and amplicon sizes corresponding to target genes are listed in **Table 1**.

Statistical Analysis

To compare the characteristics of the isolates collected from cockles and wastewater the chi-squared test, and Pearson's chi-squared test whenever needed, was used.

RESULTS

Characterization and Enumeration of *Vibrio cholerae*

None of the 216 isolates (cockles: 116, wastewater: 100) characterized for the MPN estimation—one isolate by positive enrichment broth—belonged to the O1 or the O139 serogroup (No agglutination was observed; absence of the *rfb* sequence gene), and carried neither the *ctxA* gene, nor the El Tor or Classical variants of the *tcpA* gene. All *V. cholerae* isolates were therefore non toxigenic.

The frequency of the hemolysin gene variants El Tor or Classical was 52.3% (113 strains), without any difference between wastewater and cockle isolates (59.0 vs. 46.5%). The El Tor variant was detected in 66 isolates (30.5%) and the Classical variant in 47 isolates (21.8%).

In 2000, *V. cholerae* densities were recorded in cockles and in treated wastewater. In cockles, between June and September, concentrations of *V. cholerae* ranged from 2.5 to 230 MPN/100 g of flesh plus intra-valve liquid. The highest density was observed in September and *V. cholerae* was detected in all seven samples (**Table 3**). At the outlet of the biological aerobic treatment plan, *V. cholerae* was detected in 75% of the samples (9/12), and densities ranged from 0.075 MPN/L to 78.2 MPN/L (**Figure 2**). Abundance of *V. cholerae* was below the detection limit of the method once in September (09/25/2000–detection limit: 0.198 MPN/L) and twice in October (10/09/2000 and 10/23/2000—detection limit: 0.181 MPN/L). The highest densities were observed in August. The geographic mean is 0.6 $\log_{10}$ (MPN/L). The temperature followed the seasonal variation and ranged from 22.2°C in August to 16°C in October (**Figure 2**). The pH ranged from 6.31 to 7.51 with an average of 7.40 ± 0.43. The salinity ranged from 0.2 to 0.9‰. The densities of *E. coli* and intestinal *Enterococci* were relatively stable during the sampling period with geometric mean value of $4.9 \times 10^5 \pm 0.5$ $\log_{10}$ (MPN/100 mL) and 4.5 ± 0.6 $\log_{10}$ (MPN/100 mL) respectively. Means densities per liter of *E. coli* were 5.3 $\log_{10}$ unit higher than *V. cholerae* ones and intestinal *Enterococci* were 4.8 $\log_{10}$ unit higher than *V. cholerae* densities (**Figure 2**).

In 2001, sampling dates were chosen in August and September to optimize the collection of isolates from cockles and wastewater.

Antimicrobial Susceptibility

All the 99 isolates studied were susceptible to the 11 following antimicrobial agents: amoxicillin-clavulanic acid, cefotaxime, imipenem, chloramphenicol, nalidixic acid, ciprofloxacin, norfloxacin, amikacin, gentamicin, tetracycline, trimethoprim-sulfamethoxazole association (**Table 2**).

For doxycycline and erythromycin, no breakpoint were available, based on the distribution of the diameter of inhibition

TABLE 3 | Densities of *V. cholerae* in cockle samples (MPN per 100 g of flesh plus intra-valve liquid) according to sampling dates.

Sampling dates (month/day)	08/24/2000	09/04/2000	09/17/2000	09/18/2000	09/24/2000	10/02/2000	10/16/2000
MPN	3.8×10^1	2.3×10^2	1.0×10^2	3.4×10^2	8.0×10^1	2.5×10^0	1.5×10^2

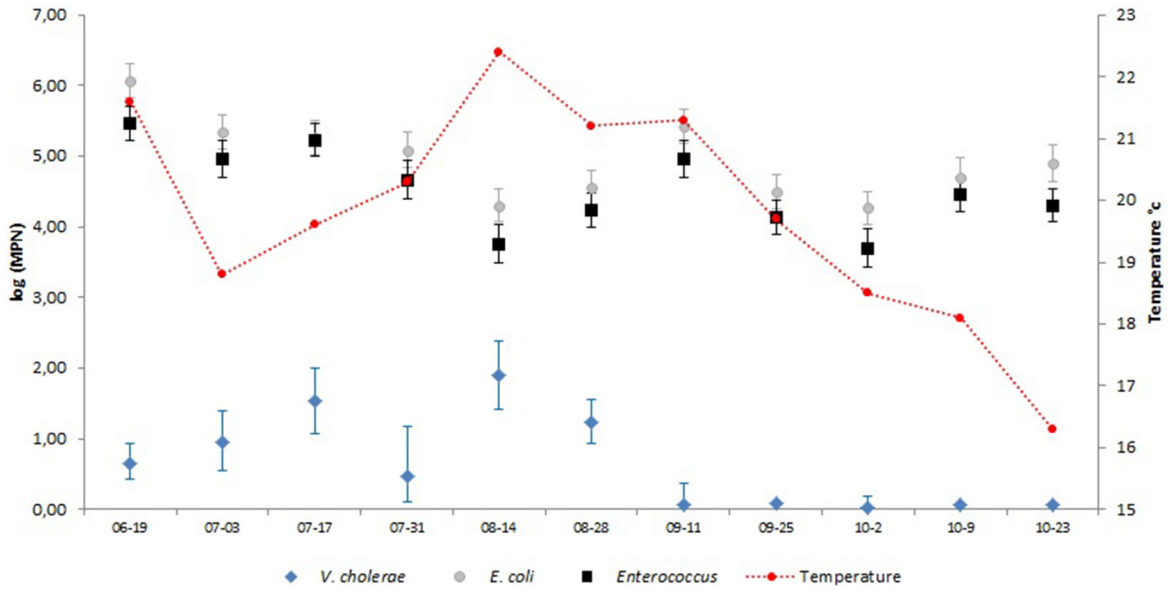

FIGURE 2 | Densities of *V. cholerae*, *E. coli* and intestinal *Enterococci* in treated wastewater. Densities of *E. coli* and intestinal *Enterococci* were expressed by 100 mL of water and *V. cholerae* per 1 liter of water.

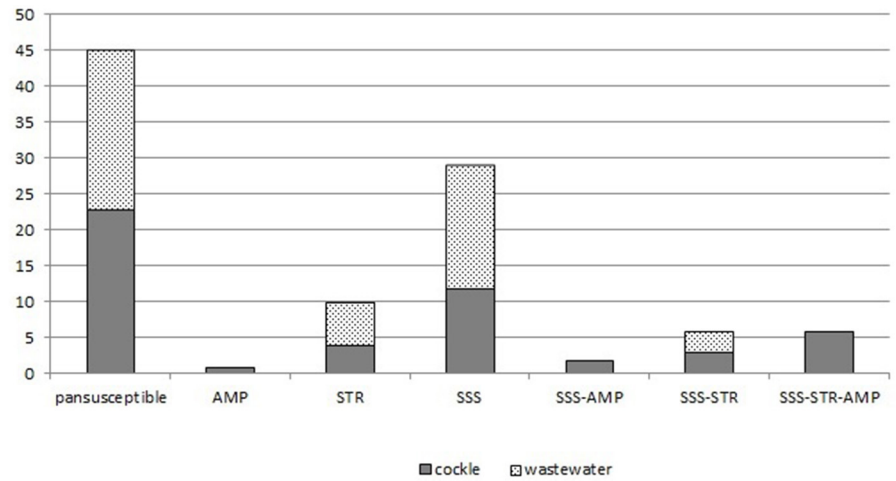

FIGURE 3 | Distribution of resistance profiles among the 99 isolates of non-O1/non-O139 *Vibrio cholerae*.

(**Figures 4A,B**), only one population could be observed. All the strains were considered as susceptible (Baron et al., 2016).

Among the 54 isolates (54.6%) that were resistant to at least one antimicrobial agent, 28 were collected in cockles and 26 in wastewater. The most frequent resistance was to sulfonamides (44 isolates), followed by streptomycin (22 isolates) and ampicillin (9 isolates). No significant difference was observed for the frequency of resistance to either sulfonamides or streptomycin between the isolates from cockles and those from wastewater. The nine isolates resistant to ampicillin were collected from cockles in 2000.

The most frequent resistance profile was susceptibility to the 16 antimicrobial agents tested ($n = 45$), then resistance to sulfonamides only ($n = 29$), followed by resistance to streptomycin only ($n = 10$). Six multi-drug resistant isolates were detected in cockles in 2000; none in wastewater. They were resistant to sulfonamides, ampicillin and streptomycin (**Figure 3**).

No difference was observed in the frequency of associated resistance genes between cockles and wastewater. Among the 99 isolates, neither the *erm*B nor the bla_{CTX-M} gene was detected. The most frequent gene was *erm*A ($n = 97$); *sul1* and *sul2* were detected in 86 and 49 isolates respectively (**Table 4**). None of the three classes of integron were detected in the 99 *V. cholerae* strains (**Table 2**).

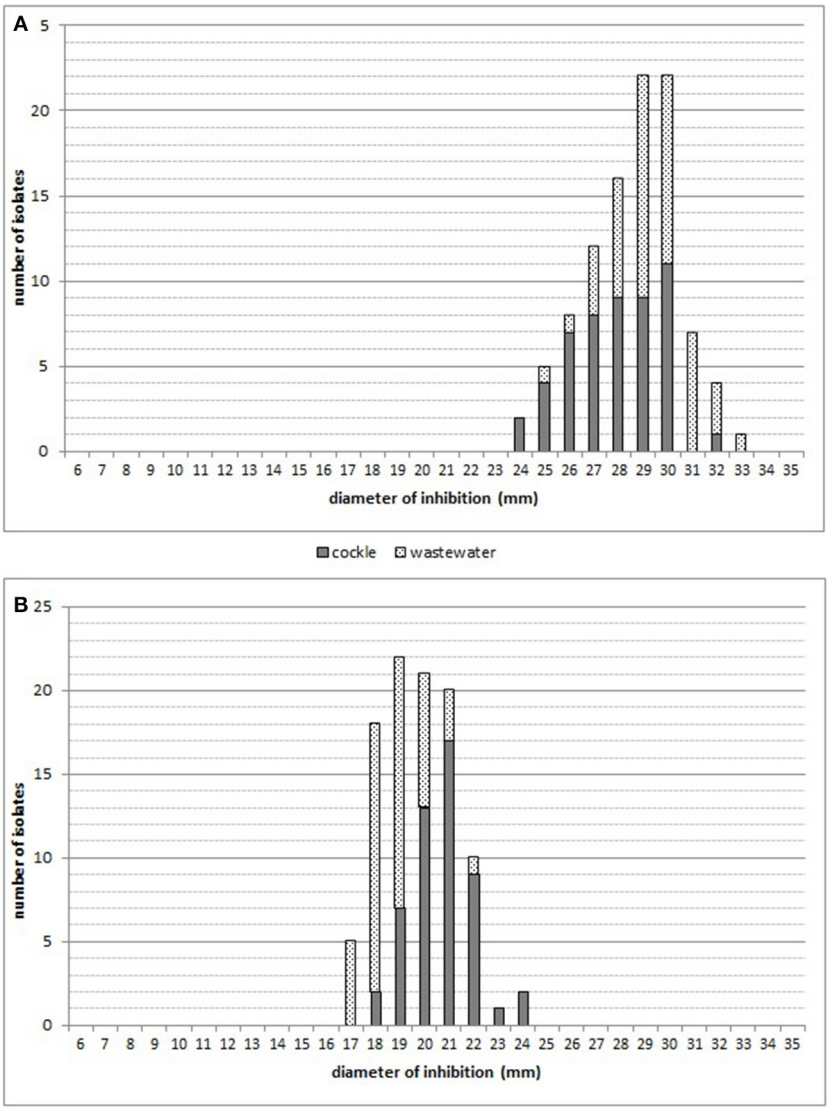

FIGURE 4 | Distribution of diffusion zone diameters: **(A)** doxycycline (30 μg); **(B)** erythromycin (15 μg).

Among the 45 isolates harboring *sul1* and *sul2*, 10 were resistant to sulfonamides and 35 were susceptible. Similarly, for the isolates harboring *str*A and *str*B, two were resistant and four were susceptible to streptomycin. All the isolates harbored at least one of the eight targeted genes and 52.3% of the isolates harbored at least three genes. Only two isolates harbored simultaneously *sul*2, *str*A and *str*B (**Table 4**).

DISCUSSION

The objective of this baseline study was to characterize the susceptibility to antibiotics of non-toxigenic *V. cholerae* collected in 2000/2001 in a cholera-free area, at two points of interface between the aquatic environment and the human population—a discharge of urban wastewater and shellfish harvested from the shore downstream.

Abundance of *Vibrio cholerae*

The presence of *V. cholerae* in treated wastewater had never been described in France. Non-O1/non-O139 *V. cholerae* was detected in 75% of the wastewater samples at the outlet of the treatment plant of the city of Dinan. An investigation from our team conducted at the same time in stabilization ponds at Saint-Helen, a village in the watershed of La Rance estuary, confirmed the persistent presence of *V. cholerae* in wastewater at another location (data not shown). To our knowledge, it can only be compared in Europe to the results of an Italian study: 19 strains were isolated from 69 samples of wastewater at the inlet and the outlet of a treatment plant (Gatti et al., 1997).

The levels of abundance of non-O1/non-O139 *V. cholerae* observed in the treated wastewater from Dinan (0.075 MPN/L to 78 $\times$ 10^1 MPN/L) were much lower than those calculated with a comparable method of analysis, outside the context

TABLE 4 | Distribution of resistance-associated genes among the 99 isolates of non-O1/non-O139 *Vibrio cholerae*.

	Cockle	Wastewater	Total
*erm*A	2	7	9
*sul*1 *erm*A	17	20	37
*sul*2 *erm*A	1		1
*sul*1 *sul*2 *erm*A	11	9	20
*sul*1 *str*B *erm*A	3		3
*sul*1 *sul*2 *str*B		2	2
*sul*2 *erm*A *mef*	1		1
*sul*2 *str*B *erm*A	1		1
*sul*1 *sul*2 *erm*A *mef*	8	5	13
*sul*1 *sul*2 *str*B *erm*A	5	1	6
*sul*2 *str*A *str*B *erm*A		1	1
*sul*1 *str*A *str*B *erm*A		1	1
*sul*1 *sul*2 *str*A *erm*A	1	2	3
*sul*1 *sul*2 *str*A *str*B *erm*A	1		1
Total	51	48	99

of cholera, in Mediterranean Europe (Yugoslavia, wastewater discharge of a coastal town with 600,000 inhabitants, 1990: range from 2.0×10^2 to 2.1×10^5 MPN/L) (Muic, 1990), or in North Africa (Marrakesh, at the outlet of experimental stabilization ponds, from January 1986 to December 1987: range from $<3 \times 10^2$ to 2.3×10^7 MPN/L (Lesne et al., 1991) and from October 1992 to September 1993: geometric mean in winter of 2.5×10^4 MPN/L, and geometric mean in summer of 1.7×10^6 MPN/L (Mezrioui and Oufdou, 1996). The only other recent study, to our knowledge, was done in South Africa from August 2011 to May 2012 (17 dates) but in the context of a cholera epidemic (Okoh et al., 2015). The levels of abundance of *V. cholerae* observed in four wastewater treatment plants discharging into rivers at four sampling points each (influent, effluent, upstream, downstream), i.e., in 272 samples of water, were still higher (1.6×10^3 CFU/L to 6.3×10^9 CFU/L), but the method of analysis in that study is not comparable to ours. And an unspecified fraction of the counted population of *V. cholerae* is toxigenic in 40% of the samples, which is not the case in any of the other studies presented.

Sewage is oligohaline water, rich in organic matter, with a pH neutral to alkaline, characteristics which meet the requirements of *V. cholerae* for growth or survival. The abundance of *V. cholerae* also appeared higher under climates that include a hot season, which can be considered alongside the well-known favorable effect (direct or indirect) of water temperature on the growth of population of this bacterium in aquatic ecosystems. While it is well established that *V. cholerae* is autochthonous of the natural aquatic environment, its detection in sewage fed by a public water distribution network raises the question of its origin. At least two non-mutually-exclusive assumptions can be made: contribution of run-off waters and contribution of a possible healthy carriage in the gut of the human population (Morris, 1990). In any case, it seems that sewage establishes an ecosystem favorable to the installation and development of this species. We have no information about the presence of healthy carriers in the local human population, nor on local clinical cases of infection with non-O1/non-O139 *V. cholerae*. It is interesting to observe that intestinal carriage also exists in livestock (Rhodes et al., 1985) and that in our study, strains of non-O1/non-O139 *V. cholerae* have also been isolated in the wastewater treatment plants of two neighboring slaughterhouses, one for pigs and the other for calves (Sandrine Baron, personal communication).

Between June and September 2000, *V. cholerae* was detected in all seven samples of cockles. The field of naturally-growing cockles was located in brackish water area (salinity between 0.5 and 30‰) in the upper estuary. Similar results were obtained in a field of mussels (*Mytilus edulis*) located in a zone where sea water (Northern Sea) mixes with brackish water (Baltic Sea), in The Sound (between Sweden and Denmark) during summer 2006: 100% ($n = 19$) of samples contained non-toxigenic *V. cholerae* (Collin and Rehnstam-Holm, 2011). In other salinity conditions, such as marine waters (salinity ≈33‰) or polyhaline waters (salinity between 18 and 30‰), the frequencies of detection are much lower. In France, in summer 1999, only two strains of non-O1/non-O139 *V. cholerae* were detected in two samples of mussels from the Channel, and two strains in one sample of mussels from the Atlantic Ocean (Hervio-Heath et al., 2002). Again in France, between 2006 and 2007, *V. cholerae* was not detected in mussels and clams of two lagoons which are open onto the Mediterranean Sea (Cantet et al., 2013). In Italy, during a national study from May to September 2006 on the Ligurian Adriatic and Tyrrhenian coasts, non-O1/non-O139 *V. cholerae* was present in only 7.8% ($n = 90$) of mussel samples and never detected in the clam samples ($n = 56$) (Ottaviani et al., 2009). In 2011–2014, another study detected *V cholerae* $ctx^- tcp^-$ in only one sample of clams out of 112 collected (Passalacqua et al., 2016). In Germany, during a study of non-O1/non-O139 *V. cholerae* in mussels produced in the Wadden Sea, the frequencies of presence were 16% ($n = 46$) in 2012 and 11% ($n = 71$) in 2013 (Huehn et al., 2014). On the coast of the Atlantic Ocean, north of São-Paulo (Brazil), in mussels cultivated in sea water, less than 10% of samples collected between February 1989 and February 1990 were positive for non-O1/non-O139 *V. cholerae*. On the Northeast Atlantic Coast in Long Island Sound (USA) *V. cholerae* was detected in 8.8% ($n = 68$) of oyster samples (*Crassostea gigas*) and in 3.3% ($n = 30$) in clams. All these data show a higher frequency of detection of *V. cholerae* in brackish waters than in marine waters.

The following studies are the only ones that supply quantitative data of *V. cholerae* in shellfish. In Brazil, with a method similar to ours, the abundances are lower, varying between 7 and 2.3×10^1 MPN/100 g (Matté et al., 1994). In the USA, by using a PCR multiplex on enrichment broth, the abundances observed were between 3.6×10^1 and 3.0×10^3 MPN/100 g for oysters and 3.0×10^2 in the only sample of positive clams (Jones et al., 2014). This difference of method might explain the higher abundances.

Characterization of the Isolates

In France, the rare cases of cholera are imported (Geneste et al., 2000; Tarantola et al., 2007), thus the presence of the main virulence factors of cholera, *ctxA* and *tcpA,* is very improbable

in the aquatic population of *V. cholerae*. The products of the *ctxA* gene are involved in interactions with the host that are responsible for pathological damage (Wassenaar and Gaastra, 2001). This gene encodes the cholera toxin A subunit. The *tcpA* gene is a colonization gene encoding the principal subunit of the toxin-coregulated pilus (type IV), which appears to facilitate the interaction between bacteria and the intestinal epithelium surface (Reidl and Klose, 2002). In our collection, the *ctxA* gene and the *tcpA* gene were never detected. Similarly, none of 11 isolates of non-O1/non-O139 *V. cholerae* collected from seafood samples (Italian coastal waters), harbored either the *ctxA* or the *tcpA* genes (Ottaviani et al., 2009). Also in a collection of 395 strains of non-O1/non-O139 *V. cholerae* collected in Chesapeake Bay, Maryland (USA), in water, sediment and oyster samples during a *Vibrio* surveillance program (2009 to 2012), only four isolates harbored the *ctxA* gene and none the *tcpA* gene (Ceccarelli et al., 2015).

Inversely, *hlyA* genes are frequently harbored by environmental isolates of non-O1/non-O139 *V. cholerae*. The *hlyA* gene encodes hemolysin, the production of which was originally used to differentiate Classical and El Tor *V. cholerae* O1 biotypes. Its role in virulence is not clearly established. However, it does not seem to be an essential factor for cholera-type human pathogenesis, since the hemolytic function is absent in clinical isolates of the 6th pandemic (Classical biotype), and disappears gradually from isolates of the 7th (El Tor biotype) (Barrett and Blake, 1981). By contrast, in non-O1/non-O139 strains associated with cases of gastro-enteritis and diarrhea, but lacking virulence factors associated with cholera, hemolysin production might constitute an important virulence factor. In our study, 52.3% of the *V. cholerae* isolates harbored the hemolysin gene (variant El Tor or Classical without distinction) and no significant difference between cockles and wastewater was noticed. In comparison, all the 13 isolates of *V. cholerae* collected from seafood in Italy harbored *hlyA* (Ottaviani et al., 2009). Among 39 non-O1/non-O139 *V. cholerae* strains from Brazil, 94.9% showed homology to El Tor hemolysin, 2.6% were associated with Classical hemolysin, and 2.5% were negative for both genes (Rivera et al., 2001). Lastly, among the 395 environmental isolates collected by Ceccarelli et al. (2015), 83% were *hlyA* positive, including the seven oyster isolates. In these studies, the El Tor variant was thus either the only variant detected or the most abundant. In our study, the El Tor variant was found in only 30.5% ($n = 66$) of the isolates and the Classical variant in 21.8% ($n = 47$). The Classical biotype has an 11 bp deletion within the *hlyA* coding region, compared to the El Tor biotype, resulting in a truncated and non-hemolytic *hlyA* product which may have another, as yet unknown, function (Rader and Murphy, 1988; Alam et al., 1997). It suggests that a part of our isolates would not be capable of producing a functional hemolysin, or at best a less active one. It could be verified by carrying out *in vitro* tests following the example of those performed by Ottaviani et al. (2009).

Other genes, such as *rtxA*, *chxA, T6SS*, *hapA, nanH,* etc... are suspected of being involved in the virulence of toxigenic *V. cholerae*; but this does not mean that their role is well defined. Their detection in environmental isolates helps to better characterize these isolates and to estimate the genetic reservoir associated with virulence, but does not permit any conclusion concerning the pathogenicity of a strain. Indeed, their presence can be interpreted by precaution as enabling the bacterium to induce acute gastroenteritis if present in shellfish (Ceccarelli et al., 2015). But at present, there are still no markers of virulence validated for the pathogenic strains of non-O1/non-O139 *V. cholerae*. In view of the variety of the pathologies caused by non-O1/non-O139 *V. cholerae*, very numerous markers will probably be necessary to cover all the mechanisms of pathogenicity. These data would be essential to better manage the hazard for human health represented by the environmental isolates, in the context of the increase of cases of human vibriosis in Europe (Baker-Austin et al., 2016).

Antimicrobial Susceptibility

The existence of environmental strains of non-O1/non-O139 *V. cholerae* presenting acquired resistances was reported in the literature in cholera-free regions. Therefore, we undertook the study of the antibiotic resistance by choosing two compartments exposed to different pressures of antibiotics: wastewater coming from the sewage system of an urban area containing a hospital, and a field of wild shellfish located downstream in the estuary. This estuary is characterized by a low pressure of ground breeding and a few zones contaminated by farm effluent, but no fish farming.

In case of infection by non-O1/non-O139 *V. cholerae*, ciprofloxacin, or/and doxycycline or extended spectrum cephalosporin are the recommended treatments (Daniels and Shafaie, 2000). As all isolates tested here were susceptible to these antimicrobial agents, the loss of efficacy of such first line treatments was not to be feared.

Among the 99 environmental isolates tested, resistances were observed for only three antimicrobial agents: ampicillin, streptomycin and sulfonamides, and no significant difference was observed between wastewater ($n = 48$) or cockles ($n = 51$) origin These molecules are considered as "old antibiotics." In a similar context (cholera free area, temperate climate, and labeled use of antibiotics), recent studies on environmental isolates–collected in the Baltic Sea (Germany) ($n = 131$ isolates, between 2008 and 2014) (Bier et al., 2015) and in the Chesapeake Bay (USA) ($n = 307$ isolates, between 2009 and 2014) (Ceccarelli et al., 2015) were performed between 2008 and 2014. In our study, 9.0% of the isolates were resistant to ampicillin, result in agreement with those of the two previous ones (16.3% in the Chesapeake Bay and 7.6% in the Baltic Sea).

The resistance profiles observed in our strains collected in wastewater were very similar to those observed in *V. cholerae* isolated at the outlet of stabilization ponds in Marrakesh 10 years previously, except for streptomycin. The highest observed resistance rates were for streptomycin (11.8%), and ampicillin (9.1%) (Imziln and Hassani, 1994). All the 120 isolates of non-O1/non-O139 *V. cholerae* tested were susceptible to quinolones (nalidixic acid) and phenicols (chloramphenicol) and only one isolate was resistant to trimethoprim-sulfamethoxazole association. More recently, in Tanzania, all the strains of *V. cholerae* collected in wastewater were resistant to ampicillin

and to tetracycline but quite susceptible to phenicol or quinolone (Hounmanou et al., 2016).

Wastewater is considered to be hot spots for the acquisition of resistance. Nevertheless, in our study, it is worth noting that there was not more resistance in wastewater than in shellfish. One of the explaining hypotheses could be the relatively low abundance of this bacterium in wastewater, compared to enteric species (like *E. coli* or intestinal *Enterococci*).

Six multidrug resistant strains were collected from cockles; they were resistant to ampicillin, streptomycin and sulfonamides (6% of the isolates). The multi-resistant strains of environmental origin are rare in cholera-free areas. Bier et al. (2015) and Ceccarelli et al. (2015) thus detected no multi-resistant strains among 131 and 307 isolates respectively. But these authors did not test sulfonamides, which in our study accounted for the most frequent resistance.

All our isolates were susceptible to extended spectrum cephalosporins and none harbored the bla_{CTX-M} gene, one of the most important genes conferring this resistance to *Enterobacteriaceae* in France. These isolates were collected in 2000 and 2001; the dissemination of this gene in environmental strains appeared later and so its non-detection in our strains was not surprising. Even though 29.5% (62/210) of non-O1/non-O139 *V. cholerae* isolated from tropical seafood in Cochin (India) in 2013 were resistant to cefpodoxime (Kumar and Lalitha, 2013), clearly, extended spectrum β-lactamase enzymes are uncommon in *V. cholerae* (Ceccarelli et al., 2016).

All isolates of *V. cholerae* collected in 2000–2001 in La Rance estuary were also susceptible to carbapenem, another critical antimicrobial agent. Since the beginning of the twenty-first century, environmental *V. cholerae* harboring carbapenemase genes have been reported several times in the literature, but this could be a bias linked to the interest focused on this antimicrobial resistance. Indeed, a gene conferring resistance to carbapenems has been detected in environmental isolates of non-O1/non-O139 *V. cholerae*, even in Europe. In France, one strain of *V. cholerae* isolated from cloacal swab samples from juvenile unfledged yellow-legged gulls (*Larus michahellis*) coharbored the bla_{VIM-1} and bla_{VIM-4} carbapenemase genes (Aberkane et al., 2015). Bier et al. (2015) identified a non-toxigenic *V. cholerae* harboring a carbapenemase gene that could not be identified by standard PCR typing. In Canada, a novel Amber class A carbapenemase was found in non-toxigenic *V. cholerae* strains isolated from shrimp intended for human consumption (Mangat et al., 2016). Given the potential ability of conjugative plasmids to transfer naturally between enterobacterial populations in the intestinal gut (Rashid and Rahman, 2015), the aquatic environment is now considered as an ideal setting for acquisition and dissemination of antibiotic resistance (Marti et al., 2014) and the horizontal transfer of ESBL/carbapenemase genes to *V. cholerae* cannot be ruled out.

On the basis of the distribution of a limited set of resistance genes (eight), our study shows that *V. cholerae* can constitute an environmental reservoir for these genes. However, none of the 99 isolates studied harbored integron. This result is in agreement with those of Bier et al. (2015) and Ceccarelli et al. (2015). As such mobile genetic elements play an important role in the acquisition and dissemination of antimicrobial resistance; this provides some confirmation for the conclusion drawn above from our susceptibility data, that, in our geographic context at least, non-O1/non-O139 *V. cholerae* seems not to have significant role in the dissemination of antibiotic resistance in the environment.

Nevertheless, integrons are not the only medium by which *V. cholerae* acquires antimicrobial resistance genes. Presence of integrative and conjugative element (ICE) has been regularly reported since the first description of the so-called SXT element in *V. cholerae* (Waldor et al., 1996) confering resistance to sulfonamides, trimethoprim, streptomycin and chloramphenicol. Further study should probably also target detection of specific markers to evaluate the presence of the SXT element in this non-O1/non-O139 *V. cholerae* collection. But, since multiple studies investigating either clinical or non-pathogenic collections of *V. cholerae* (Ceccarelli et al., 2006; Mala et al., 2016, 2017; Wang et al., 2016) had demonstrated that antimicrobial resistance genes carried by the SXT element are various, and *sul2, strA* and *strB* appeared to be the most commonly encountered resistance genes. We might hypothesize that the proportion of strains from our collection harboring the SXT element might be quite low.

CONCLUSION

In France, a cholera-free country, the presence of *V. cholerae* non-O1/non-O139 in treated wastewater of an urban area (11,000 inhabitants), and in cockles had never been investigated simultaneously in a same geographic area.

In similar epidemiological contexts, without any history of cholera cases but potentially different local antibiotic pressures, this study and two others (Bier et al., 2015; Ceccarelli et al., 2015) performed later on cast doubt on the capacity of *V. cholerae* non-O1/non-O139 to acquire resistance-associated genes and its potential role as indicator for the dissemination of antimicrobial resistance in the aquatic environment. However, *V. cholerae* presents the advantage of being a bacterium able to develop in the aquatic estuarine environment, unlike enteric bacteria which only survive for a limited time in receiving surface waters. Moreover, *V. cholerae* is known to be susceptible to carbapenems and to third generation cephalosporins, unlike *Aeromonas* spp., which is also more and more often proposed as candidate to be an indicator bacterium of antimicrobial resistance circulating in the aquatic environment (Usui et al., 2016; Varela et al., 2016; Baron et al., 2017). So, detection of *V. cholerae* harboring a mechanism of resistance to antibiotic critically important for Public health, such as carbapenems or third generation cephalosporins, could establish an alert on contaminated ecosystems.

Finally, those historical data would deserve now to be refreshed in order to determine if there has been a shift in the antimicrobial resistance genes harbored by the population of *V. cholerae* non-O1/non-O139 from La Rance Estuary over the past 20 years.

AUTHOR CONTRIBUTIONS

SB, EL, EJ, SC, and JL contributed to the design of the study. SB, EL, SC, and JL produced data. All authors contributed to the

analysis of the data, to the redaction and/or the edition of the article.

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SUPPLEMENTARY MATERIAL

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Evidence for Cleavage of the Metalloprotease Vsm from *Vibrio splendidus* Strain JZ6 by an M20 Peptidase (PepT-like Protein) at Low Temperature

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Metalloprotease Vsm is a major extracellular virulence factor of *Vibrio splendidus*. The toxicity of Vsm from *V. splendidus* strain JZ6 has been characterized, and production of this virulence factor proved to be temperature-regulated. The present study provides evidence that two forms (JZE1 and JZE2) of Vsm protein exist in extracellular products (ECPs) of strain JZ6, and a significant conversion of these two forms was detected by SDS-PAGE and immunoblotting analyses of samples obtained from cells grown at 4, 10, 16, 20, 24, and 28°C. Mass spectroscopy confirmed that JZE1 was composed only of the peptidase_M4 domain of Vsm, and JZE2 contained both the PepSY domain and the peptidase_M4 domain. An M20 peptidase T-like protein (PepTL) was screened from the transcriptome data of strain JZ6, which was considered as a crucial molecule to produce the active Vsm (JZE1) by cleavage of the propeptide. Similar to that of Vsm, PepTL mRNA accumulation was highest at 4°C (836.82-fold of that at 28°C), decreased with increasing of temperature and reached its lowest level at 28°C. Deletion of the gene encoding the PepTL resulted in a mutant strain that did not produce the JZE1 cleavage product. The peptidase activity of PepTL recombinant protein (rPepTL) was confirmed by cleaving the Vsm in ECPs with an *in vitro* degradation reaction. These results demonstrate that PepTL participates in activating Vsm in strain JZ6 by proteolytic cleavage at low temperature.

Keywords: *Vibrio splendidus*, metalloprotease Vsm, peptidase T-like protein, extracellular products, temperature regulation

INTRODUCTION

Proteolytic degradation plays important roles in physiological processes of bacteria, including protein maturation, signal peptide modification, gene expression, and nutrients acquisition (Lazdunski, 1989; Charlier et al., 2000). Peptidases have been grouped into 86 families according to their evolutionary relationship, such as serine, cysteine, aspartic, threonine, glutamic-acid and metallo peptidases (Rawlings and Barrett, 1993; Wu and Chen, 2011; Oda, 2012). Among them, the M20 family contains many important members, including metallopeptidases and non-peptidase homologs (amidohydrolases), which are involved in the catalyzed reaction for the release of an

N-terminal amino acid from a polypeptide (Rawlings and Barrett, 1995). Members in M20 family commonly exist as homodimers, which contain a zinc-binding domain and a second domain mediating dimerization (Lindner et al., 2003; Chen et al., 2008; Chang et al., 2010). Many M20 peptidases have attracted attention due to their biological functions. For instance, aminopeptidase V (PepV) from *Lactobacillus* sp. and peptidase T (PepT) from *Salmonella typhimurium* are involved in amino acid utilization, and allantoate amidohydrolase from *Escherichia coli* and β-alanine synthase (βAS) from yeast are enzymes of the nucleotides catabolic pathway (Håkansson and Miller, 2002; Jozic et al., 2002; Lundgren et al., 2003; Agarwal et al., 2007).

Vibrio splendidus is a pathogen that causes fatal diseases of larval and juvenile marine animals, including turbots (Gatesoupe et al., 1999; Thomson et al., 2005), oysters (Waechter et al., 2002; Garnier et al., 2007), clams (Gómez-León et al., 2005; Kesarcodi-Watson et al., 2009) and scallops (Nicolas et al., 1996; Liu et al., 2013). As an opportunistic pathogen, the virulence of *V. splendidus* is mainly regulated by environmental factors, most importantly by temperature (Crapoulet et al., 2006; Wu et al., 2012). *V. splendidus* JZ6 is a pathogenic agent of Yesso scallop at the low temperature, and its pathogenicity is significantly reduced when the temperature increases (Liu et al., 2013). The toxicity and the expression of metalloprotease Vsm in extracellular products (ECPs) of *V. splendidus* JZ6 are higher at 10°C than that at 28°C (Binesse et al., 2008; Hasegawa et al., 2009; Liu et al., 2016). Furthermore, the toxicity of Vsm was associated with its two protein sizes at different temperatures in ECPs of *V. splendidus* JZ6 (Liu et al., 2016), suggesting temperature-dependent post-translational modification of the protease.

Vsm is a multi-domains metalloprotease, containing a signal peptide, a FTP domain, a PepSY domain and two peptidase_M4 domains. The FTP is a fungalysin/thermolysin propeptide domain, which is found in both the bacterial M4 peptidase propeptide and the fungal M36 propeptide to prevent premature activation of proteases (Tang et al., 2003). The PepSY domain is also found in the peptidase M4 family and likely has protease inhibitory activity (Yeats et al., 2004). The peptidase_M4 domain is the metalloprotease activity motif in MEROPS peptidase M4 family with a metal ion binding site (Rawlings and Barrett, 1995). According to its structural feature, the active protein of Vsm must be cleaved to produce the mature enzyme by other peptidase.

In the present study, an M20 peptidase T-like protein (PepTL) was hypothesized to be involved in Vsm maturation in *V. splendidus* JZ6 since mRNA encoding this peptidase was up-regulated under low-temperature conditions. The recombinant protein of PepTL was obtained and its proteolytic activity on Vsm was demonstrated *in vitro*. A *ΔpepTL* mutant was constructed to elucidate the function of PepTL in toxicity regulation of Vsm at different temperatures.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The Yesso scallop (*Patinopecten yessoensis*) pathogen, *V. splendidus* strain JZ6 (Liu et al., 2013), was cultivated on Zobell 2216E agar at 20°C for 24 h. *E. coli* strains DH5α, BL21 (DE3), SY327 and S17-1 (**Table 1**) were cultured on LB agar at 37°C for 24 h.

Analysis of Extracellular Products (ECPs) from *V. splendidus* JZ6 at Different Temperatures

ECPs of *V. splendidus* JZ6 wild-type and mutant strains were obtained by using the cellophane method (Balebona et al., 1998). Strains were grown in Tryptic Soy Broth (TSB) medium supplemented with 2% w/v sodium chloride (NaCl) at 20°C for 12 h, and 200 μL of the cultures was spread onto Zobell 2216E agar plates, overlayed with sterile cellophane sheets and plates were incubated at 4, 10, 16, 20, 24, and 28°C for 24 h. Bacterial

TABLE 1 | Bacterial strains and plasmids used in the present study.

Strans/plasmids	Description[a]	References
***VIBRIO SPLENDIDUS* STRAINS**		
JZ6	*V. splendidus* JZ6, a pathogenic agent of scallop	Liu et al., 2013
Δ*pepTL*	*pepTL* gene deletion mutant of *V. splendidus* JZ6	This study
***ESCHERICHIA COLI* STRAINS**		
DH5α	F$^-$, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*) U169, *deoR*, *recA*1, *endA*1, *hsdR*17 (r_K^-, m_K^+), *phoA*, *supE44*, λ$^-$, *thi*-1, *gyrA*96, *relA*1	Tiangen
BL21 (DE3)	F$^-$, *ompT*, *hsdSB* (r_B^-, m_B^-), *gal*, *dcm* (DE3)	Tiangen
SY327	Δ(*lac pro*), *argE*(Am), *recA56*, *rpoB*, λ *pir*	Ma et al., 2009
S17-1	Tp^r, Sm^r, *recA*, *thi*, *pro* (r_K^-, m_K^-), *RP4:2-Tc:* MuKm, Tn7, λ *pir*	Ma et al., 2009
PLASMIDS		
pMD19-T simple	High copy number cloning vector, Amp^r	TaKaRa
pET28a (+)	Prokaryotic expression vector, Kan^r	Novagen
pK18mobsacB-Ery	Widely used gene knockout vector, Kan^r, Ery^r	Wang et al., 2015
pK18Ery-*pepTL*	pK18mobsacB-Ery containing the homologous fragment of *pepTL* gene of JZ6, Kan^r, Ery^r	This study

[a] *Amp^r, ampicillin resistance; Ery^r, erythromycin resistance; Kan^r, kanamycin resistance.*

cells were harvested with sterile saline, and the cell suspensions were centrifuged at 12,000g, at 4°C for 10 min. The bacterial cells were collected and stored at −80°C prior to RNA extraction. The supernatants were filtered through 0.22 μm membrane filters and used as the crude ECPs (designated as P_4, P_{10}, P_{16}, P_{20}, P_{24}, and P_{28}). The total protein contents of the ECPs were measured following the bicinchoninic acid (BCA) method (Smith et al., 1985).

The concentration of the six ECPs samples were adjusted at 20 μg/μL, and 10 μL were loaded onto a 12% SDS-polyacrylamide gel for SDS-PAGE. Following electrophoresis, protein bands were visualized with Coomassie bright blue R250. Vsm in ECPs samples was detected by immunoblotting using a monoclonal antibody of JZ6 Vsm (Liu et al., 2016). The ECPs samples were transferred onto a sheet of nitrocellulose transfer membrane (Millipore, USA). The membrane was blocked with 5% skim milk powder solution overnight and incubated with anti-Vsm solution (1:1000, v/v) at room temperature (RT) for 1 h. After being washing with TBST (10 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl and 0.05% (w/v) Tween 20), the membrane was incubated with 1:2000 (v/v) horseradish peroxidase-conjugated anti-mouse IgG (Life Technologies, USA) at RT for 1 h. Finally, the membrane was incubated with SuperSignal®West Pico (Thermo Scientific, USA) and exposed to film. Mouses pre-immune serum was used as negative control.

Mass Spectrometry Analysis of Proteins in ECPs

After SDS-PAGE, protein bands from the ECPs of strain JZ6 were excised and sent to BGI (BGI technology service co., LTD, China) for mass spectrometry (MS) analysis. Proteins were digested with 0.01 μg/μL trypsin, and the resulting peptides were subjected to nanoelectrospray ionization followed by tandem mass spectrometry (MS/MS) in a LTQ Orbitrap Velos (Thermo, USA) coupled online to the HPLC. Raw data files acquired from the Orbitrap were converted into MGF files using Proteome Discoverer 1.2 (Thermo, USA), and protein identification was performed by using Mascot search engine 2.3.02 (Matrix Science, United Kingdom).

RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was extracted with TRIzol reagent (Invitrogen, USA). Based on Promega M-MLV RT Usage information, the first-strand cDNA synthesis was carried out with the DNase I (Promega, USA)-treated total RNA and random primers (TaKaRa, Japan). Synthesis was performed at 42°C for 1 h, then terminated by heating at 95°C for 5 min.

The mRNA levels of the *vsm* and *pepTL* at different temperatures were validated by quantitative real-time PCR (qRT-PCR). Specific primers (P1/P2 for *vsm* and P3/P4 for *pepTL*) were designed according to the corresponding sequences in the genome of strain JZ6 (**Table 2**). The comparative C_T method ($2^{-\Delta\Delta CT}$ method) was used to analyze the expression level (Livak and Schmittgen, 2001). Two 16S rDNA primers for *V. splendidus*, P5 and P6 (**Table 2**), were used as internal control to verify successful transcription and to calibrate the cDNA template for corresponding samples. qRT-PCR was performed using Applied Biosystems 7500 (Life technologies, USA), and the collected data were analyzed with 7500 System SDS Software. The assay was conducted in a volume of 20 μL consisting 10 μL of 2 × SYBR® Premix Ex Taq™ II (TaKaRa, Japan), 0.8 μL of each forward and reverse primer (10 μmol/L), 0.4 μL of 50 × ROX reference dye, 2 μL of DNA extract (10 ng/μL) and 6 μL of nuclease-free water. The reaction was performed at 95°C for 30 s, 40 cycles of primer annealing at 95°C for 5 s, primer extension at 60°C for 31 s. Dissociation curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. All data were given in terms of relative mRNA expressed as mean ± S.E. ($N = 4$).

TABLE 2 | The primers used in the present study.

Primer name	Sequence (5′-3′)
Vsm qRT-PCR	
P1	AAGTCGCCCAAGTGGTGTATCT
P2	CGATGGGAAAGCTAGGGAAGT
PepTL qRT-PCR	
P3	TTCACAAACTGGCTGTCCCTAC
P4	GCCGATACCTGGCGTTACTG
16S qRT-PCR	
P5	TCGTGTYGTGARATGTTGGGT
P6	CCACCTTCCTCCRGTTTRTCA
Vsm EXPRESSION	
P7	GAATTCATGGCAGAAATGGTCAGAGTCG
P8	GAATTCATGCCAAAGCTGAATCGAGAACAAGC
P9	GAATTCATGGGACTAAACCACGCTAAAGCATTAG
P10	CTCGAGGACACCACAACTCGCATTAACG
PepTL EXPRESSION	
P11	CCATGGATATGGAACAACGTCTTGTAGAACATTTC
P12	GAATTCATGGAACAACGTCTTGTAGAACATTTC
PepTL KNOCKOUT	
P13	CTAGTCTAGAGAACAACGTCTTGTAGAACATTTCT
P14	GATGACCACCCCCGAGCGTAGATGTTGAAACCGT
P15	GGGGTGGTCATCTCACCCGCACATTCTTTCTATC
P16	ACGCGTCGACCGTCACGTAAGCCACAACAAA

Expression and Purification of Recombinant Proteins

Genomic DNA of *V. splendidus* JZ6 was extracted from 5 mL of an overnight culture using the DNeasy Blood and Tissue Kit according to the manufacturer's protocol (Qiagen, Germany). Specific primers P7, P8, and P9 (**Table 2**) were designed from the nucleotide sequences encoding the upstream fragment of Vsm's fungalysin/thermolysin propeptide (FTP) domain, protease inhibitory function (PepSY) domain and peptidase M4 domain, respectively. An *Eco*RI site sequence was added at 5′ end. P10 (**Table 2**) was designed as a reverse primer with an *Xho*I site sequence. PCR products were digested with *Eco*RI and *Xho*I (NEB, USA), and ligated into the same restriction enzymes sites of expression vector pET28a (+) (Merck, Germany).

The complete open read frame (ORF) encoding PepTL was amplified with primers P11 and P12 (**Table 2**) containing *Nco*I and *Eco*RI recongnition sequences at their 5′ end, respectively. The PCR product was digested with *Nco*I and *Eco*RI (NEB, USA), and ligated into the same restriction enzymes sites of expression vector pET22b (+) (Merck, Germany).

The recombinant plasmids were transformed into competent cell *E. coli* BL21 DE3 (Tiangen, China), and transformants were incubated in LB medium at 37°C with shaking at 220 rpm for 4 h. IPTG was added to the cell cultures to a final concentration of 1 mmol/L once the cultures reach at an OD_{600} of 0.4–0.6, and incubated at 18°C with shaking at 150 rpm for 24 h. The cultures were sonicated (200 W for 30 min) and centrifuged (12,000g, 4°C for 10 min) to obtain the supernatant containing soluble target proteins. Recombinant proteins (rVsmP1, rVsmP2, rVsmP3, and rPepTL) were purified with a Ni Sepharose column (Roche, Switzerland), and dialyzed against Tris buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 8.0) for 24 h. The resultant proteins were separated by SDS-PAGE, and visualized with Coomassie Bright Blue R250. The concentrations of purified soluble proteins were quantified by BCA method (Smith et al., 1985).

Structure Analysis and Interaction Prediction of Protein PepTL and Vsm

The protein domains of PepTL and Vsm were predicted by the simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/). The presumed structures of both PepTL and Vsm were modeled by using the prediction algorithm I-TASSER (http://zhanglab.ccmb.med.umich.edu/) and displayed by Jmol Viewer (version 14.4.4). The interaction between PepTL and Vsm was *in silico* performed and displayed by AutoDock Tools version 1.5.6.

In vitro Cleavage of Vsm in ECPs with rPepTL

The *in vitro* assay was carried out to assess the proteolytic activity of rPepTL. Three concentrations of rPepTL (5, 10 and 20 μg/μL) were screened in preliminary experiment, and 10 μg/μL of protein was determined to be an optimal concentration. For the experimental group, 50 μL of rPepTL solution containing 10 mmol/L $ZnCl_2$ was mixed with 50 μL of ECPs P_{28} (20 μg/μL) at 20°C for 1 h. Fifty microliter of ECPs P_{28} and ECPs P_{10} was used as negative and positive control, respectively. After the reaction, the mixtures were separated by SDS-PAGE and analyzed for reaction products as described in Section Analysis of Extracellular Products (ECPs) From *V. splendidus* JZ6 at Different Temperatures.

Construction of a *pepTL* Gene Deletion Mutant of *V. splendidus* JZ6

The suicide plasmid, pK18mobsacB-Ery (Wang et al., 2015), was used for deletion of the region encoding the active site in PepTL. Two pairs of primers (P13/P14 and P15/P16) were used to amplify the upstream and downstream DNA sequences of the target region from strain JZ6 genomic DNA. The PCR fragments of 170 and 220 bp were purified and fused in an overlap PCR reaction using primers P13 and P16 (**Table 2**). The fused segment was sequenced and digested with *Xba*I/*Sal*I (NEB, USA), cloned into the same sites of pK18mobsacB-Ery, and then transformed into *E. coli* stains SY327 and S17-1. Suicide plasmid pK18Ery-*pepTL* was mobilized from *E. coli* S17-1 into *V. splendidus* JZ6 by intergeneric conjugation.

After mating, cells were spread on 2216E plates containing erythromycin (25 μg/mL) to select for clones in which the suicide vector pK18Ery-*pepTL* had been integrated into the JZ6 genome via a single crossover event. The mutants were then grown at 20°C with shaking in 2216E medium without any antibiotics for 8 h. To select mutants in which a second recombination event had occurred, the culture was diluted and spread on 2216E medium containing 10% sucrose and incubated at 20°C for 24–36 h. Single colonies were replica-plated onto 2216E and erythromycin containing 2216E plates, and colonies sensitive to erythromycin (25 μg/mL) were collected and confirmed by PCR followed by DNA sequencing.

Statistical Analysis

The significant differences among groups were subjected to one-way analysis of variance (one-way ANOVA) and multiple comparisons by using SPSS 16.0 program. Statistically significant difference was designated at $p < 0.05$ or $p < 0.01$.

RESULTS

Vsm in ECPs from Strain JZ6 Is Present in Two Forms

In our previous study, we demonstrated that Vsm from strain JZ6 was presented in the extracellular protein fraction in two forms differing in their molecular weight (Liu et al., 2016). In the present study, the differences in ECPs from strain JZ6 cultured at different temperatures (4, 10, 16, 20, 24, and 28°C) were monitored by SDS-PAGE. Two bands (JZE1:∼35kDa and JZE2: ∼45kDa) were observed in these ECPs, and their relative quantities were assessed with Image Lab Software (Bio-Rad, USA). At 4°C, JZE1 was the dominant band observed in ECPs P_4, with an intensity 5.88-fold that of JZE2 (∼45kDa) (**Figures 1A,C**). As the temperature increased, the relative quantity of JZE1 increased at 10°C (1.54-fold of JZE1 in ECPs P_4) and then decreased from 0.89- to 0.11-fold of the level of JZE1 in ECPs P_4 as the temperature increased from 16 to 28°C (**Figures 1A,C**). The concentration of JZE2 in ECPs increased 0.42-, 0.49-, 0.51-, 0.57-, and 0.67-fold that of JZE1 in ECPs P_4 at 10, 16, 20, 24, and 28°C, respectively (**Figures 1A,C**). Western-blotting with a monoclonal antibody against JZ6 Vsm also revealed two main bands in ECPs of strain JZ6 (**Figure 1B**), and their intensity changes were consistent with the tendency observed in the SDS-PAGE assay.

Two bands of JZE1 and JZE2 were analyzed by LC-ESI-MS/MS mass spectrometry. Eight and eleven specific peptide fragments detected in JZE1 and JZE2, respectively, and all of them were identified as Vsm from strain JZ6. The eight specific peptide fragments from JZE1 were located in the M4 domain,

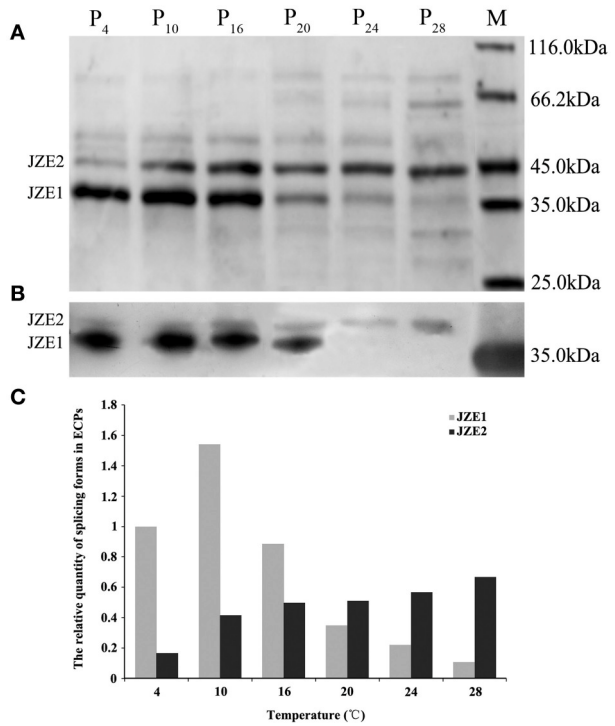

FIGURE 1 | Extracellular products (ECPs) of *V. splendidus* JZ6 cultured at 4, 10, 16, 20, 24, and 28°C, respectively, analyzed by SDS-PAGE and Western-blotting. (A) P_4, ECPs of JZ6 cultured at 4°C. P_{10}, ECPs of JZ6 cultured at 10°C. P_{16}, ECPs of JZ6 cultured at 16°C. P_{20}, ECPs of JZ6 cultured at 20°C. P_{24}, ECPs of JZ6 cultured at 24°C. P_{28}, ECPs of JZ6 cultured at 28°C. Lane M, protein molecular weight standards. **(B)** Western-blotting detected the extracellular metalloprotease (Vsm) of *V. splendidus* with monoclonal antibody of JZ6 Vsm diluted 1:1000. **(C)** The relative quantities of two bands assessed with Image Lab Software.

and so were eight of the eleven fragments from JZE2. The other three peptides from JZE2 were assigned to the PepSY domain (**Table 3**). Thus, JZE1 was generated from JZE2 by cleavage.

The *Vsm* and *pepTL* mRNA Levels Decrease with Increasing Temperatures

The relative expression levels of *vsm* mRNA at 4, 10, 16, 20, 24°C were significantly higher ($p < 0.01$) than that at 28°C, with the highest level at 4°C (836.82-fold of that at 28°C), and then gradually decreased with the temperature increasing from 10 to 24°C (**Figure 2**). Similarly, the relative expression level of *pepTL* was highest at 4°C (191.38-fold, $p < 0.01$), and gradually decreased from 29.25-fold ($p < 0.01$) at 10°C to 2.59-fold ($p < 0.05$) at 24°C, finally reaching its lowest level at 28°C (**Figure 2**).

Modeling the Structure of PepTL and Vsm

PepTL is predicted to perform its proteolytic activity as a homodimer (**Figure 3A**). A structure similar to that of *S. typhimurium* peptidase T (PepT, PDB No.1fno), was predicted with 10 α-helix and 15 β-sheet elements, but the conformation of dimerization domain from Ala176 to Phe291 in PepTL was significantly different from that of PepT (Glu206-Asn321) (**Figure 3B**). The 3D structure of Vsm consisted of the FTP domain, PepSY domain and two M4 domains (**Figure 3C**). *In silico* analysis of interaction by AutoDock Tools revealed that the homodimer of PepTL could tightly bind to Vsm, and the binding sites were located in the gap between PepSY and M4 domains of Vsm (**Figure 3C**).

In vitro Proteolytic Activity of PepTL

Three protein segments of Vsm containing different functional domains were expressed to confirm the composition and molecular weight of JZE1 and JZE2. Segment 1 of Vsm (rVsmP1) consisted of 478 amino acids, containing FTP, PepSY and two Peptidase_M4 domains with a predicted molecular weight of ∼51.50 kDa (**Figure 4A**). Segment 2 (rVsmP2) was composed of PepSY and two Peptidase_M4 domains (389 amino acids with a predicted molecular weight of ∼44.93 kDa). Segment 3 (rVsmP3) only had two Peptidase_M4 domains (312 amino acids), and its predicted molecular weight was ∼34.90 kDa. These recombinant proteins were expressed in *E. coli* BL21 (DE3) and purified by Ni Sepharose column. After SDS-PAGE analysis, the molecular weights of purified proteins were consistent with their theoretical values (**Figure 4B**), indicating that rVsmP3 shared a similar protein size with JZE1, and rVsmP2 had the same domain architectures as JZE2.

The full-length ORF of *pepTL* was of 1107 bp, encoding a polypeptide of 368 amino acids with a predicted molecular weight of ∼39.30 kDa. Four different domain architectures of PepTL were analyzed by SMART, and the Peptidase_M20 domain was considered as the PepTL with *E*-value 3.8×10^{-13}. Recombinant PepTL was expressed in *E. coli* BL21 (DE3) utilizing pET22b (+). After 24 h IPTG induction, the whole cell lysate was analyzed by SDS-PAGE and a distinct band was revealed with a molecular weight of ∼40 kDa (**Figure 5**, Lane 2) which was in agreement with the predicted molecular mass. The purified and refolded rPepTL was of the same molecular weight (**Figure 5**, Lane 3). After incubation of ECPs P_{28} with rPepTL at 20°C for 1 h, the intensity of the band corresponding to JZE2 dramatically decreased and the intensity of the JZE1 band increased (**Figure 6**). Furthermore, the quantity of rPepTL was less than that in the rPepTL only group (**Figure 6**). The changes in the intensity of the bands corresponding to JZE1 were also observed in immunoblotting with the monoclonal antibody of Vsm (**Figure 6**). No autocatalytic activity of ECPs P_{10} and P_{28} was observed.

Participation of PepTL in Activation of Vsm *In vivo*

The function of PepTL was also tested by deleting the *pepTL* gene from strain JZ6. ECPs from both wide type and *ΔpepTL* mutant were collected and subjected to SDS-PAGE post-culture at 10 and 28°C. Compared with the wild type (**Figure 7**, Line 1 and 2), the bands for Vsm in ECPs were not significantly different when the *ΔpepTL* mutant was cultured at 10 and 28°C (**Figure 7**, Line 3 and 4). The banding patterns of JZE1 and

TABLE 3 | The peptide fragments identified by mass spectrum analysis.

Source	Peptide fragment	Protein	Location[a]	Domain[b]
JZE1				
	AAADMGYVVADVEDAFNTVGVNASCGVTPPTGNVLTK	Vsm	477–513	M4
	GNVDWVVGSDIFK	Vsm	382–394	M4
	KGFEIFTVANQLYWTANSTFDAGACGVAK	Vsm	448–476	M4
	SEGGLRYFDQPSK	Vsm	395–407	M4
	SIDHASQYYDGLNVHLSSGVYNR	Vsm	411–433	M4
	YEYGSDFPSFPIDK	Vsm	212–225	M4
	YFDQPSK	Vsm	401–407	M4
	YINGAYSPLNDAHYFGNVVFDMYK	Vsm	267–290	M4
JZE2				
	AAADMGYVVADVEDAFNTVGVNASCGVTPPTGNVLTK	Vsm	477–513	M4
	DGRSIDHASQYYDGLNVHLSSGVYNR	Vsm	408–433	M4
	EWMNTSPLTFQLTMR	Vsm	291–305	M4
	GKKSIENKNAKLMVRLDENQTA	Vsm	136–157	PepSY
	GNVDWVVGSDIFKSEGGLR	Vsm	382–400	M4
	SIDHASQYYDGLNVHLSSGVYNR	Vsm	411–433	M4
	TTRYEYGSDFPSFPIDK	Vsm	209–225	M4
	YFDQPSK	Vsm	401–407	M4
	YFIDATTGDVLQKWNGLNHAK	Vsm	177–197	PepSY
	YINGAYSPLNDAHYFGNVVFDMYK	Vsm	267–290	M4
	YLVDFFIASSMPERPF	Vsm	161–176	PepSY

[a] *The location of peptide fragments in Vsm protein. The full-length of Vsm protein was 607 amino acids.*
[b] *The identified peptide fragments located in domains of Vsm.*

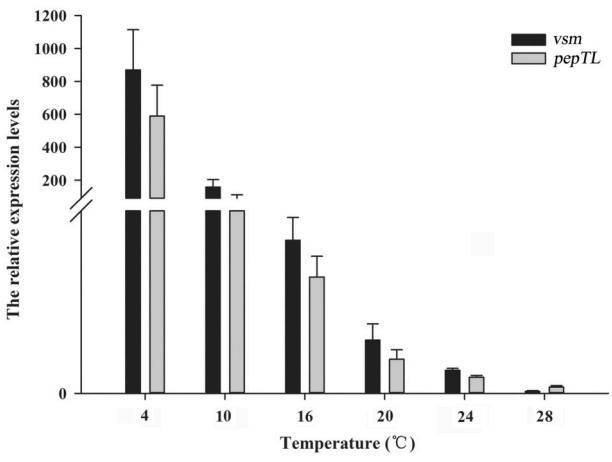

FIGURE 2 | Relative *vsm* and *pepTL* mRNA expression levels in strain JZ6 at 4, 10, 16, 20, 24, and 28°C as determined by qRT-PCR.

JZE2 were completely consistent with ECPs P_{28} of wild type, indicating that *pepTL* gene was crucial for the transformation from JZE2 to JZE1.

DISCUSSION

V. splendidus JZ6 was isolated from a diseased Yesso scallop (*P. yessoensis*) during winter and shown to cause higher mortality of scallops at 10°C than at 28°C (Liu et al., 2013). Its unique biological properties, such as the regulation of its pathogenicity by temperature distinguish this strain from other *V. splendidus* strains and *Vibrio* spp. (Liu et al., 2013, 2016). Our previous studies indicated that the virulence of strain JZ6 was mainly determined by the quantity and toxicity of Vsm, which was dependent on the temperature (Liu et al., 2016). Although comparative transcriptome analysis was able to demonstrate temperature regulation of Vsm expression, it did not reveal the mechanism of its post-translational modifications.

Vsm of *V. splendidus* strain JZ6 shared high sequence and structural similarities with thermolysin-like metalloproteases (TLPs) in M4 family, such as vibriolysin of pathogenic *Vibrio* spp. (Peters et al., 1982; Miyoshi et al., 1987; Lutfullah et al., 2008; Iqbal et al., 2011). Extracellular metalloproteases are usually synthesized as inactive zymogens with the catalytic domain inhibited by a propeptide. Removal of the propeptides is dependent on other proteases or an autocatalytic process (Nickerson et al., 2008; Gao et al., 2010). Unlike most of TLPs, a typical autocatalytic process was not observed during the maturation of Vsm, and another peptidase was predicated to involve in this process of Vsm. In the present study, two forms (JZE1 and JZE2) of Vsm were identified in ECPs of strain JZ6. JZE1 only included the activity region (peptidase_M4 domain) of Vsm with a molecule weight of ∼35kDa. JZE2 was containing the activity region of Vsm and the PepSY domain (∼45kDa). JZE1 was expressed at higher levels in ECPs

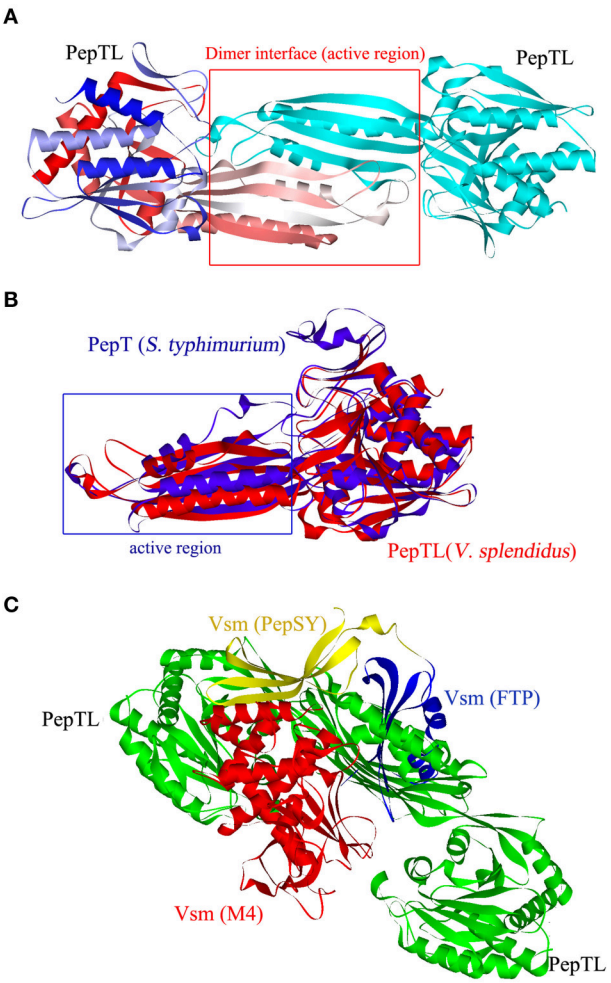

FIGURE 3 | Three dimensional structures of PepTL and Vsm protein. **(A)** Ribbon diagram of the PepTL homodimer. **(B)** The superposition view of *S. typhimurium* PepT (blue) and *V. splendidus* JZ6 PepTL (red) structures. **(C)** The interaction of PepTL and Vsm displayed by AutoDock Tools.

at low temperatures (highest at 10°C), and its concentrations gradually decreased with an increase in temperatures. On the contrary, JZE2 was detected with the highest concentration at 28°C and lowest at 4°C. As a low-temperature pathogenic bacterium, the pathogenicity of strain JZ6 and the toxicity of Vsm at 10°C were higher than those at 28°C (Liu et al., 2013, 2016). These observations indicated that JZ1 containing only M4 domain was likely the key modular part of Vsm with toxicity.

PepTL was annotated as a peptidase T-like protein of *V. splendidus* by BlastP analysis. It displayed a parallel expression pattern with Vsm at 10 and 28°C in strain JZ6 (Liu et al., 2016). PepTL containes similar secondary structure elements as PepT of *S. typhimurium* (Håkansson and Miller, 2002), thus it possesses

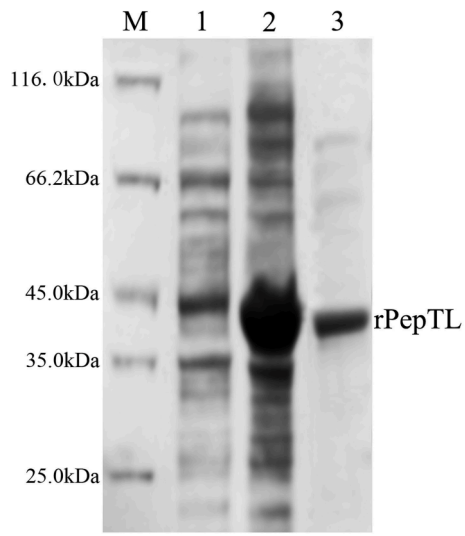

FIGURE 5 | SDS-PAGE analysis of the recombinant PepTL protein (rPepTL). Lane M, protein molecular standard. Lane 1, uninduced rPepTL (the whole cell lysate). Lane 2, induced rPepTL. Lane 3, purified rPepTL.

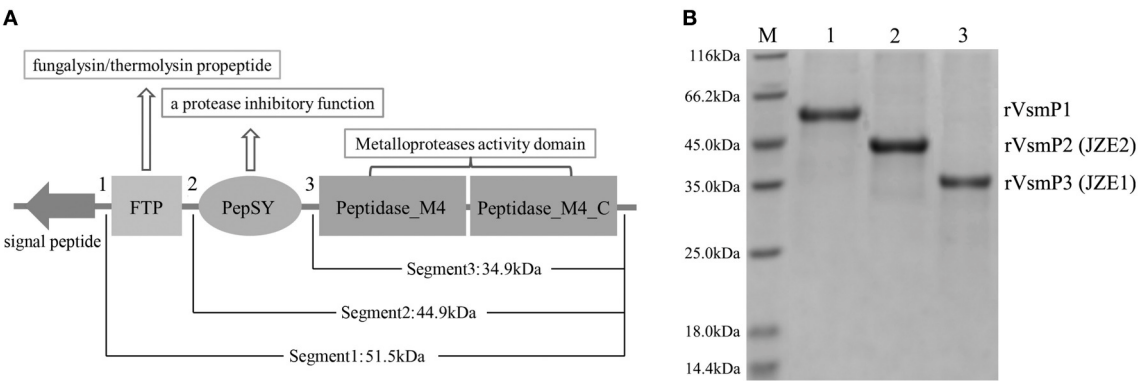

FIGURE 4 | Structure characteristics of the different protein segments of Vsm in *V. splendidus* JZ6. (A) Scheme showing protein domains present in full length of Vsm. **(B)** purified recombinant proteins of the different Vsm forms analyzed by SDS-PAGE. Line M, protein molecular standard. Line 1, rVsmP1. Line 2, rVsmP2. Line 3, rVsmP3.

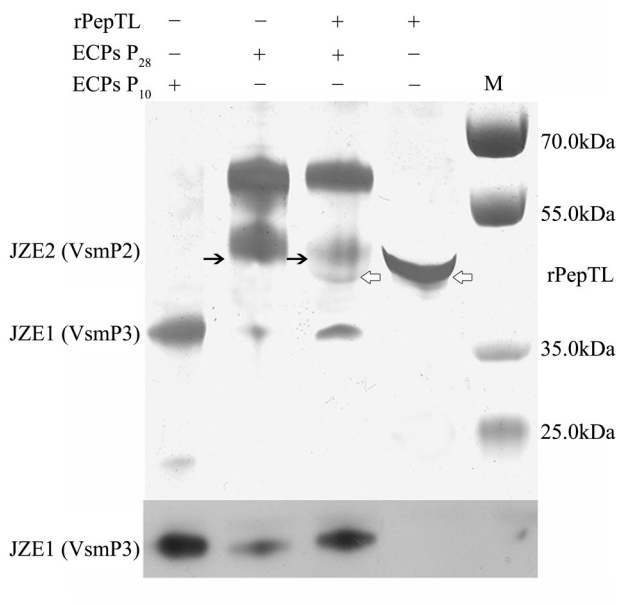

FIGURE 6 | rPepTL-induced cleavage of Vsm in ECPs as determined by SDS-PAGE. Line 1, sample with only ECPs of strain JZ6 cultured at 10°C. Line 2, sample with only ECPs of strain JZ6 cultured at 28°C. Line 3, sample with both rPepTL and ECPs of strain JZ6 cultured at 28°C. Line 4, sample with only rPepTL. Line M, protein molecular weight standards.

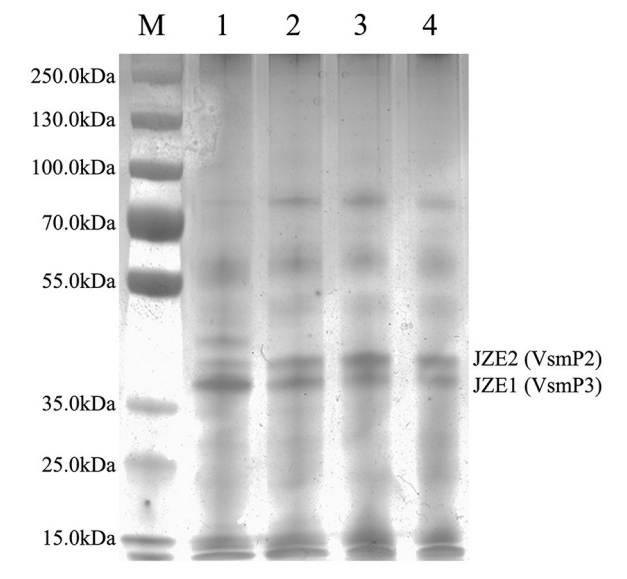

FIGURE 7 | ECPs of *V. splendidus* JZ6 and *ΔpepTL* mutant cultured at 10 and 28°C, respectively, analyzed by SDS-PAGE. Lane M, protein molecular weight standards. Line 1, ECPs of JZ6 cultured at 10°C. Line 2, ECPs of JZ6 cultured at 28°C. Line 3, ECPs of *ΔpepTL* mutant cultured at 10°C. Line 4, ECPs of *ΔpepTL* mutant cultured at 28°C.

the structural base of peptidase T proteins although the tertiary structures of PepTL and PepT are different from each other in their dimerization domain according to molecular overlay analysis. The conformation of PepTL from Ala176 to Phe291 was predicted to deviate significantly from the corresponding motif (Glu206-Asn321) of PepT. This observation suggests a different function of PepTL compared with PepT (Lindner et al., 2003). The 3D structures of PepTL and Vsm were predicted with homology modeling, and the interaction between PepTL and Vsm was analyzed *in silico* by AutoDock Tools. Although the dimerization domain of PepTL was different from that of PepT, the homodimer was still predicted to exist in PepTL. After the analog calculation, the dimer interface of PepTL was found to closely bind to the Vsm and to fit into the space between PepSY and M4 domains. This result is accordant with the observation of most M20 peptidases which commonly form the homodimers to bind polypeptides in some proteins, and whose active region is situated mainly at the interface between two protein molecules (Lindner et al., 2003). Therefore, PepTL possesses the structural foundation for binding and catalyzing the Vsm cleavage.

In order to determine the proteolytic activity of PepTL, Vsm degradation was analyzed via *in vitro* and *in vivo* experiments. In the *in vitro* reaction, rPepTL degraded JZE2 in ECPs P_{28}, and the concentration of JZE1 increased considerable compared to the negative control. The function of PepTL was further verified with the *ΔpepTL* mutant *in vivo*. Compared with the wild type of strain JZ6, there was no difference in the bands corresponding to Vsm forms in ECPs of the *ΔpepTL* mutant at 10 and 28°C. These results demonstrate that PepTL of strain JZ6 indeed catalyzes the maturation of Vsm by removing the PepSY inhibitor domain from the M4 domain. Furthermore, since the deleted fragment of *pepTL* gene encodes the dimer interface, it is likely that homodimer formation is crucial for the peptidase activity of PepTL.

AUTHOR CONTRIBUTIONS

RL is the first author of this manuscript, who is mainly responsible for the experimental design and most of the experimental results. LQ is the corresponding author, who is responsible for the guidance and the modification of this manuscript. QC is one of coauthors for this manuscript, who is responsible for the experiment of proteins. HZ is one of coauthors for this manuscript, who is responsible for the experiment of microbial genetic operations. LW is one of coauthors for this manuscript, who is responsible for the experiment of structural analysis. LS is the corresponding author, who is responsible for the guidance and the modification of this manuscript.

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DATA REPORT
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Genome Sequence of *Vibrio parahaemolyticus* VP103 Strain Isolated from Shrimp in Malaysia

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Keywords: ***Vibrio parahaemolyticus***, **seafood, genome,** ***Penaeus indicus***, **antibiotic resistance**

BACKGROUND

Vibrio parahaemolyticus is a Gram-negative bacterium that widely inhabits the marine and estuarine environments worldwide (Letchumanan et al., 2014). While the majority of strains isolated from environmental sources are innocuous members of marine microbiota, a small number of *V. parahaemolyticus* strains is capable of causing human illness and often associated with food borne gastroenteritis or diarrhea (Hazen et al., 2015; Raghunath, 2015). This organism has caused the highest number of seafood associated gastroenteritis cases in many countries including United States and Asian countries (Scallan et al., 2011; Newton et al., 2012).

In addition, there have been many reports of multidrug antibiotic resistance in *V. parahaemolyticus* worldwide (Odeyemi and Stratev, 2016). Our dependence on antibiotics to control this bacterial infections in humans, aquaculture, agriculture, veterinary medicine, and clinical setting has resulted in indiscriminate use which in turn led to the emergence of multidrug resistant strains in the biosphere (Letchumanan et al., 2015b, 2016a; Rao and Lalitha, 2015). Multidrug resistant *V. parahaemolyticus* strains have been isolated and detected from shrimp in Thailand (Yano et al., 2014), Malaysia (Al-Othrubi et al., 2011; Sani et al., 2013; Letchumanan et al., 2015a,c), and China (Peng et al., 2010; Xu et al., 2014). Resistance toward clinically used antibiotics will eventually hamper the treatment of bacterial infections in humans and potentially increase the fatality rate (Daniels et al., 2000). Therefore, monitoring *Vibrio* species in aquaculture surroundings is crucial for both human health and the aquaculture industry.

In order to gain better understanding of the multidrug resistance pattern, we studied the genome sequence of *V. parahaemolyticus* VP103 strain which was isolated from our previous study (Letchumanan et al., 2015a). *V. parahaemolyticus* VP103 strain was isolated from *Penaeus indicus* (Banana prawn) and originated from a fishery market in Malaysia. This strain exhibited multidrug resistance profiles toward 5/14 antibiotics tested. Based on the antibiotic susceptibility phenotype, the strain exhibited multiple-antibiotic resistance toward ampicillin, 3rd generation cephalosporins (cefotaxime and ceftazidime), and aminoglycosides (amikacin and kanamycin) (Letchumanan et al., 2015a).

This is a worrying situation as the antibiotic resistant profiles shown by *V. parahaemolyticus* VP103 include the recommended antimicrobial agents used in treatment of *Vibrio* spp. infections, including 3rd generation cephalosporin, fluoroquinolones, aminoglycosides, tetracycline, gentamicin, trimethoprim/sulfamethoxazole (Daniels and Shafaie, 2000; Shaw et al., 2014). Therefore, the whole genome sequence of *V. parahaemolyticus* VP103 was studied with respect to the multidrug resistance profiles to gain a better understanding of the antibiotic resistant patterns. The availability of this genome sequence of *V. parahaemolyticus*

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VP103 will aid as a basis for further in-depth analysis of the antibiotic resistance profile of *V. parahaemolyticus*.

MATERIALS AND METHODS

Genome Sequencing and Assembly and Annotation

Genomic DNA of VP103 was extracted using Masterpure™ DNA purification kit (Epicenter, Illumina Inc, Madison, WI, USA) followed by RNase (Qiagen, USA) treatment (Ser et al., 2015; Letchumanan et al., 2016b). The DNA quality was quantified using NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA), and a Qubit version 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). Illumina sequencing library of genomic DNA was prepared using Nextera™ DNA Sample Preparation kit (Illumina, San Diego, CA, USA) and library quality was validated by a Bioanalyzer 2100 high sensitivity DNA kit (Agilent Technologies, Palo Alto, CA) prior to sequencing. The genome of VP103 strain was sequenced on MiSeq platform with MiSeq Reagent Kit 2 (2 × 250 bp Illumina Inc, San Diego, CA, USA). The trimmed sequences were *de novo* assembled with CLC Genomic Workbench version 5.1 (CLC Bio, Denmark).

TABLE 1 | General features of *Vibrio parahaemolyticus* VP103 genome.

	Vibrio parahaemolyticus VP103
Genome size (bp)	4,988,425
Contigs	180
Contigs N_{50} (bp)	508,838
G + C content %	53.37
Protein coding genes	4648
RNA genes	91
rRNA	10
tRNA	81

Genome Annotation

Gene prediction was carried out using Prodigal 2.6, while rRNA and tRNA were analyzed using RNAmmer and tRNAscan SE version 1.21 (Lowe and Eddy, 1997; Lagesen et al., 2007; Hyatt et al., 2010). Gene prediction and annotation were performed using Rapid Annotation Search Tool (RAST, Aziz et al., 2008). Antibiotic resistance genes were analyzed using antibiotic resistance genes-ANNOTation (ARG-ANNOT, Gupta et al., 2014).

RESULTS

Genome Characteristics

The genome of *V. parahaemolyticus* VP103 consists of 4,988,425 bp with a mean genome coverage of 177.8-fold and with an average G + C content of 53.37% (**Table 1**). A total of 4820 genes was predicted of which 4648 genes were identified as protein coding genes. There are 91 RNA genes consisting of 10 rRNAs and 81 tRNAs. This genome sequence data of VP103 strain sequenced under this study has been deposited in DDBJ/EMBL/GenBank under Accession No. LBDB00000000. The version described in this paper is the first version, LBDB01000000. The genome sequences data are available in FASTA, annotated GenBank flat file, graphical, and ASN.1 formats.

Virulence and Antimicrobial Resistance Genes

The analysis obtained from RAST server revealed 573 subsystems (**Figure 1**). The annotated genome has 97 genes responsible

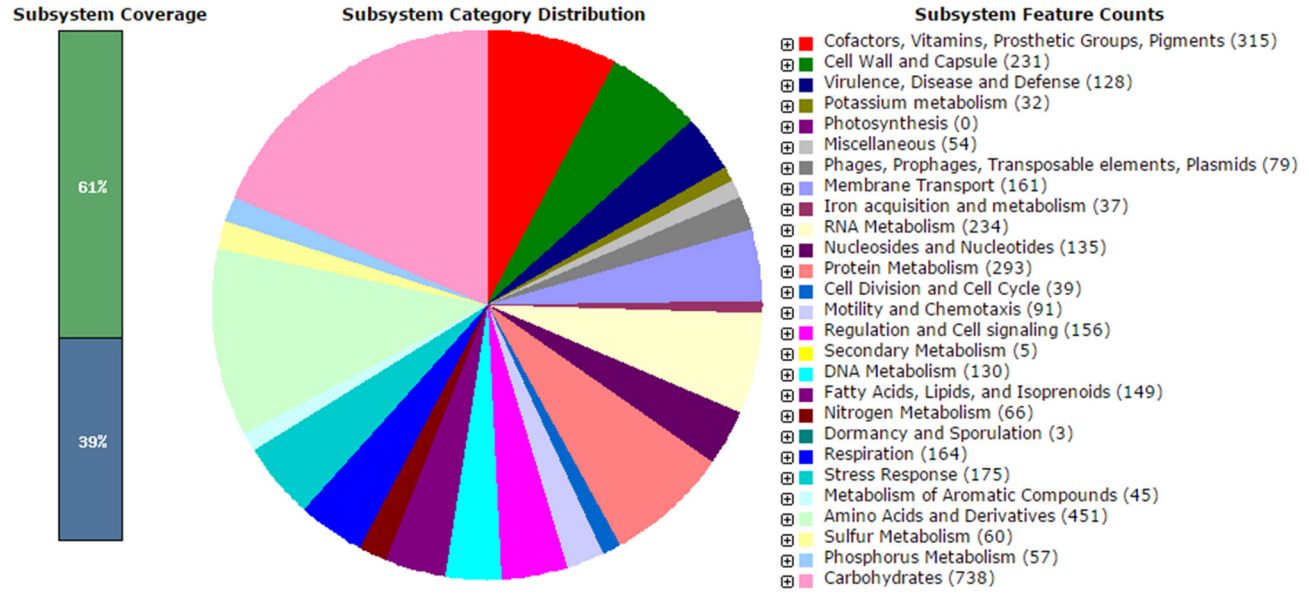

FIGURE 1 | Subsystem category distribution of *Vibrio parahaemolyticus* VP103 (based on RAST annotation server).

for resistance to antibiotic and toxic compounds including 19 genes for multidrug resistance efflux pumps, 4 genes for Beta-lactamase, 4 genes for multiple antibiotic resistance MAR locus, and 2 genes for aminoglycosides adenylyltransferase. The hemolysin gene was present in *V. parahaemolyticus* VP103 strain genome. The genome analysis on ARG-ANNOT noted the presences of β-lactam resistant gene, *bla* gene within the genome at 99% similarities when compared to other *V. parahaemolyticus* strains. The phenotypic resistance shown by *V. parahaemolyticus* VP103 toward ampicillin, cefotaxime, and ceftazidime is closely related to the gene coding Beta-lactamase in the genome. The gene coding aminoglycosides adenylyltransferase of *V. parahaemolyticus* VP103 confers resistance phenotypic observed toward amikacin and kanamycin.

Multidrug resistance profile seen in the phenotype and genes of *V. parahaemolyticus* VP103 genome illustrates how extensive antibiotics have been used in the aquaculture. Although antibiotics namely oxytetracycline, tetracycline, quinolone, sulphonamides, and trimethoprim are allowed in the Asian aquaculture industry (Rico et al., 2012; Yano et al., 2014), the extensive use of these antimicrobials has led to emergence of multidrug resistant strains in the environment. As the efficiency of clinical antibiotics has declined, the extensive use of antibiotics in the aquaculture and humans are in distress conditions due to spread of multidrug resistant strains (Letchumanan et al., 2015b). This situation is a definite cause of concern and warrants more stringent surveillance in the use of antibiotics. In summary, the whole genome sequence of *V. parahaemolyticus* VP103 will be useful in future studies to determine antimicrobial resistance and virulence attributes as well as mechanisms that enhance its environmental or host fitness.

AUTHOR CONTRIBUTIONS

The experiments, data analysis and manuscript writing were performed by VL and HS, while KC, BG, and LL provided vital guidance and technical support. LL founded the research project.

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Multiple Cross Displacement Amplification Combined with Gold Nanoparticle-Based Lateral Flow Biosensor for Detection of *Vibrio parahaemolyticus*

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Vibrio parahaemolyticus (*V. parahaemolyticus*) is a marine seafood-borne pathogen causing severe illnesses in humans and aquatic animals. In the present study, multiple cross displacement amplification was combined with a lateral flow biosensor (MCDA-LFB) to detect the *toxR* gene of *V. parahaemolyticus* in DNA extracts from pure cultures and spiked oyster homogenates. Amplification was carried out at a constant temperature (62°C) for only 30 min, and amplification products were directly applied to the biosensor. The entire process, including oyster homogenate processing (30 min), isothermal amplification (30 min) and results indicating (~2 min), could be completed within 65 min. Amplification product was detectable from as little as 10 fg of pure *V. parahaemolyticus* DNA and from approximately 4.2×10^2 CFU in 1 mL of oyster homogenate. No cross-reaction with other *Vibrio* species and with non-*Vibrio* species was observed. Therefore, the MCDA-LFB method established in the current report is suitable for the rapid screening of *V. parahaemolyticus* in clinical, food, and environmental samples.

Keywords: *Vibro parahaemolyticus*, multiple cross displacement amplification, lateral flow biosensor, MCDA-LFB, limit of detection

INTRODUCTION

Vibrio parahaemolyticus (*V. parahaemolyticus*) is a Gram-negative halophilic bacterium that is widely distributed in marine, estuarine, and coastal environments (Letchumanan et al., 2014). The organism is a major food-borne pathogen and frequently isolated from a variety of raw seafoods, such as shellfish, oysters, shrimp, crab, fish and lobster (Wang R. et al., 2015; Letchumanan et al., 2016). In humans, *V. parahaemolyticus* is able to cause acute gastroenteritis after the consumption of raw, undercooked or mishandled seafood (Su and Liu, 2007). The typical clinical symptoms of *V. parahaemolyticus* infection are abdominal pain and acute dysentery, accompanied by fever, chills, headache, nausea, vomiting, diarrhea, and water-like stools (Shimohata et al., 2010). In rare

cases, the bacterium is responsible for ear infection, wound infection, or septicaemia that may be life-threatening to populations belonging to special at-risk groups, such as people with immune disorders or liver disease (Centers for Disease Control and Prevention, 2005). In aquatic animals, *V. parahaemolyticus* has the ability to cause serious illnesses in shellfish, fish and penaeid shrimp, resulting in significant losses in aquaculture industries (Tran et al., 2013).

Two hemolysins, thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH), are major virulence factors for *V. parahaemolyticus* (Letchumanan et al., 2015). Most *V. parahaemolyticus* strains in seafood samples and environment do not harbor these two hemolysin genes, but virulent strains are often found within larger populations of avirulent strains (Su and Liu, 2007; Velazquez-Roman et al., 2012). Since avirulent and virulent isolates have similar growth characteristics, it is difficult to distinguish them phenotypically and therefore, the presence of *V. parahaemolyticus* in general has been as an indicator of seafood contamination.

Traditionally, culture and biochemical methods for identification and detection of *V. parahaemolyticus* from seafood samples can take more than 3 days to complete (Cai et al., 2006). Although PCR-based assays offer more rapid detection, they require instrumentation that might not be readily available in many settings (Levin, 2006; Letchumanan et al., 2014; Law et al., 2015). Multiple cross displacement amplification (MCDA), a novel nucleic acid amplification technique, has been applied in detecting many bacterial agents (Wang Y. et al., 2015; Wang et al., 2016b,c,d). MCDA assay was conducted under isothermal conditions (60–65°C), thus a simple heater or water bath that maintained a uniform temperature was sufficient. MCDA methods are simple, rapid, highly specific and sensitive, and yield amplifcons from as few as three bacterial cells. Detection of these amplicons can be achieved with disposable lateral flow biosensors (LFB; Wang et al., 2016b,d).

In the current report, a MCDA-LFB assay was established for the rapid detection of *V. parahaemolyticus* strains carrying *toxR* gene (*V. parahaemolyticus*-specific gene; Kim et al., 1999). The analytical sensitivity and specificity were determined in pure cultures and in spiked oyster samples.

TABLE 1 | Bacterial strains used in this study.

Bacteria	Strain no. (source of strains) [a]	No. of strains
Vibrio parahaemolyticus	ICDC-NVP001	1
	Isolated strains	99
Vibrio vulnificus	ATCC27562	1
	Isolated strains	4
Vibrio cholerae	ATCC14035	1
	Isolated strains	4
Vibrio mimicus	Isolated strains	1
Vibrio fluvialis	Isolated strains	1
Vibrio alginolyticus	Isolated strains	1
Plesiomonas shigelloides	Isolated strains	1
Aeromonas hydrophila	Isolated strains	1
Enterohemorrhagic E. coli	EDL933	1
Enteropathogenic E. coli	Isolated strains	1
Enterotoxigenic E. coli	Isolated strains	1
Enteroaggregative E. coli	Isolated strains	1
Enteroinvasive E. coli	Isolated strains	1
Shigella dysenteriae	Isolated strains	1
Shigella boydii	Isolated strains	1
Shigella flexneri	Isolated strains	1
Shigella sonnei	Isolated strains	1
Salmonella	Isolated strains	1
Enterococcus faecalis	ATCC35667	1
Enterococcus faecium	Isolated strains	1
Listeria monocytogenes	EGD-e	1
Listeria ivanovii	ATCCBAA-678	1
Listeria grayi	ATCC25402	1
Listeria innocua	Isolated strains	1
Listeria welshimeri	Isolated strains	1
Listeria seeligeri	Isolated strains	1
Yersinia enterocolitica	ATCC23715	1
Enterobacter cloacae	Isolated strains	1
Bntorobater sakazakii	Isolated strains	1
Bacillus cereus	Isolated strains	1
Campylobacter jejuni	ATCC33291	1
Pseudomonas aeruginosa	Isolated strains	1
Staphylococcus aureus	Isolated strains	1
Staphylococcus epidermidis	Isolated strains	1
Staphylococcus saprophyticus	Isolated strains	1
Klebsiella pneumoniae	Isolated strains	1

[a]*ATCC, American Type Culture Collection; ICDC, National Institute for Communicable Disease Control Disease Control and Prevention, Chinese Center for Disease Control and Prevention.*

MATERIALS AND METHODS

Reagents and Instruments

Rabbit anti-fluorescein antibody (anti-FITC Ab) and biotinylated bovine serum albumin (biotin-BSA) were purchased from the Abcam Co., Ltd. (Shanghai, China). Streptavidin-immobilized 30-nm gold nanoparticles (SA-G) was purchased from the Resenbio Co., Ltd. (XiAn, China). Membrane backing materials, sample and conjugate pads, nitrocellulose membrane (NC), and absorbent pads were purchased from the Jie Yi Biotechnology Co., Ltd. (Shanghai, China). The Loopamp kits were purchased from Eiken Chemical Co., Ltd. (Beijing, China). QIAamp DNA Mini Kit (QIAamp DNA minikits; Qiagen, Hilden, Germany) was purchased from Qiagen Co., Ltd. (Beijing, China). The visual detection reagent (Hydroxynaphthol blue, HNB) was purchased from BeiJing-HaiTaiZhengYuan Technology Co., Ltd. (Beijing, China).

Preparation of Gold Nanoparticle-Based Dipstick Biosensor

The dry-reagent strips (4 mm × 60 mm) were prepared as previously described with some modifications (Wang et al., 2016b,d). In brief, the sample pad, conjugate pad, NC membrane and absorbent pad were laminated onto a plastic adhesive backing card. The anti-FITC Ab (0.15 mg/ml) and biotin-BSA

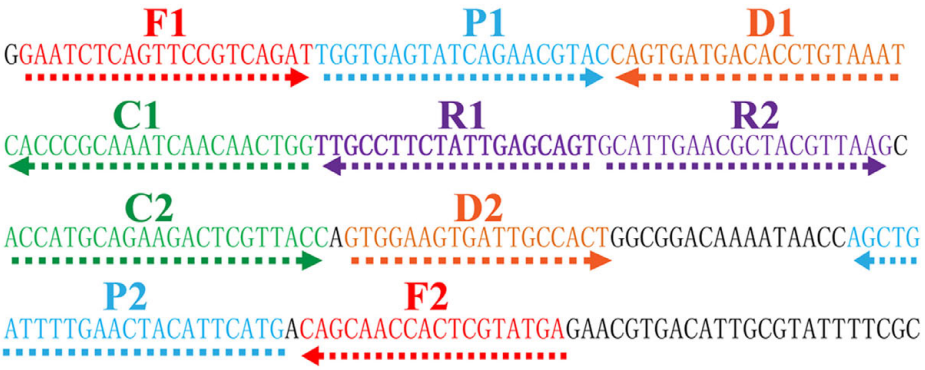

FIGURE 1 | Location and sequence of *toxR* gene (*Vibrio parahaemolyticus*-specific gene) used to design multiple cross displacement amplification primers. The nucleotide sequence of the sense strand of *toxR* gene was shown. Right arrows and left arrows indicate sense and complementary sequences that were used.

TABLE 2 | The primers used in this study.

Primers[a]	Sequences and modifications (5′-3′)	Length[b]	Gene
F1	GAATCTCAGTTCCGTCAGAT	20 nt	*toxR*
CP1	CCAGTTGTTGATTTGCGGGTGTGGTGAGTATCAGAACGTAC	41 mer	
CP1*	Biotin-CCAGTTGTTGATTTGCGGGTGTGGTGAGTATCAGAACGTAC	41 mer	
C1	CCAGTTGTTGATTTGCGGGTG	21 nt	
C1*	FITC-CCAGTTGTTGATTTGCGGGTG	21 nt	
D1	ATTTACAGGTGTCATCACTG	20 nt	
R1	ACTGCTCAATAGAAGGCAA	19 nt	
R2	GCATTGAACGCTACGTTAAG	20 nt	
D2	GTGGAAGTGATTGCCACT	18 nt	
C2	ACCATGCAGAAGACTCGTTACC	22 nt	
CP2	ACCATGCAGAAGACTCGTTACCCATGAATGTAGTTCAAAATCAGCT	46 mer	
F2	TCATACGAGTGGTTGCTG	18 nt	

[a]*CP1*, 5′-labeled with biotin when used in MCDA-LFB assay; C1*, 5′-labeled with FITC when used in MCDA-LFB assay.* [b]*nt, nucleotide; mer, monomeric.*

(2.5 mg/ml) conjugates were sprayed onto NC membrane to form the test line (TL) and control line (CL), with each line separated by 5 mm. SA-G in 0.01M PBS (PH 7.4) was deposited on the conjugate pad of the strip. The assembled cards were cut into 4-mm wide strips (Deli No. 8012). The assembled biosensors were packaged in a plastic box containing a desiccant gel and stored at the room temperature.

Bacterial Strains and Genomic Template Preparation

The strains employed in this study (**Table 1**) were stored in 10% (w/v) glycerol broth at −70°C. The *Vibrio* isolates were cultured three times on thiosulfate citrate bile salt sucrose agar (TCBS agar, Eiken Chemical) at 35°C and the non-*Vibrio* strains were cultured three times on nutrient agar plate at 37°C. Genomic DNA was extracted from all culture strains using the QIAamp DNA Mini Kit according to the manufacturer's instructions and quantified using a Nano drop ND-1000 instrument (Calibre, Beijing, China). *V. parahaemolyticus* ICDC-NVP001 was serially diluted (10 ng, 10 pg, 10 fg, 1 fg, and 0.1 fg) for sensitivity analysis of *V. parahaemolyticus*-MCDA-LFB detection.

Design of MCDA Assay Primers

The MCDA primer pairs (F1, F2, CP1, CP2, C1, C2, D1, D2, R1 and R2) were designed using PrimerExplorer V4 (Eiken Chemical, Japan) and primer software PRIMER PREMIER 5.0. All primers were analyzed for hairpin structures and hybrids using the Integrated DNA Technologies design tools[1]. Blast analysis was used to verify that the MCDA primers were specific for *V. parahaemolyticus*. The CP1 (C1+P1) and C1 primers were labeled at their 5′ end with biotin and fluorescein isothiocyanate (FITC), respectively. The sequences, positions and modifications of the primer pairs are displayed in **Figure 1** and **Table 2**. All of the oligomers were synthesized and purified by TsingKe Biotech Co., Ltd. (Beijing, China) at HPLC purification grade.

The Standard MCDA Assay

Multiple cross displacement amplification reactions were carried out in 25 μl amplification mixtures as previous studies (Wang Y. et al., 2015; Wang et al., 2016b,c,d). Briefly, each reaction contained 0.4 μM each of displacement primers F1 and F2, 0.8 μM each of amplification primers C1* and C2, 1.2 μM each

[1]http://www.idtdna.com/pages/scitools

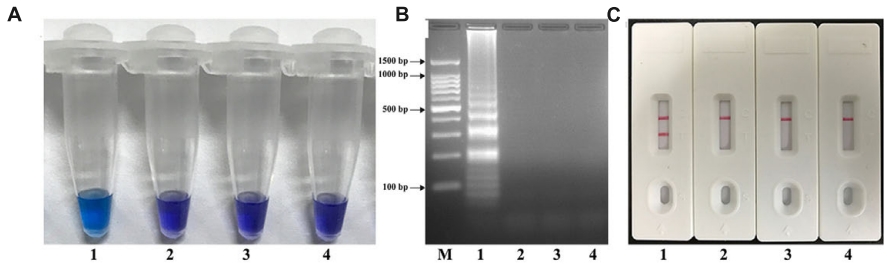

FIGURE 2 | Confirmation and Detection of *V. parahaemolyticus*-MCDA products. (A) By Hydroxynaphthol blue (HNB), amplification products of *V. parahaemolyticus*-MCDA assay were visually analyzed by observation of the color change. **(B)** Agarose gel electrophoresis of *V. parahaemolyticus*-MCDA products was shown. **(C)** Lateral flow biosensor applied for visual detection of *V. parahaemolyticus*-MCDA products. Tuble 1/Lane 1/Biosensor 1, positive amplification of *V. parahaemolyticus* strain (ICDC-NVP001); Tuble 2/Lane 2/Biosensor 2, negative control of *E. faecalis* strain (ATCC35667); Tuble 3/Lane 3/Biosensor 3, negative control of *S. flexneri* strain (ICDC-NPS001); Tuble 4/Lane 4/Biosensor 4, blank control (DW). Lane M, DNA maker DL 100.

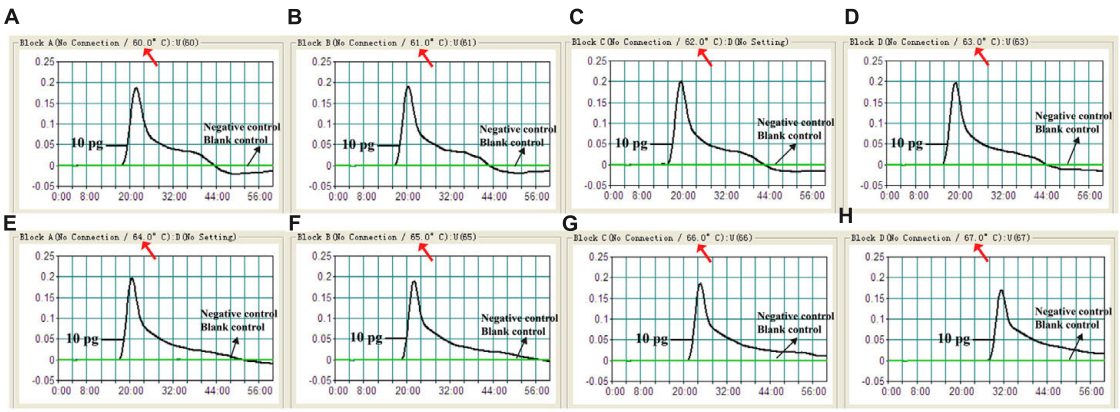

FIGURE 3 | Optimal reaction temperature for *V. parahaemolyticus*-MCDA primer sets. The standard MCDA reactions for detection of *V. parahaemolyticus* were monitored by real-time measurement of turbidity and the corresponding curves of concentrations of DNA were marked in the figures. The threshold value was 0.1 and the turbidity of >0.1 was considered to be positive. Eight kinetic graphs **(A–H)** were generated at various temperatures (60–67°C, 1°C intervals) with target pathogens DNA at the level of 10 pg per reaction. The graphs from **B–E** showed robust amplification.

of amplification primers R1, R2, D1 and D2, 1.2 μM each of cross primers CP1 and CP1*, 2.4 μM cross primer CP2, 12.5 μl 2× reaction mix (Loopamp DNA amplification Kit), 1.25 μl of *Bst* DNA polymerase (10 U) and 1 μl DNA template.

A total of four monitoring methods, including colorimetric indicator (HNB), gel electrophoresis, turbidimeter (LA-320C) and LFB detection, were used for analyzing the amplicons. When employing HNB, the amplified products caused a color change from violet to sky blue, while the negative controls and blank control remained violet. MCDA products were analyzed by 2% agarose gel electrophoresis, the specific ladder of multiple bands should be seen for positive amplifications, but not in the negative and blank controls. By LFB, two visible red lines (TL; CL) should be observed in positive reactions, and only the control lines were visual in negative and blank controls.

The optimal reaction temperature was determined in the range of 60 to 67°C for 60 min. Mixtures with 1 μl genomic template of *Enterococcus faecalis* strains (*E. faecalis*, ATCC35667) and *Shigella flexneri* (*S. flexneri*, ICDC-NPS001) strains were used as negative controls, and mixtures with 1 μl double distilled water (DW) were selected as a blank control.

Specificity and Sensitivity of the *V. parahaemolyticus*-MCDA-LFB Assay

The specificity of *V. parahaemolyticus*-MCDA-LFB was analyzed with DNA templates from 143 bacterial strains (**Table 1**). The assays were repeated at least twice. The limit of detection (LoD), which was tested using serial dilutions (10 ng, 10 pg, 10 fg, 1 fg and 0.1 fg per microliter), was defined by genomic DNA amount of the template. Detection by *V. parahaemolyticus*-MCDA-LFB was compared to that with a colorimetric indicator (HNB), real time turbidity and 2% agarose gel electrophoresis. Three replicates of each dilution were tested.

Optimization the Amplification Time of the *V. parahaemolyticus*-MCDA-LFB Assay

The optimal time for *V. parahaemolyticus*-MCDA-LFB was determined by increasing the reaction time from 10 to 40 min at 10 min intervals. The MCDA products were analyzed using LFB detection and two replicates of each amplification time were determined.

FIGURE 4 | Analytical specificity of *V. parahaemolyticus*-MCDA-LFB assay for different strains. The MCDA reactions were carried out using different genomic DNA templates and were analyzed by means of visual format. Biosensor 1, *V. parahaemolyticus* strain (ICDC-NVP001); biosensors 2–20, nineteen isolated strains of *V. parahaemolyticus*; biosensor 21, *V. Cholerae* strain (ATCC14035); biosensors 22–23, two isolated strains of *V. cholerae*; biosensor 24, *V. vulnificus* (ATCC27562); biosensors 25–26, two isolated strains of *V. vulnificus*; biosensor 27 isolated strain of *V. mimicus*; biosensor 28, isolated strain of *V. fluvialis*; biosensor 29, isolated strain of *V. alginolyticus*; biosensors 30–59, *Plesiomonas shigelloides*, *Aeromonas hydrophila*, *Enteropathogenic E. coli*, *Enterotoxigenic E. coli*, *Enteroaggregative E. coli*, *Enteroinvasive E. coli*, *Enterohemorrhagic E. coli*, *Shigella dysenteriae*, *Shigella boydii, Shigella flexneri*, *Shigella sonnei*, *Salmonella*, *Enterococcus faecalis*, *Enterococcus faecium*, *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria grayi*, *Listeria innocua*, *Listeria welshimeri*, *Listeria seeligeri*, *Yersinia enterocolitica*, *Enterobacter cloacae*, *Bntorobater sakazakii*, *Bacillus cereus*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Klebsiella pneumoniae*, biosensor 60, blank control (DW).

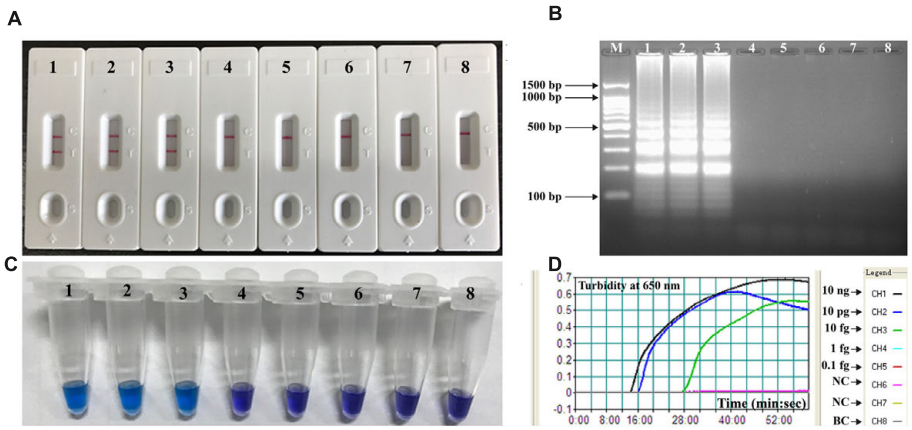

FIGURE 5 | Analytical sensitivity of MCDA-LFB assay using serially diluted genomic DNA with *V. parahaemolyticus* strain ICDC-NVP001. A total of four monitoring techniques, including later flow biosensor **(A)**, gel electrophoresis **(B)**, colorimetric indicator (HNB; **C)** and real time turbidity **(D)**, were applied for analyzing the amplification products. The serial dilutions (10 ng, 10 pg, 10 fg, 1 fg, and 0.1 fg) of target templates were subjected to standard MCDA reactions. Biosensors **(A)**/ Lanes **(B)**/Tubes **(C)**/ Turbidity signals **(D)** 1–8 represented the DNA levels of 10 ng, 10 pg, 10 fg, 1 fg and 0.1 fg per reaction, negative control (10 pg of *E. faecalis* genomic DNA), negative control (10 pg of *S. flexneri* genomic DNA) and blank control (DW). The genomic DNA levels of 10 ng, 10 pg and 10 fg per reaction produced the positive reactions.

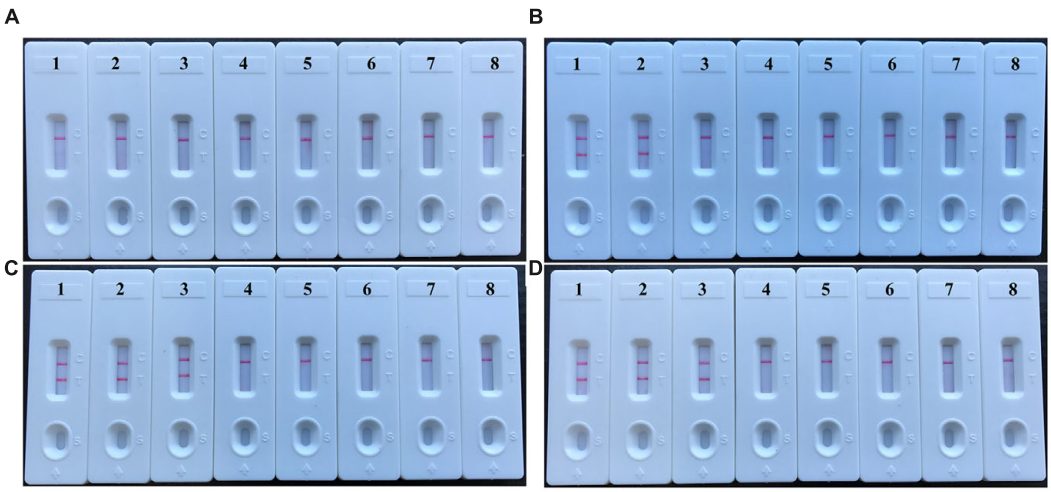

FIGURE 6 | The optimal duration of time required for *V. parahaemolyticus*-MCDA-LFB assay. Four different reaction times (**A**, 10 min; **B**, 20 min; **C**, 30 min; **D**, 40 min) were evaluated and compared at 62°C. Biosensors 1, 2, 3, 4, 5, 6, 7, and 8 represent DNA levels of 10 ng of *V. parahaemolyticus* templates, 10 pg of *V. parahaemolyticus* templates, 10 fg of *V. parahaemolyticus* templates, 1 fg of *V. parahaemolyticus* templates, 0.1 fg *V. parahaemolyticus* templates per tube, negative control (*E. faecalis*, 10 pg per reaction), negative control (*S. flexneri*, 10 pg per reaction) and blank control (DW). The best sensitivity was observed when the amplification lasted for 30 min **(C)**.

V. parahaemolyticus-MCDA-LFB Detection in Oyster Samples

Vibrio parahaemolyticus ICDC-NVP001 was added into oyster samples obtained from local seafood restaurants in Beijing. Only oyster samples that tested negative for *V. parahaemolyticus* according to Kim et al. (1999) were spiked. *V. parahaemolyticus* cultures were serially diluted (10^{-1} to 10^{-8}), and 100-μl aliquots (appropriate dilution:10^{-6}) were placed in triplicate on brain heart infusion (BHI). CFUs were counted after 24 h at 37°C. Simultaneously, 0.1 mL of diluted *V. parahaemolyticus* cultures (10^{-3} to 10^{8}) with known amounts (4.2×10^{5} to 4.2×10^{0} CFU/mL) was inoculated into 900 μl of oyster homogenates and mixed well. The spiked oyster samples were centrifuged at 200 g for 5 min, and the supernatant was placed into a new tube, and the was centrifuged at 18000 g for 5 min. The supernatant was removed and the pellet was subjected to extract genomic DNA, and the DNA templates were eluted in 20 μl of elution buffer. For the MCDA-LFB assay, 1 μl of the extracted DNA was used as template and non-contaminated oyster samples were served as negative control. Three independent assays were performed.

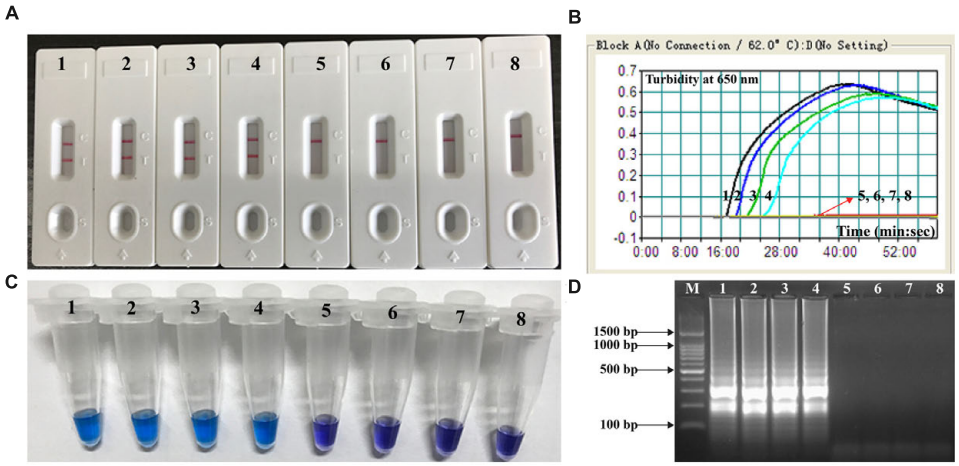

FIGURE 7 | Analytical sensitivity of *V. parahaemolyticus*-MCDA-LFB for detecting target pathogen in oyster samples. Four monitoring techniques, including later flow biosensor **(A)**, real time turbidity **(B)**, colorimetric indicator (HNB; **C)** and gel electrophoresis **(D)**, were applied for analyzing the amplification products. The serial dilutions of target templates were subjected to standard MCDA reactions. Strips **(A)**/Turbidity signals **(B)**/ Tubes **(C)**/Lanes **(D)** 1–8 represented the DNA levels of 2100 CFU, 210 CFU, 21 CFU, 2.1 CFU, 0.21 CFU, and 0.021 CFU per reaction, negative control (non-contaminated oyster sample) and blank control (DW). The genomic DNA levels of 2100 CFU, 210 CFU, 21 CFU, and 2.1 CFU per reaction produced the positive reactions.

RESULTS

Confirmation and Detection of *V. parahaemolyticus* MCDA Products

To determine the availability of *V. parahaemolyticus*-MCDA primers (**Table 2**), *V. parahaemolyticus* MCDA assays with DNA from pure cultures were carried out at 62°C for 1 h. Amplification occurred with DNA from *V. parahaemolyticus* (ICDC-NVP001), but not with *E. faecalis* (ATCC 35667), *S. flexneri* (ICDC-NPS001) DNA and the DW control (**Figure 2**). Therefore, the *V. parahaemolyticus*-MCDA primer set was a good candidate for development of the MCDA-LFB assay for *V. parahaemolyticus* detection.

Optimization of the Temperature for *V. parahaemolyticus*-MCDA-LFB Assay

To verify the optimum amplification temperature, *V. parahaemolyticus* strain (ICDC-NPV001) was used as the positive control at the level of 10 pg per reaction and the reactions were monitored by the real time turbidity method. Carrying out MCDA at temperatures from 60 to 67°C at 1°C increments confirmed that 62°C was the most suitable temperature for amplification as indicated by the kinetics graphs displayed in **Figure 3**. At all tested temperatures, the typical kinetics graphs were generated, with the faster amplification obtained from assay temperatures of 62°C.

Specificity and Sensitivity for *V. parahaemolyticus* Detection by MCDA-LFB

When DNA from the bacteria listed in table 1 was used in MCDA-LFB assays, only the DNA from the *V. parahaemolyticus* strains provided results. DNA from other *Vibrio* species and from all non-*Vibrio* isolates did not lead to the production of detectable amplification products (**Figure 4**). Two red lines, including TL and CL, appeared on the strips for the positive tests, and only a red line (CL) appeared on the biosensors, suggesting negative results for non-*V. parahaemolyticus* isolates and blank control.

Serial dilution of the *V. parahaemolyticus* genomic DNA in triplicate and use in MCDA assays demonstrated that as little as 10 fg of template DNA produced sufficient amplified DNA for detection by the four monitoring methods employed (**Figure 5**). The results obtained using biosensor were in complete accordance with real time turbidity, HNB reagent and agarose gel electrophoresis analysis (**Figure 5**). Moreover, it was possible to detect the amplicons when the amplification reaction was carried out for 30 min (**Figure 6**).

Application of MCDA to *V. parahaemolyticus*-Spiked Oyster Homogenates

The lowest number of *V. parahaemolyticus* CFUs could be detected in 1 mL of spiked oyster homogenate was approximately 4.2×10^2 CFU/mL (~2.1 CFU per reaction; **Figure 7**). The *V. parahaemolyticus*-MCDA assay produced negative results at the concentrations lower than 4.2×10^1 CFU/mL (~0.21 CFU per reaction), negative control and blank control. As seen with MCDA assays utilizing DNA from pure cultures, detection of the amplicons was as sensitive with the LFB method with the other three methods (**Figure 7**).

DISCUSSION

The present study demonstrated that multiple cross displacement amplification combined a lateral flow biosensor (MCDA-LFB) utilizing the *toxR* gene as amplification target is capable of

detection *V. parahaemolyticus* with excellent specificity and sensitivity. The high level of specificity of the assay is likely due to the utilization of *toxR* as target gene. Although the sequences of housekeeping genes (*rpoD*, *rctB*, *pyrH*, *recA*, and *gyrB*) and 16S rRNA gene have also been considered as possible targets, *toxR* is regarded as gene providing the highest level of discrimination according to phylogenetic tree analysis (Yamamoto and Harayama, 1998; Le Roux et al., 2005; Pascual et al., 2010). Then, the assay's specificity was successfully examined using pure cultures and oyster samples. The test was positive for all *V. parahaemolyticus* isolates, but negative for other *Vibrio* spp. and non-*Vibrio* isolates (**Figure 4**). Hence, the *V. parahaemolyticus*-MCDA-LFB method provided a high degree of selectivity for identifying *V. parahaemolyticus* strains.

In additional to its sufficient specificity, the newly established *V. parahaemolyticus*-MCDA-LFB method was able to detect as little as 10 fg of *V. parahaemolyticus* DNA isolated from a pure culture (**Figure 5**). The *V. parahaemolyticus*-MCDA-LFB assay was 25-fold more sensitive than *V. parahaemolyticus*-LAMP method, which only detected 250 fg of template DNA per reaction (Wang et al., 2016a). The detection limit of approximately 4.2×10^2 CFU in 1 mL (∼2.1 CFU per reaction) of oyster homogenate was also lower than that of the *V. parahaemolyticus*-LAMP assay (92 CFU per reaction in spiked oyster samples; **Figure 7**) (Wang et al., 2016a). Although the amplification products could be detected equally with other three methods employed in the current study, LFB is likely the preferred method as reading the results is less subjective and does not require instrumentation.

The *V. parahaemolyticus*-MCDA-LFB assay only required a simple incubation at 62°C for 30 min. A variety of portable user-friendly instruments adapted for MCDA reaction exist, the dry block heater (HDT-100C, HengAo, Tianjing, China) being one example. The portable (18 cm × 22 cm), battery-powered device supports 96 MCDA reactions per assay. The MCDA amplification can be conducted using the commercial isothermal amplification kits (such as Eiken Loopamp kits and NEB Warmstart kits), and an MCDA reaction costs approximately $3.5 USD. The cost of LFB is estimated to be $2 USD per test. Combined with the elimination of labor costs because of the requirements for trained personnel in a certified laboratory, our assay becomes more cost-effective.

The MCDA products were directly analyzed using the biosensor (**Figures 2** and **4–7**). The entire procedure, including specimen processing (30 min), isothermal reaction (30 min) and detection (1 min), could be finished with 65 min. Detection of amplification products with a lateral flow device is not only fast, but also simpler and less error-prone than detection by the other methods employed in the current study (Zhang et al., 2014).

CONCLUSION

A reliable *toxR*-MCDA-LFB assay was successfully established for identification of *V. parahaemolyticus*, causing seafood-borne gastroenteritis in human, which could facilitate investigations to detect the etiological agent of food poisoning, surveillance for *V. parahaemolyticus* contamination in seafood, as well as ecological studies related with regions, practices and environmental factors. The MCDA-LFB approach devised here was sensitive, specific and simple, and did not rely on complicated instrument and expensive reagents. The use of lateral flow biosensor could provide an objective, rapid and easily interpretable readout of the method's results. Therefore, the *toxR*-MCDA-LFB method could be regarded as a valuable tool for the rapid screening of *V. parahaemolyticus* isolates in clinical, food and environmental samples, especially, in resource-limited areas of developing countries during epidemic periods.

AUTHOR CONTRIBUTIONS

YiW, JX, and CY conceived and designed the experiments. YiW, HL, DL, KL, and YaW performed the experiments. YiW, HL, and DL analyzed the data. YiW, HL, DL, KL, and YaW contributed reagents/materials/analysis tools. YiW performed the software. YiW, JX, and CY wrote the paper.

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Diversity of *Vibrio navarrensis* Revealed by Genomic Comparison: Veterinary Isolates Are Related to Strains Associated with Human Illness and Sewage Isolates While Seawater Strains Are More Distant

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Strains of *Vibrio navarrensis* are present in aquatic environments like seawater, rivers, and sewage. Recently, strains of this species were identified in human clinical specimens. In this study, *V. navarrensis* strains isolated from livestock in Germany were characterized that were found in aborted fetuses and/or placentas after miscarriages. The veterinary strains were analyzed using phenotypical and genotypical methods and compared to isolates from marine environments of the Baltic Sea and North Sea. The investigated phenotypical traits were similar in all German strains. Whole genome sequencing (WGS) was used to evaluate a phylogenetic relationship by performing a single nucleotide polymorphism (SNP) analysis. For the SNP analysis, WGS data of two American human pathogenic strains and two Spanish environmental isolates from sewage were included. A phylogenetic analysis of concatenated sequences of five protein-coding housekeeping genes (*gyrB*, *pyrH*, *recA*, *atpA*, and *rpoB*), was additionally performed. Both phylogenetic analyses reveal a greater distance of the environmental seawater strains to the other strains. The phylogenetic tree constructed from concatenated sequences of housekeeping genes places veterinary, human pathogenic and Spanish sewage strains into one cluster. Presence and absence of virulence-associated genes were investigated based on WGS data and confirmed by PCR. However, this analysis showed no clear pattern for the potentially pathogenic strains. The detection of *V. navarrensis* in human clinical specimens strongly suggests that this species should be regarded as a potential human pathogen. The identification of *V. navarrensis* strains in domestic animals implicates a zoonotic potential of this species. This could indicate a potential threat for humans, as according to the "One Health" concept, human, animal, and environmental health are linked. Future studies are necessary to search for reservoirs of these bacteria in the environment and/or in living organisms.

Keywords: *Vibrio* spp., pathogen, genome, diversity, whole genome sequencing, virulence-associated factors

INTRODUCTION

Vibrio navarrensis was first described as a species isolated from sewage and river water in the Spanish province Navarra in 1991 (Urdaci et al., 1991). Later, some strains from the Baltic Sea were reported that differed in some biochemical reactions to the Spanish strains. However, DNA-DNA hybridization and fatty acid analysis revealed them as *V. navarrensis* and they were classified as *V. navarrensis* biotype *pommerensis* (Jores et al., 2007). All *V. navarrensis* strains showed hemolytic activity on blood agar containing different types of erythrocytes, e.g., human, sheep, horse or cattle blood cells (Jores et al., 2003, 2007). The strains were regarded as environmental strains and found during surveys to determine the occurrence and distribution of pathogenic *Vibrio* species like *Vibrio cholerae* (Urdaci et al., 1991) and *Vibrio vulnificus* (Jores et al., 2007) in aquatic environments.

In 2014, the characterization of *V. navarrensis* isolates associated with human illness was reported in a publication of the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia (Gladney and Tarr, 2014). Most of the strains had been in the CDC strain collection for some time and could not be characterized to the species level by phenotypical methods at the time of isolation. By applying multilocus sequence analysis (MLSA), the strains could be placed in a phylogenetic framework and were assigned to the species *V. navarrensis* (Gladney and Tarr, 2014).

Our laboratory received *Vibrio* strains from a veterinary lab in Saxony, Germany, collected between 1990 and 2011 which contained strains that had been isolated from domestic animals like pig and cattle after abortions. The isolates had been found in animals intended for food production in farms of the German state Saxony that does not border on marine environments. The strains were recovered after abortions from placentas and aborted fetuses (Stephan et al., 2002; Schirmeister et al., 2014). At first, some of these strains had been classified as *V. vulnificus* by phenotypic characterization or were assigned *Vibrio* spp., but could be assigned to *V. navarrensis* by sequencing of the *rpoB* gene coding for the ß-subunit of RNA polymerase (Tarr et al., 2007; Adékambi et al., 2009; Dieckmann et al., 2010). As the animal source of these strains is an unusual source of *Vibrio* bacteria, we compared them to environmental *V. navarrensis* strains of the North Sea and Baltic Sea by studying genotypic and phenotypic traits to find out if the veterinary strains may originate from this environment. Additionally, by including published whole genome sequences of two human pathogenic strains (Gladney et al., 2014) the aim of the study was also to find out if the veterinary isolates are related to these strains which could indicate a pathogenic potential.

The occurrence of *V. navarrensis* strains in freshwater and seawater as well as the isolation from humans and domestic animals reveals a broad ecological range of habitats, which may show a wide genetic diversity of the species. For this purpose, WGS data and sequences of housekeeping genes were applied for constructing phylogenetic trees. For the analyses, whole genome sequencing (WGS) data of four published genomes of *V. navarrensis* strains consisting of two human pathogenic strains from the U.S. and two environmental strains from Spain were included. A number of genes associated with virulence in other human pathogens were found in the *V. navarrensis* genome sequence (Gladney et al., 2014). Presence or absence of some of these virulence-associated genes were investigated by genome comparison and confirmed by PCR analyses.

MATERIALS AND METHODS

Bacterial Strains

In total, 19 *V. navarrensis* isolates from German sources and one reference strain (CIP 103381 from Spain, isolate from sewage) were investigated in this study (**Table 1**). Ten strains were obtained from an official veterinary laboratory in Saxony (Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen, Dresden) and were mostly isolated from domestic animals after abortions. The veterinary strains were recovered from aborted fetuses or placentas or both. Two environmental seawater strains of the year 2011 were obtained from the Alfred Wegener Institute, Heligoland, and three environmental strains of the year 2015 from a university hospital (Medizinaluntersuchungsamt und Hygiene, Universitätsklinikum Schleswig-Holstein). *Vibrio navarrensis* biotype *pommerensis* strains came from the strain collection of the German Federal Institute for Risk Assessment (BfR). One environmental strain was isolated from a blue mussel harvested in the Wadden Sea of the North Sea.

TABLE 1 | *Vibrio navarrensis* strains used in this study.

Strain	Year of isolation	Source	Origin
CIP 103381*	1982	Reference strain/sewage	Spain
CH-271**	1996	Seawater	Baltic Sea
CH-280**	1996	Seawater	Baltic Sea
CH-291**	1996	Seawater	Baltic Sea
VN-0392	1999	Cattle/placenta	Saxony
VN-0413	2000	Cattle/placenta	Saxony
VN-0414	2000	Cattle/placenta	Saxony
VN-0415	2009	Cattle/fetus	Saxony
VN-0506	2000	Cattle/placenta	Saxony
VN-0507	2000	Cattle/placenta	Saxony
VN-0508	2000	Pig/placenta	Saxony
VN-0509	2001	Pig/fetus	Saxony
VN-0514	2007	Pig/placenta	Saxony
VN-0515	2007	Pig/placenta	Saxony
VN-0516	2015	Brackish water	Schleswig-Holstein
VN-0517	2015	Seawater	Schleswig-Holstein
VN-0518	2015	Seawater	Schleswig-Holstein
VN-0519	2011	Blue mussel	Lower Saxony
VN-3125	2011	Seawater	Kattegat
VN-3139	2011	Seawater	Kattegat

**Identical to ATCC 51183.*

***Strains of V. navarrensis biotype pommerensis. Strain CH-291 was deposited as DSM 15800.*

Biochemical Characterization

Vibrio navarrensis strains were routinely cultivated in LB medium (Merck KGaA, Darmstadt, Germany) at 37°C. Strains were characterized by biochemical tests used in routine diagnostics of the National Reference Laboratory (NRL) for Monitoring Bacteriological Contamination of Bivalve Mollusks located at the BfR. Tests included growth in 1% peptone water with 0, 3, 8, and 10% NaCl, cytochrome oxidase, sensitivity to the vibriostatic agent O/129 (10 and 150 μg), lysine decarboxylase, arginine dihydrolase, ornithine decarboxylase, nitrate reductase (all supplemented with 1% NaCl), and utilization of a number of carbohydrates (Farmer et al., 2003). Phenylalanine deamination was tested on phenylalanine agar IDM 31 (Mast Diagnostica GmbH, Reinfeld, Germany) supplemented with 1% NaCl. To ensure test results, the following Gram-negative bacterial strains served as controls: *Aeromonas hydrophila* ATCC 7966 (positive control for cytochrome oxidase test, resistance to O/129), *Escherichia coli* DSM 1103 (positive control for oxidative acid production from D-glucose, maltose, D-mannose, and trehalose; negative control for phenylalanine deaminase test, urease test, and citrate degradation test), *Klebsiella oxytoca* DSM 25736 (positive control for oxidative acid production from adonitol, L-arabinose, cellobiose, dulcitol, *myo*-inositol, lactose, D-mannitol, melibiose, raffinose, L-rhamnose, salicin, D-sorbitol, L-sorbose, sucrose, and D-xylose, esculin and citrate degradation tests), *Morganella morganii* DSM 30117 (negative control for oxidative acid production from cellobiose, trehalose, and D-xylose), *Proteus mirabilis* DSM 4479 (positive control for phenylalanine deaminase test, urease test, and H_2S production test; negative control for cytochrome oxidase test, oxidative acid production from adonitol, L-arabinose, dulcitol, *myo*-inositol, lactose, D-mannitol, D-mannose, melibiose, raffinose, L-rhamnose, salicin, D-sorbitol, L-sorbose, and sucrose, esculin degradation test), and *Shigella sonnei* DSM 25715 (negative control for oxidative acid production from D-glucose and maltose, H_2S production test). According to quality control standards of the NRL, functionality of liquid growth media supplemented with 1% NaCl was tested with the following *Vibrio* spp. strains: *V. alginolyticus* DSM 2171 (positive control for growth in 1% peptone water with 3, 6, and 8% NaCl), *V. cholerae* DSM 101014 (positive control for nitrate reductase test, lysine decarboxylase test, ornithine decarboxylase test, indole production test, growth in 1% peptone water with 0% NaCl; negative control for arginine dihydrolase test), *V. cholerae* ATCC 14035 (susceptibility to O/129), *V. furnissii* DSM 14383 (positive control for arginine dihydrolase test; negative control for lysine decarboxylase and ornithine decarboxylase tests), *V. metschnikovii* LMG 4416 (positive control for acetoin production test; negative control for nitrate reductase test, indole production test), and *V. parahaemolyticus* DSM 101031 (negative control for acetoin production test). Biochemical testing was repeated twice.

Hemolytic Activity Tests

Blood agar plates were prepared with Mueller-Hinton agar (Oxoid GmbH, Wesel, Germany) supplemented with 1% NaCl and contained 4% sheep (BfR, Berlin, Germany) or 4% human erythrocytes (German Red Cross, blood donation service, Berlin-Wannsee, Germany). Erythrocytes were washed three times in cold phosphate buffered saline and pelleted for 5 min at 400 × g and 10°C before use. Prior to hemolysis assay, bacteria were cultivated from glycerol stocks on Mueller-Hinton agar plates overnight at 37°C. Four milliliters of Mueller-Hinton broth were inoculated with one single colony and incubated for 12–14 h at 37°C with constant shaking (200 rpm). All culture media were supplemented with 1% NaCl. In order to investigate the hemolytic activity of the strains, 10 μl of the overnight cultures were spotted on a blood agar plate and incubated at 37°C to obtain macrocolonies. Zones of hemolysis around the macrocolonies were visually controlled and scored for up to 72 h. All experiments were performed twice. Strains which did not reveal hemolysis zones on sheep blood were recultivated from glycerol stocks, passaged three times on sheep blood agar plates consisting of Special Blood Agar Base DM101 (Mast Diagnostica GmbH, Reinfeld, Germany) supplemented with 5% defibrinated sheep blood (BfR, Berlin, Germany) and retested on the modified agar plates as described above.

rpoB Sequencing

Bacterial strains were grown overnight and genomic DNA was extracted using the RTP Bacteria DNA Mini Kit from Stratec Molecular, Berlin, Germany, according to the manufacturer's instructions. Analyses of the *rpoB* gene were performed using the PCR primers and sequencing primers described earlier (Mollet et al., 1997; Tarr et al., 2007; Table S1). Briefly, a 984 bp fragment of the *rpoB* coding sequence was amplified using the primers CM32b and 1110F. For sequencing of the amplification products, the PCR primers and two additional primers (1661F, 1783R) were used. PCR conditions were according to the protocol given in Tarr et al. (2007).

Whole Genome Sequencing (WGS) and Single Nucleotide Polymorphism (SNP) Analysis

Genomic DNA of *V. navarrensis* isolates was prepared using the PureLink Genomic DNA Mini Kit (Invitrogen, Karlsruhe, Germany). DNA libraries were generated with the Nextera XT DNA Sample Preparation Kit according to the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). DNA sequencing using the MiSeq Reagent v3 600-cycle Kit (2 × 300 cycles) was performed on the MiSeq benchtop (Illumina Inc., San Diego, CA, USA). For *de novo* assembling of raw sequencing reads, the SPAdes (version 3.5.0) algorithm was used. Initial annotation of the genomes was performed by using the automated Prokaryotic Genome Annotation Pipeline of the NCBI website. Further genetic features and elements of the genomes were identified using the Bacterial Analysis Pipeline and the Phage Search Tool (Zhou et al., 2011). Putative prophage sequences were recorded based on clusters of more than six phage-like genes within a sequence region of the analyzed genome. Therefore, phage-like genes were identified according to their similarity against sequences of the NCBI database. Additionally, the genome annotation of the isolates was analyzed for phage specific terms like "protease," "integrase," and "tail fiber." Predicted prophage regions were assessed according to the recommendations (Zhou

et al., 2011). Initial plasmid prediction was performed by using *de novo* assemblies of genomes with the web-based tool "PlasmidFinder" of the Center for Genomic Epidemiology (Carattoli et al., 2014). Furthermore, contigs with significantly higher sequence coverage than the rest of the genomic contigs were applied to the BLASTN search of the NCBI database and screened for similarities to known plasmids.

The SNP tree was conducted by using CSI Phylogeny 1.4 (Center for Genomic Epidemiology) under default settings and the exclusion of heterozygous SNPs. To identify SNPs, all input sequences were mapped to the *V. navarrensis* 0053-83 genome as reference (JMCF01000001) and screened for relevant nucleotide variations as previously described (Kaas et al., 2014). The following criteria for high quality SNP calling and filtering were chosen: (I) a minimum depth of 10× at SNP positions, (II) a min. relative depth of 10% at SNP positions, (III) a min. distance of 10 bp between SNPs, (IV) a min. SNP quality of 30, (V) a min. read mapping quality of 25, and (VI) a min. *Z*-score of 1.96. Site validation for each SNP position was performed. SNPs that fail the necessary requirements were excluded in the final analysis. Based on concatenated alignments of high quality SNPs, maximum likelihood trees were created using FastTree version 2.1.7 (Price et al., 2010).

The concatenated sequences derived from whole genomes were used for screening on virulence-associated genes using the BLASTN algorithm of the NCBI database (https://www.ncbi.nlm.nih.gov). The web-based tool was used with standard settings.

Multilocus Sequence Analysis (MLSA) of Housekeeping Genes

Bacterial strains were grown overnight and genomic DNA was extracted as described above. MLSA was performed on four protein-coding housekeeping genes making use of the *Vibrio* spp. MLSA website (https://pubmlst.org/vibrio/info/Vibrio_primers.pdf) developed by Keith Jolley and sited at the University of Oxford (Jolley and Maiden, 2010). The 25 µl PCR mixtures contained 1 × PCR buffer (2 mM $MgCl_2$), 0.2 mM of each dNTP, 0.2 µM of each primer, 1.5 U DreamTaq DNA Polymerase (Thermo Fisher Scientific Biosciences GmbH, St. Leon-Rot, Germany), and 1 µl of genomic DNA. For amplification of *gyrB*, the primers VigyrBF and VigyrBR were used. Amplification of *atpA* was carried out with the primers Vi_atpAdg_F and Vi_atpAdg_R. PCR reactions were performed using a Mastercycler ep gradient (Eppendorf AG, Hamburg, Germany). PCR products were purified using the MSB Spin PCRapace Kit according to the manufacturer's instructions (Stratec Molecular GmbH, Berlin, Germany) and sequenced (Eurofins Genomics GmbH, Ebersberg, Germany). The sequences were assembled and analyzed using the Lasergene software SeqMan Pro version 12.0 (DNASTAR Inc., Madison, WI, USA) and the software Accelrys Gene version 2.5 (Accelrys Inc., San Diego, CA, USA). Allele sequences including the *rpoB* sequences were concatenated in the order of loci *gyrB-pyrH-recA-atpA-rpoB* to generate a 2,893 bp concatemer for each strain. A phylogenetic tree was constructed with MEGA version 6.0 (Tamura et al., 2013) based on the alignment of the concatenated allele sequences using the neighbor-joining method with the Kimura 2-parameter model. Bootstrapping with 1,000 replications was performed to verify the robustness of the tree.

PCR Typing of Virulence-Associated Genes

Extraction of genomic DNA and PCR reactions were performed as described above with 5 ng of template DNA. PCR primers, target genes, and amplicon sizes are shown in Table S1. Primer sequences were derived from whole genome contigs of three *V. navarrensis* strains (Gladney et al., 2014) using the software Accelrys Gene version 2.5. Accession numbers of contigs are given in Table S1. The PCR running conditions were as follows: an initial denaturation step at 94°C for 4 min, 30 cycles of denaturation at 94°C for 15 s, primer annealing at 55°C for 30 s and extension at 72°C for 45 s, and a final extension step at 72°C for 7 min. PCR products were analyzed in agarose gels to determine the product lengths. Selected PCR products were sequenced for confirmation.

Accession Numbers

Nucleotide sequences were deposited in the European Nucleotide Archive (ENA) with the following accession numbers: sequences of partial *rpoB* gene accession numbers LT546547-LT546563 (*rpoB* sequences of three strains already in database, see **Figure 1**), sequences of partial *atpA* gene accession LT546564-LT546583, sequences of partial *gyrB* gene accession LT546584-LT546603, sequences of partial *pyrH* gene accession LT546604-LT546623, and sequences of partial *recA* gene accession LT546624-LT546643.

Genome sequences of *V. navarrensis* isolates have been deposited in GenBank at National Center for Biotechnology Information (NCBI) under the accession numbers MPKB00000000 to MPKU00000000 (see **Table 3**).

For *rpoB* phylogeny presented in **Figure 1**, *rpoB* sequences of non-*V. navarrensis* strains and *V. navarrensis* strains 2232, 08-2462, CIP 103381 (identical to ATCC 51183), 0053-83, VN-3125, and VN-3139 were obtained from GenBank at NCBI. Accession numbers are FN423814 (LMG 3772), FN423805 (ATCC 27562), LMYA01000047 (DAL-79040), MCJC01000044 (ATCC 14181), FN423808 (LMG 7891), FN423806 (LMG 11664), HG794494 (DSM 14383), CP014035 (ATCC 33809), FN423804 (LMG 7896), FN423803 (NCTC 4711), CP013317 (NCTC 5395), AFWH01000033 (CIP 102891), FN423816 (CCUG 16330), FN423810 (DSM 6904), FN423812 (LMG 10935), FN423802 (ATCC 17749), LVYF01000041 (VN-7501), JNUL02000012 (CFSAN007457), MVKN01000048 (VN-0293), FN423813 (DSM 507), FN423801 (CCUG 13625), EF064429 (9046-81), JMCH01000016 (2232), JMCI01000045 (08-2462), JMCG01000002 (ATCC 51183), JMCF01000001 (0053-83), KJ647757 (VN-3125), and KJ647770 (VN-3139).

RESULTS AND DISCUSSION

rpoB Phylogeny

Determination of partial *rpoB* sequences has proved a reliable method for species identification for bacteria of the family *Vibrionaceae* (Tarr et al., 2007; Adékambi et al., 2009; Dieckmann

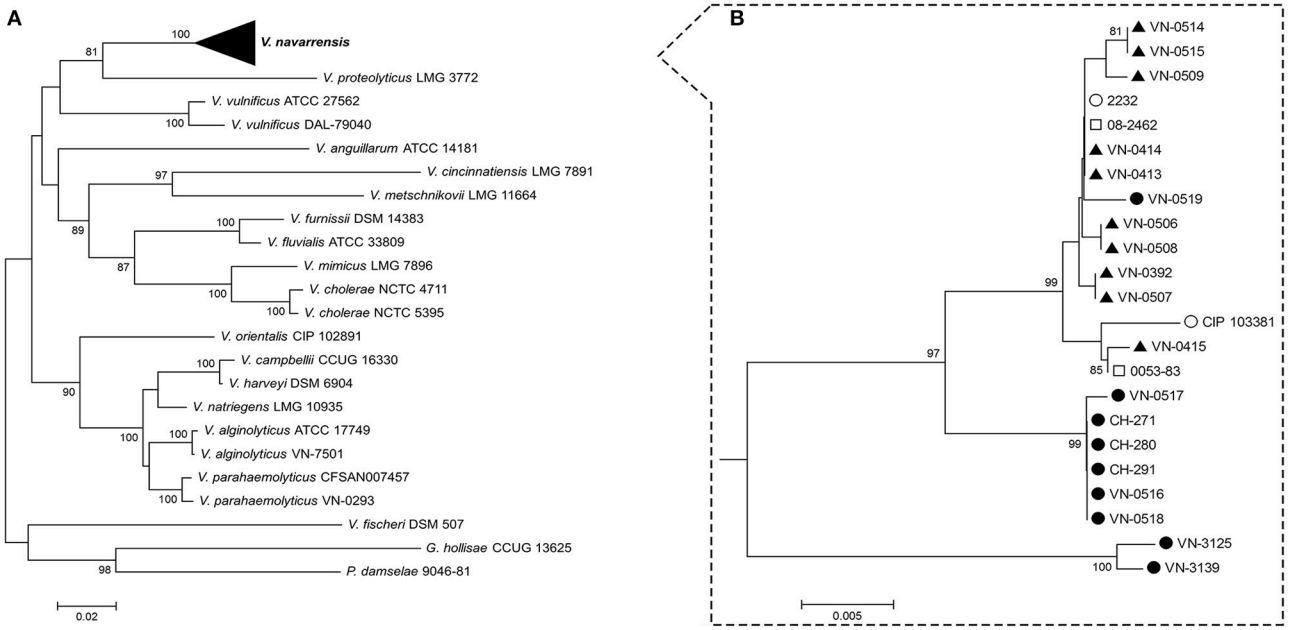

FIGURE 1 | Phylogenetic tree of different *Vibrionaceae* strains **(A)** and subtree of *V. navarrensis* strains **(B)** based on partial *rpoB* sequences (871 bp). The evolutionary history was inferred using the neighbor-joining method with Kimura 2-parameter distance model in MEGA version 6.0. Bootstrap values above 75% are shown next to the nodes ($n = 1{,}000$ replicates). Scale bars represent base substitutions per site. German veterinary (▲) and environmental (●), Spanish environmental (○), and human pathogenic *V. navarrensis* strains (□; from the U.S.) (Gladney and Tarr, 2014) form a distinct cluster within a number of different *Vibrionaceae* strains. *rpoB* sequences of non-*V. navarrensis* strains and *V. navarrensis* strains 2232, 08-2462, CIP 103381 (identical to ATCC 51183), 0053-83, VN-3125, and VN-3139 were obtained from GenBank at National Center for Biotechnology Information (NCBI). Accession numbers are given in Materials and Methods.

et al., 2010). The sequences of an 871 bp internal fragment of the coding sequence of the *rpoB* gene were identified for all isolates of this study. *rpoB* sequences of two Spanish environmental *V. navarrensis* strains [CIP 103381 (identical to ATCC 51183) and 2232] and two human pathogenic strains (0053-83 and 08-2462; Gladney et al., 2014) available in public databases were included for the construction of a phylogenetic tree (**Figure 1B**). All strains fell into a cluster that formed a distinct species among a number of different *Vibrio* species (**Figure 1**).

With the exception of two strains, the identity of the sequences of most *V. navarrensis* strains was greater than 98% in the sequenced region of 871 bp. In a previous study, we observed that in many *Vibrio* spp. the lowest sequence identity (determined by ClustalW) of this gene fragment was around 98% on species level (Dieckmann et al., 2010). The identity of the *rpoB* sequences of two *V. navarrensis* strains from seawater (VN-3125 and VN-3139) to the *rpoB* sequences of the remaining *V. navarrensis* strains was only ca. 96%. Only in three of the 40 polymorphic sites of the sequenced fragment, nonsynonymous substitutions leading to amino acid exchanges in the gene product were discovered. Two identical amino acid exchanges were observed only in the more distantly related strains VN-3125 and VN-3139.

The *rpoB* tree showed that the veterinary isolates from domestic animals clustered with two Spanish environmental strains [CIP 103381 (identical to ATCC 51183) and 2232] and two human pathogenic strains (Gladney et al., 2014; **Figure 1B**). Also one environmental strain, VN-0519 isolated from a blue mussel harvested from a mussel production area, fell into this cluster, while six environmental seawater isolates from Germany formed a separate subcluster.

Phenotypic Characteristics

All strains (**Table 1**) were phenotypically tested using a panel of standard biochemical reactions (**Table 2**). The biochemical properties were fairly homogenous with more than 90% of the strains showing the same result (only few variable reactions). Comparing to published results, biochemical characteristics were typical as described for *V. navarrensis* (Urdaci et al., 1991; Farmer and Janda, 2004; Jores et al., 2007; Gladney and Tarr, 2014; Farmer et al., 2015). The strains were negative for Voges-Proskauer test, arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase and were positive for sucrose fermentation and phenylalanine deaminase activity. The three strains of the biovar *pommerensis* (CH-271, CH-280, CH-291) were not studied in further details, as the biovar specific reactions were not part of our standard reaction panel (Jores et al., 2007).

Since the veterinary laboratory in Saxony, from which the strains were obtained, did not use molecular diagnostic techniques such as PCR and DNA sequencing, the isolates from animals had been phenotypically determined either as *Vibrio* sp. or as *V. vulnificus*. Misidentification by traditional methods happened, as phenotypic characteristics of *V. navarrensis* and *V. vulnificus* are very similar (Gladney and Tarr, 2014). The phenotypic characterization of all 20 strains was also done to

TABLE 2 | Phenotypic characterization of *V. navarrensis* strains.

Phenotypic test	No. of isolates (%)	
	+	−
Cytochrome oxidase	20 (100)	0 (0)
Nitrate reductase	20 (100)	0 (0)
Arginine dihydrolase	0 (0)	20 (100)
Lysine decarboxylase	0 (0)	20 (100)
Ornithine decarboxylase	0 (0)	20 (100)
Phenylalanine deaminase	18 (90)	2 (10)
Urease	0 (0)	20 (100)
Production of:		
Acetoin (Voges-Proskauer reaction)	0 (0)	20 (100)
H_2S	0 (0)	20 (100)
Indole	20 (100)	0 (0)
Oxidative acid production from:		
Adonitol	0 (0)	20 (100)
L-Arabinose	1 (5)	19 (95)
Cellobiose	19 (95)	1 (5)
Dulcitol	0 (0)	20 (100)
D-Glucose	20 (100)	0 (0)
myo-Inositol	0 (0)	20 (100)
Lactose	8 (40)	12 (60)
Maltose	20 (100)	0 (0)
D-Mannitol	20 (100)	0 (0)
D-Mannose	18 (90)	2 (10)
Melibiose	3 (15)	17 (85)
Raffinose	0 (0)	20 (100)
L-Rhamnose	2 (10)	18 (90)
Salicin	1 (5)	19 (95)
D-Sorbitol	0 (0)	20 (100)
L-Sorbose	0 (0)	20 (100)
Sucrose	20 (100)	0 (0)
Trehalose	20 (100)	0 (0)
D-Xylose	0 (0)	20 (100)
Degradation of:		
Esculin	4 (20)	16 (80)
Citrate (Simmons citrate reaction)	5 (25)	15 (75)
Growth in 1% peptone water		
+ 0% NaCl	0 (0)	20 (100)
+ 3% NaCl	20 (100)	0 (0)
+ 8% NaCl	0 (0)	20 (100)
+ 10% NaCl	0 (0)	20 (100)
Susceptibility to O/129 (10 μg/150 μg)	20 (100)	0 (0)
Hemolysis of:		
Human erythrocytes	19 (95)	1 (5)
Sheep erythrocytes	19 (95)	1 (5)

find out if distinct biochemical properties could be correlated to the source of the strains. The results of these investigations, however, did not reveal significant differences between the strains (see **Table 2**).

As *V. navarrensis* strains show hemolytic activity (Jores et al., 2003, 2007), we investigated all strains on agar plates containing sheep erythrocytes or human erythrocytes. Most strains did not show hemolytic activity within 24 h. However, after incubation for up to 72 h all strains but one were hemolytic against human erythrocytes. On sheep blood agar, six strains (two environmental and four veterinary strains) did not show hemolysis at first. However, after modification of the assay medium and repeated streaking on sheep blood agar also these strains (except one) displayed hemolysis zones. With sheep and human erythrocytes, hemolysis zones surrounding colonies were clear indicating a ß-hemolysis with complete degradation of hemoglobin (Zhang and Austin, 2005).

Whole Genome Sequencing

The results of genomes of ten German isolates (five veterinary and five environmental strains) are shown in **Table 3**. The genome sizes range from 4.14 to 4.90 Mbp and the GC contents of the genomes vary between 47.5 and 48.1%. The predicted number of coding sequences range from 3,559 to 4,247. The published genomes of two Spanish strains (ATCC 51183 and 2232) and two human pathogenic strains (0053-83 and 08-2462) vary between 4.2 and 4.4 Mbp (Gladney and Tarr, 2014). It was noted that the genomes of all five veterinary strains are also in this range, while the genomes of the five isolates from marine environments including the mussel isolate are larger (4.6–4.9 Mbp, **Table 3**). It is possible that the greater genome sizes of marine strains reflect a wider range of metabolic capabilities compared to the veterinary and human pathogenic strains. Some bacteria, especially those adapted to specific niches (e.g., pathogenic strains adapted to specific host environments) can lose metabolic capabilities leading to a reduction in genome size (Raskin et al., 2006). Further studies may address this question.

As bacteriophages are involved in horizontal gene transfer, the WGS data were analyzed with Phage Search Tool (Zhou et al., 2011). The search for phage sequences revealed the occurrence of several prophage sequences as expected (Table S2). Phages are one of the major forces driving horizontal gene transfer (Raskin et al., 2006). Most prophage sequences are related to giant viruses and of lower significance. In some strains, however, prophage sequences possibly encoding intact phages were detected (Enterobacteria phages HK630 and HK629, *Vibrio* phages martha 12B12 and VPUSM 8). However, no information about the phages except the genome sequences are available (Table S2). In two marine strains (VN-0516 and VN-3125), a possibly intact phage was found that is related to *Vibrio* phage VCY Φ. This phage is a small filamentous phage (approximately 7.1 kbp) and was found in association with environmental *V. cholerae* strains in ponds (Xue et al., 2012). Bioinformatics indicated the presence of a plasmid in only one strain (VN-3125). A small region of 638 bp was identified possessing high identity (>99%) to a replication region present in several *Enterobacteriaceae* plasmids [e.g., plasmid p8401 in *E. coli* (accession CP012198)]. In the four published *V. navarrensis* genomes, no plasmid sequences were reported so far.

TABLE 3 | Results of the whole genome sequence analysis of veterinary and environmental *V. navarrensis* strains.

Feature	CH-280	VN-0392	VN-0415	VN-0507	VN-0509	VN-0514	VN-0516	VN-0518	VN-0519	VN-3125
Genome size (bp)	4,899,705	4,287,414	4,138,545	4,271,170	4,356,049	4,278,964	4,605,884	4,684,360	4,788,163	4,765,427
GC content (%)	47.45	48.02	48.08	48.04	48.09	48.07	47.71	47.75	47.94	47.57
Genes (total)*	4,440	3,935	3,784	3,857	3,923	3,909	4,162	4,280	4,331	4,338
CDS (total)**	4,319	3,811	3,661	3,731	3,781	3,779	4,058	4,136	4,201	4,233
CDS (coding)	4,247	3,737	3,559	3,658	3,703	3,693	4,005	4,098	4,149	4,186
RNA genes (total)***	121	124	123	126	142	130	104	104	130	105
rRNAs (5S, 16S, 32S)***	7, 8, 7	9, 11, 10	5, 9, 7	7, 8, 7	8, 12, 11	7, 8, 11	4, 4, 4	5, 9, 1	7, 8, 9	8, 7, 7
tRNAs	95	90	98	100	107	100	88	85	101	79
ncRNAs	4	4	4	4	4	4	4	4	5	4
Pseudogenes (total)	72	74	102	73	78	86	53	38	52	47
CRISPR Arrays	2	3	3	1	1	1	1	0	1	0
Predicted prophages (no.)	7	3	7	7	2	9	1	2	4	4
intact	n.d.	1	n.d.	n.d.	1	n.d.	1	n.d.	1	3
incomplete	6	1	7	7	1	9	n.d.	1	2	1
questionable	1	1	n.d.	n.d.	n.d.	n.d.	n.d.	1	1	n.d.
Plasmids	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	ColRNAI, 88.39%
GenBank accession										
Bioproject	PRJNA353389	PRJNA353302	PRJNA353299	PRJNA353297	PRJNA353295	PRJNA353294	PRJNA353292	PRJNA353290	PRJNA353289	PRJNA353288
Biosample	SAMN06014880	SAMN06013691	SAMN06013689	SAMN06013684	SAMN06013686	SAMN06013681	SAMN06013683	SAMN06013678	SAMN06013679	SAMN06013680
Accession	MPKT00000000	MPKB00000000	MPKE00000000	MPKG00000000	MPKI00000000	MPKJ00000000	MPKL00000000	MPKN00000000	MPKO00000000	MPKP00000000

**Nucleotide sequences from the start codon (ATG) to the stop codon.*

***Nucleotide sequence that is translated to form proteins.*

****Including partial sequences of the respective element.*

n.d., not detected.

SNP Phylogeny of Whole Genome Sequences

For an SNP analysis, additional published WGS data of two human pathogenic strains (0053-83 and 08-2462) and two environmental Spanish strains (ATCC 51183 and 2232) were included. The human isolates were from human specimen isolated in the U.S. and the environmental strains were Spanish isolates from sewage (Urdaci et al., 1991). To identify SNPs, all input sequences were mapped to the *V. navarrensis* 0053-83 genome as reference (JMCF01000000) and screened for relevant nucleotide variations (Kaas et al., 2014). In total, the concatenated contigs used for the SNP analysis comprised approx. 3.688 Mbp and the number of SNPs between the strains varied between 10,000 to 63,000 (Figure S1). Using the concatenated alignments of high quality SNPs, maximum likelihood trees were created using FastTree 2 (Price et al., 2010; **Figure 2**). Based on the length of the branches, the five veterinary strains and the two Spanish environmental strains differ but are related. The two human isolates (0053-83, 08-2462) are closer related to each other (the SNP difference is around 16,000 between the two strains) but are more distant to the veterinary strains and the Spanish strains (approximately 30,000 SNPs). All marine isolates from Germany with the exception of the mussel strain VN-0519 are clearly separated from the other strains, but differ also from each other (SNP differences between 30,000 and 63,000). Only the seawater strains VN-0516 and CH-280 are closer related (difference approximately 10,000 SNPs) which indicates that VN-0516 may belong to the subspecies *V. navarrensis* biotype *pommerensis*.

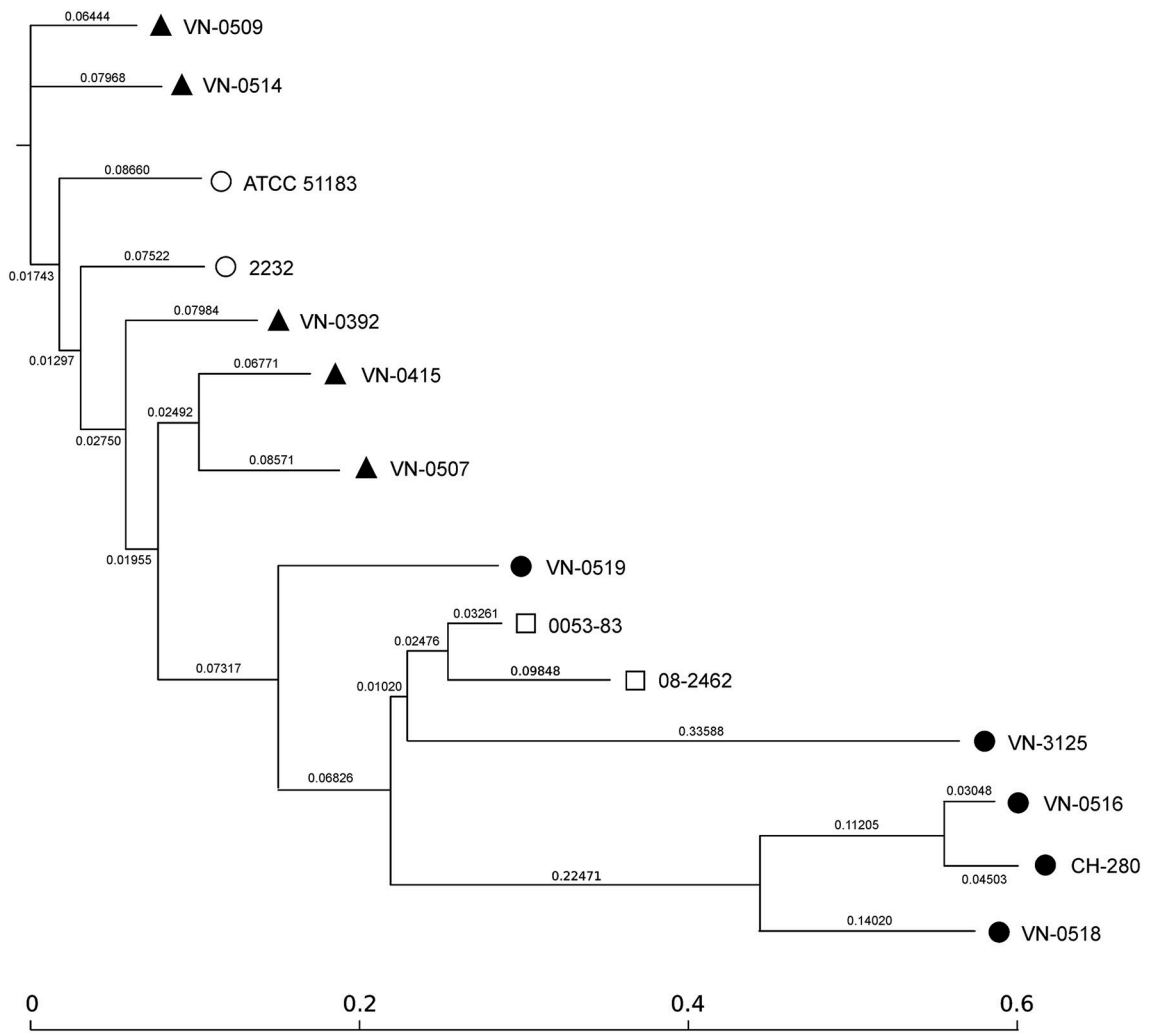

FIGURE 2 | SNP-based phylogeny tree of German veterinary (▲) and environmental (●) *V. navarrensis* isolates, two human (□) isolates from the U.S., and two Spanish environmental (○) strains [CIP 103381 (identical to ATCC 51183) and 2232 (see text)]. SNP-tree was conducted by using CSI Phylogeny 1.4 under default settings. Single nucleotide polymorphisms (SNPs) were called by mapping to the *V. navarrensis* 0053-83 genome as reference (JMCF01000000). Criteria for high quality SNP calling and filtering are described in Material and Methods. Based on concatenated alignments of high quality SNPs, maximum likelihood trees were created using FastTree version 2.1.7. Scale bar represents the number of nucleotide substitutions per site and numbers indicate branch length.

The SNP analysis revealed clearly that the seawater strains are distant to the remaining *V. navarrensis* strains. In *E. coli*, SNP analysis of the core genome consisting of 1,429 genes revealed 128,214 variable sites for a pathovar (Von Mentzer et al., 2014) and the average SNP differences between two related clades of isolates from different hosts were below 100 within a clade and below 1,800 between two clades (Schaufler et al., 2016). The SNP calculation for the *V. navarrensis* strains (without the seawater strains) vary from 18,000 to 32,000 variable sites. Thus, a close relatedness of all strains cannot be deduced from SNP data (Figure S1).

Phylogenetic Relationship from Multilocus Sequence Analysis of Housekeeping Genes

We applied the *Vibrio*-MLSA scheme available on the PubMLST website and included sequences from published WGS data of the four *V. navarrensis* strains (Gladney et al., 2014) into the phylogenetic analysis. To increase the depth of the analysis, the *rpoB* sequences were added and the sequences of the concatemer were arranged in the order *gyrB-pyrH-recA-atpA-rpoB*. Concatemers of sequences of housekeeping genes were created, as comparison of housekeeping genes is used for infra-species resolution and determination of the clonality of strains (Glaeser and Kämpfer, 2015).

The phylogenetic analysis of the concatemers displayed one subcluster containing the veterinary isolates, the mussel isolate, and the reference strain ATCC 51183 (identical to CIP 103381; **Figure 3**). The sewage strain 2232 and the two human isolates were also placed in this cluster. It should be noted that the two Spanish environmental strains were isolated from sewage in towns of the Spanish province Navarra away from the coast (Urdaci et al., 1991). The concatemers of the German seawater isolates split into three branches and were clearly distant to the subcluster formed by the remaining strains. The MLSA tree indicates a stronger relationship between the veterinary strains, the Spanish strains and the human pathogenic strains than the SNP phylogenetic tree. This discrepancy is likely to be explained by the fact that the housekeeping genes are encoding essential

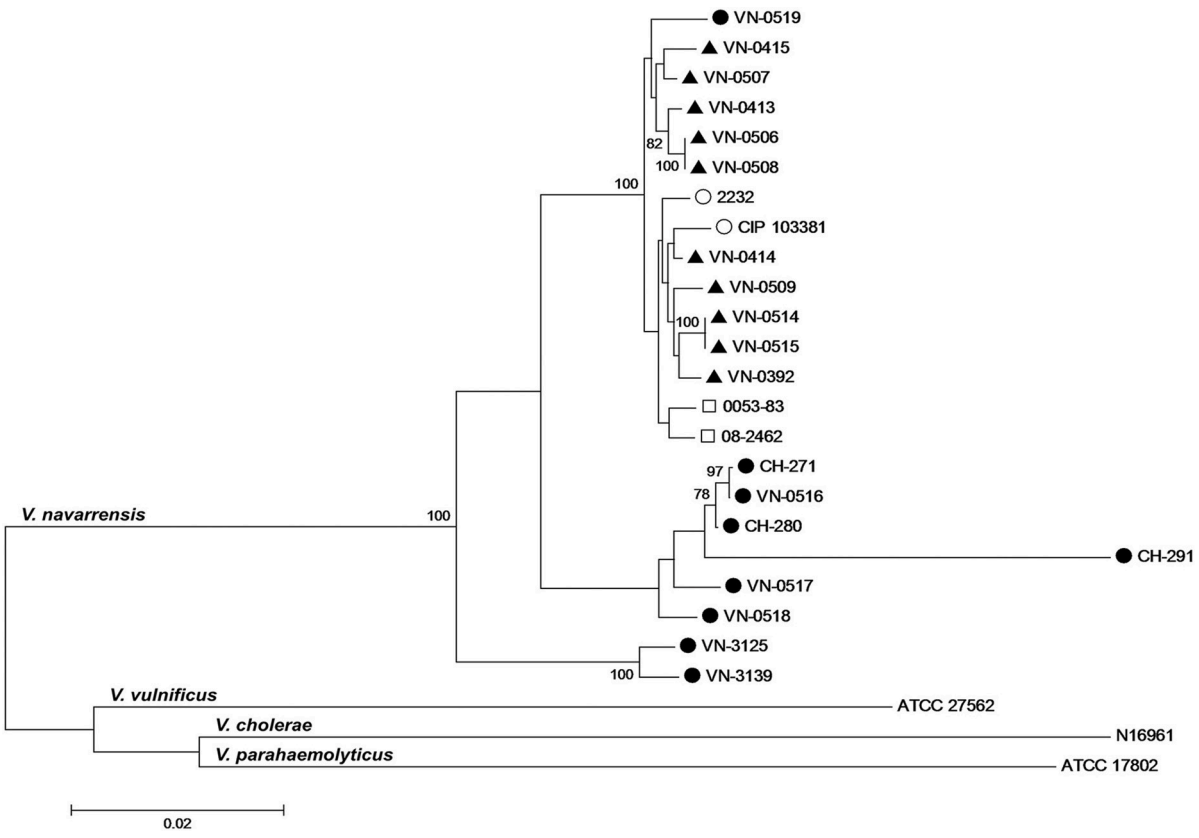

FIGURE 3 | Phylogenetic relationship of German veterinary (▲) and environmental (●) *V. navarrensis* isolates, human (□) isolates from the U.S., and Spanish environmental (○) strains based on concatenated sequences of five protein-coding housekeeping genes (*gyrB-pyrH-recA-atpA-rpoB*; 2,893 bp). Sequences of *V. navarrensis* 0053-83, 08-2462, CIP 103381 (identical to ATCC 51183), and 2232 were obtained from previous studies (Gladney and Tarr, 2014; Gladney et al., 2014). Sequences of *V. vulnificus* ATCC 27562, *V. cholerae* N16961, and *V. parahaemolyticus* ATCC 17802 from *Vibrio* spp. MLSA website (http://pubmlst.org/vibrio/) and GenBank at NCBI, respectively, were included for comparison. The evolutionary history was inferred using the neighbor-joining method with Kimura 2-parameter distance model in MEGA version 6.0. Bootstrap values above 75% are shown next to the nodes ($n = 1{,}000$ replicates). Scale bar represents 0.02 base substitutions per site.

cellular functions and are therefore more conserved and evolve relatively slowly (Hanage et al., 2006).

The observation that the environmental Spanish strains are related to the veterinary strains is a remarkable observation. The Spanish strains were isolated from low salinity aquatic environments away from the coast (Urdaci et al., 1991) in rivers and sewage. As bacteria of the genus *Vibrio* are regarded as environmental aquatic bacteria, it seems possible that *V. navarrensis* may also occur in freshwater in regions of Germany and that the veterinary isolates were ingested by the animals through uptake of surface water. This hypothesis is supported by the results of the phylogenetic analyses which show that the veterinary strains are distinctly different from the German seawater strains. Unfortunately, there is no knowledge concerning a possible origin of the veterinary strains as respective investigations were not undertaken. The phylogenetic studies also indicate that the human strains from the U.S. are more related to the veterinary ones. However, no further information concerning the American strains is available.

The mussel strain (VN-0519) is the only strain from a marine environment that shows a stronger relationship to the veterinary and human isolates. It should be noted that knowledge on natural habitats of *V. navarrensis* are fragmentary, as only few publications on this species are available. In one report from Thailand, partial sequences of 16S rDNA of uncultured bacteria recovered from the gut of marine shrimps were 99% identical to 16S rDNA sequences of *V. navarrensis* (Rungrassamee et al., 2013). In another recent paper, the occurrence of *V. navarrensis* in larval midgut of the date palm root borer *Oryctes agamemnon* in Saudi-Arabia (El-Sayed and Ibrahim, 2015) was detected based on sequence analysis of the gut microbiome. According to this paper, the endosymbiotic bacterial community was dominated by *Vibrionaceae* revealing that these bacteria can be prevalent in an insect environment.

Presence/Absence of Virulence-Associated Factors

To find out if environmental *V. navarrensis* strains can be distinguished from the veterinary strains and human pathogenic strains, the presence or absence of genes coding for virulence-associated factors were investigated by BLASTN searches of the WGS data and by PCR genotyping of the 20 available strains. A number of putative candidate genes were selected based on the published genomes of the reference strain ATCC 51183 (identical to CIP 103381) and the two human *V. navarrensis* strains 0053-83 and 08-2462. PCR primers were designed for a number of genes using the WGS data of these strains (Table S1). The genes targeted were coding for potential virulence factors with cytolytic or hemolytic activities and parts of secretion systems (Gladney et al., 2014). **Table 4** summarizes the results of these investigations for all *V. navarrensis* strains including the four published strains.

For genes encoding hemolytic and cytolytic proteins, we chose homologs of *vvhA*, *tlh*, δ-*vph*, *hlyIII*, and *osmY*. The *vvhA* gene of *V. vulnificus* encodes a potent cytolytic hemolysin whose role in pathogenicity has been under debate (Jones and Oliver, 2009; Lee et al., 2013). The gene is present in clinical and environmental *V. vulnificus* strains and is used for species identification (Campbell and Wright, 2003). Similarly, a *tlh* homolog encoding a putative thermolabile hemolysin is found in many *Vibrio* species. Its role in pathogenicity is unclear (Zhang and Austin, 2005) and it is used in *V. parahaemolyticus* for species identification (Jones et al., 2014). WGS data indicated the presence of the two genes in all genomes and PCR assays for gene homologs of *vvhA* and *tlh* were positive in the 20 *V. navarrensis* strains of this study, which indicates that these genes might also be suitable for identification of this species (**Table 4**).

A gene encoding a putative hemolysin III family protein (HlyIII) with a size of 214 amino acids is annotated in the WGS data of all *V. navarrensis* strains (except VN-0507). Due to nucleotide sequence variations, PCR amplicons of this gene were not obtained for all strains (data not shown). In case of *V. vulnificus*, a homolog of the HlyIII protein was investigated in more detail (Chen et al., 2004). As a *hlyIII* mutant of *V. vulnificus* exhibited attenuated virulence in a mouse model compared with the wild-type strain, a role of HlyIII in virulence was suggested (Chen et al., 2004; Zhang and Austin, 2005). Another thermostable hemolysin, δ-VPH, with unclear role in pathogenicity has been found in *V. parahaemolyticus* and *V. cholerae* (Zhang and Austin, 2005). In contrast to *tlh*, *vvhA*, and *hlyIII*, the putative δ-*vph* gene was only detected in strains from marine environments (seawater strains and blue mussel strain) and in the two human pathogenic strains (WGS data). The *osmY* gene homolog encoding a putative hemolysin (accession KGK22069) with a domain for attachment to phospholipid membranes was present in all strains (**Table 4**).

WGS data of all strains showed the presence of a *hlyD* gene encoding a hemolysin D protein and an *rtx* gene encoding a repeats-in-toxin protein. HlyD proteins are involved in transport of hemolysins through the bacterial inner membrane (Pimenta et al., 2005; Linhartová et al., 2010), while secreted RTX proteins mostly exhibit pore-forming activity visible as hemolytic halo surrounding bacterial colonies on blood agar (Linhartová et al., 2010).

Jores et al. cloned a 15.6 kbp DNA fragment of *V. navarrensis* biotype *pommerensis* CH-291 into the plasmid pVH that upon introduction into *E. coli* strain DH5α conferred hemolytic properties. DNA hybridization experiments of the whole fragment were positive only with strains of the biotype *pommerensis* and were suggested to be specific for the biotype. The hemolytic properties were found on two neighboring regions of the 15.6 kbp fragment, each containing more than one open reading frame (ORF) (Jores et al., 2003). ORF12, the largest ORF conferring hemolytic properties, was only present in four strains (CH-271, CH-280, CH-291, VN-0516; **Table 4**). The significance of this region for identification of a subpopulation of *V. navarrensis* strains requires the study of more strains.

Type IV pilins of Gram-negative bacteria play various roles in pathogenicity (Giltner et al., 2012). In toxigenic *V. cholerae*, a type IV pilus is a major virulence factor that functions as an essential colonization factor and acts as cholera toxin phage receptor (Karaolis et al., 1998; Rivera et al., 2001). Two genes, *pilW* and *pilV*, coding for type IV pilus assembly or pilus biosynthesis proteins were present in most strains. The *pilV* gene was absent

TABLE 4 | Presence/absence of virulence-associated traits in veterinary, human, and environmental *V. navarrensis* isolates based on WGS data.

Strain	Source code	Virulence-associated genotypic traits*													
		cps	T6SS *DUF877*	T6SS *DUF770*	T6SS *vasD*	*pilV*	*pilW*	*tlh*	*osmY*	*vvhA*	δ-*vph*	*hlyD*	*hlyIII***	*rtx***	ORF12
VN-0392	vet	■	■	■	■	■	■	■	■	■		■	■	■	
VN-0413	vet	■	■	■	■		■	■	■	■		■	■	■	
VN-0414	vet	■	■	■	■	■	■	■	■	■		■	■	■	
VN-0415	vet	■	■	■	■	■	■	■	■	■		■	■	■	
VN-0506	vet	■	■	■	■	■	■	■	■	■		■	■	■	
VN-0507	vet	■	■	■	■	■	■	■	■	■		■		■	
VN-0508	vet	■	■	■	■	■	■	■	■	■		■	■	■	
VN-0509	vet	■	■	■	■	■	■	■	■	■		■	■	■	
VN-0514	vet	■	■	■	■	■	■	■	■	■		■	■	■	
VN-0515	vet	■	■	■	■	■	■	■	■	■		■	■	■	
08-2462**	hum	■				■	■	■	■	■	■	■	■	■	
0053-83**	hum	■				■	■	■	■	■	■	■	■	■	
CIP 103381***	env-Sp	■	■	■	■	■	■	■	■	■		■	■	■	
2232**	env-Sp	■	■	■	■		■	■	■	■		■	■	■	
CH-271	env-G	■				■	■	■	■	■	■	■	■	■	■
CH-280	env-G	■					■	■	■	■	■	■	■	■	■
CH-291	env-G	■				■	■	■	■	■	■	■	■	■	■
VN-0516	env-G	■				■	■	■	■	■	■	■	■	■	■
VN-0517	env-G	■				■	■	■	■	■	■	■	■	■	
VN-0518	env-G	■				■	■	■	■	■	■	■	■	■	
VN-0519	env-G	■	■	■	■	■	■	■	■	■	■	■	■	■	
VN-3125	env-G	■	■	■	■		■	■	■	■	■	■	■	■	
VN-3139	env-G	■	■	■	■	■	■	■	■	■	■	■	■	■	

vet, veterinary; hum, human; env-Sp, environmental-Spain; env-G, environmental-Germany.

**In WGS analysis, gene sequences of V. navarrensis 08-2462 (cps, osmY, vvhA, δ-vph), 0053-83 (pilV, pilW, tlh, hlyD, hlyIII, rtx), CIP 103381 (T6SS vasD), and 2232 (T6SS DUF877, T6SS DUF770) as well as ORF12 of CH-291 were used as reference sequences. Strains showing 90–100% sequence similarity to the specific reference sequence were defined as positive for the respective virulence-associated trait. WGS data were confirmed by PCR assays. VN-0392, VN-0507, and VN-0519 were PCR-negative for cps, T6SS DUF770 and pilW, respectively. WGS data showed primer mismatches.*

***No verification of the WGS data by PCR assays.*

****Identical to ATCC 51183.*

in three out of the 20 strains (WGS and PCR), whereas the *pilW* gene was found in all strains, although in one strain (VN-0519), a PCR to confirm the gene failed. A gene coding for a putative protein of capsule biosynthesis (designated as *cps*) was detected in all strains (**Table 4**).

Type VI secretion systems (T6SS) have attracted attention, as they play important roles in virulence of a number of Gram-negative bacteria by translocating effector proteins into eukaryotic cells. Recently, T6SS have also been shown to transport proteins into prokaryotic cells showing bactericidal activity against competitors (Ho et al., 2014). Three genes of putative T6SS proteins were tested for presence in *V. navarrensis* strains. Two of the genes encode proteins associated with the baseplate containing domains of unknown functions (T6SS DUF877 and T6SS DUF770) and one gene encodes a VasD protein homolog which is a lipoprotein tethered to the outer membrane. Interestingly, six of eight seawater strains were negative for these genes indicating that the T6SS is not present in these environmental strains. In contrast, the mussel strain as well as the reference strain CIP 103381 from sewage and all veterinary strains harbor the T6SS (**Table 4**). The WGS analysis confirmed the PCR results. WGS data of the two human pathogenic strains 0053-83 and 08-2462 revealed the absence of the three selected genes and indicate the lack of the T6SS in these strains.

In summary, no clear discrimination based on virulence-associated factors was observed between the strains, as most of the investigated genes were present in all strains. Some

genes encoding virulence-associated traits may be useful for further analysis of strains from different origins. Candidate genes identified in this study are the δ-*vph* gene and genes encoding components of the T6SS and the hemolytic activity encoding region of biotype *pommerensis* strains. However, evidence if some of these genes contribute to a pathogenic potential do require additional research. It is feasible that discrimination of environmental and potentially pathogenic strains requires the identification of allelic variants of specific genes as it is the case for clinical strains of *V. vulnificus* (Jones and Oliver, 2009).

CONCLUSION

This study was initiated by a recent publication about *V. navarrensis* strains recovered from human specimens. The strains originated from diverse human sources (blood, wound, ear, stool) suggesting that this species is a human pathogen. The veterinary isolates of this study were isolated from animals intended for food production in farms of the German state Saxony that has no border to marine environments. The strains were recovered after abortions from placentas and some strains were isolated directly from inner organs of the aborted fetuses. A pathogenic potential of these isolates seems likely. However, it cannot be excluded that the strains were purely commensals, as no further investigations regarding pathogenicity were performed. The animal source of the strains is unusual, as *Vibrio* bacteria are mostly found in marine environments and are commonly associated with marine organisms. The uptake through feed of marine origin (seafeed) was discussed; however, no satisfying explanation for the occurrence of *Vibrio* strains in domestic animals was found. Cases of human vibriosis result either through contact to seawater or by uptake of contaminated seafood. The occurrence of possibly pathogenic *Vibrio* strains in mammalian hosts intended for food production is of great interest, as it could indicate a so far unrecognized source of *Vibrio* infections.

The isolation of *V. navarrensis* from domestic animals after miscarriages and from diseased humans suggests a pathogenic potential of these bacteria and could mean that this species is a so far unrealized zoonotic agent. The "One Health" concept acknowledges that human, animal, and environmental health are linked. Further research is necessary to identify reservoirs, sources, and ways of transmission of this species to determine a possible role as zoonotic agent.

AUTHOR CONTRIBUTIONS

KS, NB, KT, and ES designed the study. KS, CK, and NB performed the experiments. KS, CK, NB, JH, KT, and ES analyzed the data. KS, JH, and ES prepared the tables and figures, wrote the manuscript. All authors edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01717/full#supplementary-material

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Bile Sensing: The Activation of *Vibrio parahaemolyticus* Virulence

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Bacteria must develop resistance to various inhospitable conditions in order to survive in the human gastrointestinal tract. Bile, which is secreted by the liver, and plays an important role in food digestion also has antimicrobial properties and is able to disrupt cellular homeostasis. Paradoxically, although bile is one of the guts defenses, many studies have reported that bacteria such as *Vibrio parahaemolyticus* can sense bile and use its presence as an environmental cue to upregulate virulence genes during infection. This article aims to discuss how bile is detected by *V. parahaemolyticus* and its role in regulating type III secretion system 2 leading to human infection. This bile–bacteria interaction pathway gives us a clearer understanding of the biochemical and structural analysis of the bacterial receptors involved in mediating a response to bile salts which appear to be a significant environmental cue during initiation of an infection.

Keywords: bacteria, human gastrointestinal tract, bile, *Vibrio parahaemolyticus*, type III secretion system 2

INTRODUCTION

Humans have a complex digestive system that not only aids in digestion of food but also has a role in self-defense against microorganisms in the body. Microorganisms such as bacteria have to tolerate various extreme environments in order to survive in the human gastrointestinal tract (Begley et al., 2005). Bile is an alkaline substance that is continuously secreted by liver and stored in the gall bladder in humans; the presence of bile plays an important role in the digestive system process. During the digestive process, the lipids are emulsified and solubilized by bile. In addition, bile has the capability to affect the cell membranes proteins and phospholipid structures and cause cellular homeostasis. Bile aids in the emulsification and solubilization of lipids in the gastrointestinal tract. In addition, it has the capability to affect the phospholipids and proteins of cell membranes and disrupt cellular homeostasis. Hence the ability to overcome the potentially lethal effects of bile is important for bacteria in order to survive and subsequently colonize the gastrointestinal tract (Begley et al., 2005; Hung and Mekalanos, 2005; Edwards and Slater, 2009). Recently, there has been increased evidence showing bile is been used as a signaling cue by enteric

bacteria to initiate virulence genes in host infection (Pope et al., 1995; Krukonis and DiRita, 2003; Prouty et al., 2004). Bile acids are a major component of crude bile that triggers the expression of bacterial virulence in the body. In this article, we aim to discuss how *Vibrio parahaemolyticus* senses bile in the human GI tract to regulate type III secretion system 2. This bile–bacteria interaction pathway gives us a clearer understanding of the biochemical and structural analysis of the bacterial receptors that takes action upon sensing the bile salts during an infection.

VIRULENCE FACTORS OF *Vibrio parahaemolyticus*

The bacterial protagonist in this story is *V. parahaemolyticus*, a Gram-negative, halophilic bacterium which naturally inhabits marine and estuarine environments worldwide (Zhang and Orth, 2013; Letchumanan et al., 2014). *V. parahaemolyticus* is recognized as the causative agent of foodborne gastroenteritis, a disease often associated with consumption of raw or undercooked seafood (Raghunath, 2015). Global climate change and rising ocean temperatures have led to the increase in the distribution of this pathogen worldwide (O'Boyle and Boyd, 2014). This is of concern as approximately half the reported foodborne cases in Asian countries are caused by *V. parahaemolyticus* (Alam et al., 2002; Bhuiyan et al., 2002). Frequent outbreaks of *V. parahaemolyticus* cases have also been reported in the United States and coastal countries of Europe such as Spain, Italy, and Norway (Caburlotto et al., 2008; Scallan et al., 2011; Ottaviani et al., 2013).

V. parahaemolyticus possess wide range of virulence factors that enables them to cause a gastrointestinal infection including adhesin (Liu and Chen, 2015), toxins, and secreted effectors (Zhang and Orth, 2013). These virulence factors play a vital role in the pathogenesis of the disease. During the initial host cell binding, adhesion is the first important step in bacterial pathogenesis (Liu and Chen, 2015). This factor is present on the surface of all *V. parahaemolyticus* to form a platform for them to attach onto host cell and secrete toxins during an infection (Broberg et al., 2011; Zhang and Orth, 2013; Letchumanan et al., 2014). The thermostable direct hemolysin (*tdh*) and TDH related hemolysin (*trh*) are the two major toxins found in *V. parahaemolyticus* (Honda et al., 1988; Nishibuchi et al., 1992; Okada et al., 2009). These two virulence toxins are believed to cause hemolysis and cytotoxic activity in a host cell (Broberg et al., 2011; Ceccarelli et al., 2013). The *tdh* is a pore-forming toxin which forms pores in the erythrocyte's membrane (Matsuda et al., 2010). The large pore size enables both water and ions to flow through the membrane (Honda and Iida, 1993). The subsequent alterations in ion flux in the intestine causes the diarrhea which is observed during an infection (Raghunath, 2015). Similar to the *tdh* gene, the *trh* gene also triggers cl^- channels resulting in altered ion flux during an infection (Takahashi et al., 2000). Both the *tdh* and *trh* are correlated with pathogenic *V. parahaemolyticus* strains, however, these genes do not completely account for the pathogenicity of *V. parahaemolyticus* (Lynch et al., 2005). There are several studies have reported that even in the absence of *tdh* and/or *trh* genes, *V. parahaemolyticus* strains remain virulent indicating the existence of other virulence factors (Jones et al., 2012; Pazhani et al., 2014). The thermolabile hemolysin (*tlh*), a type of phospholipase is another virulence toxin found in *V. parahaemlyticus* (DePaola et al., 2003; Zhang and Austin, 2005). Although the specific function of this gene in human infection remains unclear, *tlh* gene expression is upregulated under conditions mimic the intestinal environmental of human (Broberg et al., 2011; West et al., 2013). Hence, in the process of infection, *tlh* gene may be equally important as the *tdh* and *trh* genes.

The type III secretion system (T3SSs) is another important virulence factor of *V. parahaemolyticus* which is responsible for its pathogenicity (Broberg et al., 2011). This protein like structure has a secretion apparatus consisting of three main parts: the basal body that extends into the inner and outer membranes; a needle like structure that allows toxins to travel; and the translocon which is a pore injected into a target cell membrane (Izore et al., 2011). The T3SS1 and T3SS2 are the two main T3SSs encoded by *V. parahaemolyticus*. The cytotoxic T3SS1 is reported to be present in all *V. parahaemolyticus* and causes mouse lethality and possible initiation of autophagy (Park et al., 2004; Burdette et al., 2009; Hiyoshi et al., 2010). The enterotoxin T3SS2, on the other hand plays a vital part in determining the environmental fitness of strains (Hiyoshi et al., 2010; Matz et al., 2011). The T3SS2, *tdh* and *trh* are also known to be encoded on the pathogenicity island (Vp-PAI), signifying that *V. parahaemolyticus* acquires virulence determines through horizontal gene transfer (Okada et al., 2009; Matz et al., 2011). It is believed that the progression and severity of infection in humans are effected by the *V. parahaemolyticus* T3SS toxins (Ono et al., 2006). The strains that possess this needle-like T3SSs have the advantage of being able to secrete bacterial protein effectors directly into the host cell membrane and cytoplasm without facing the extracellular environment (Cornelis, 2006). In addition, the T3SS2 is suggested to be associated with *tdh*- and/or *trh*-positive *V. parahaemolyticus* strains (Raghunath, 2015). There are two distinct lineages of T3SS2 that have been described and associations were demonstrated of *tdh* with T3SSα and *trh* with T3SSβ (Park et al., 2004; Noriea et al., 2010). This could suggest that *V. parahaemolyticus* strains with the *tdh* and/or *trh* genes and T3SSs system have better ability to overcome host defenses in humans, conferring virulence that facilitates the development of infection.

Further analysis on the virulence properties has led to the discovery of type VI secretion systems encoded by T6SS1 and T6SS2 in *V. parahaemolyticus*. The T6SS1 is located on chromosome 1 where else, T6SS2 is located on chromosome 2 on *V. parahaemolyticus* RIMD 2210633 (Boyd et al., 2008; Izutsu et al., 2008). Salomon et al. (2013) proposed the role of T6SSs in *V. parahaemolyticus*. The T6SS1 is very active under warm marine-like conditions where else, T6SS2 is active under low salt conditions. It is also noted that surface sensing and quorum sensing differentially regulate both systems (Salomon et al., 2013). The T6SS2 and T3SS2 co-exist, suggesting the both systems may cooperate during an infection. T6SS2 takes the first step of

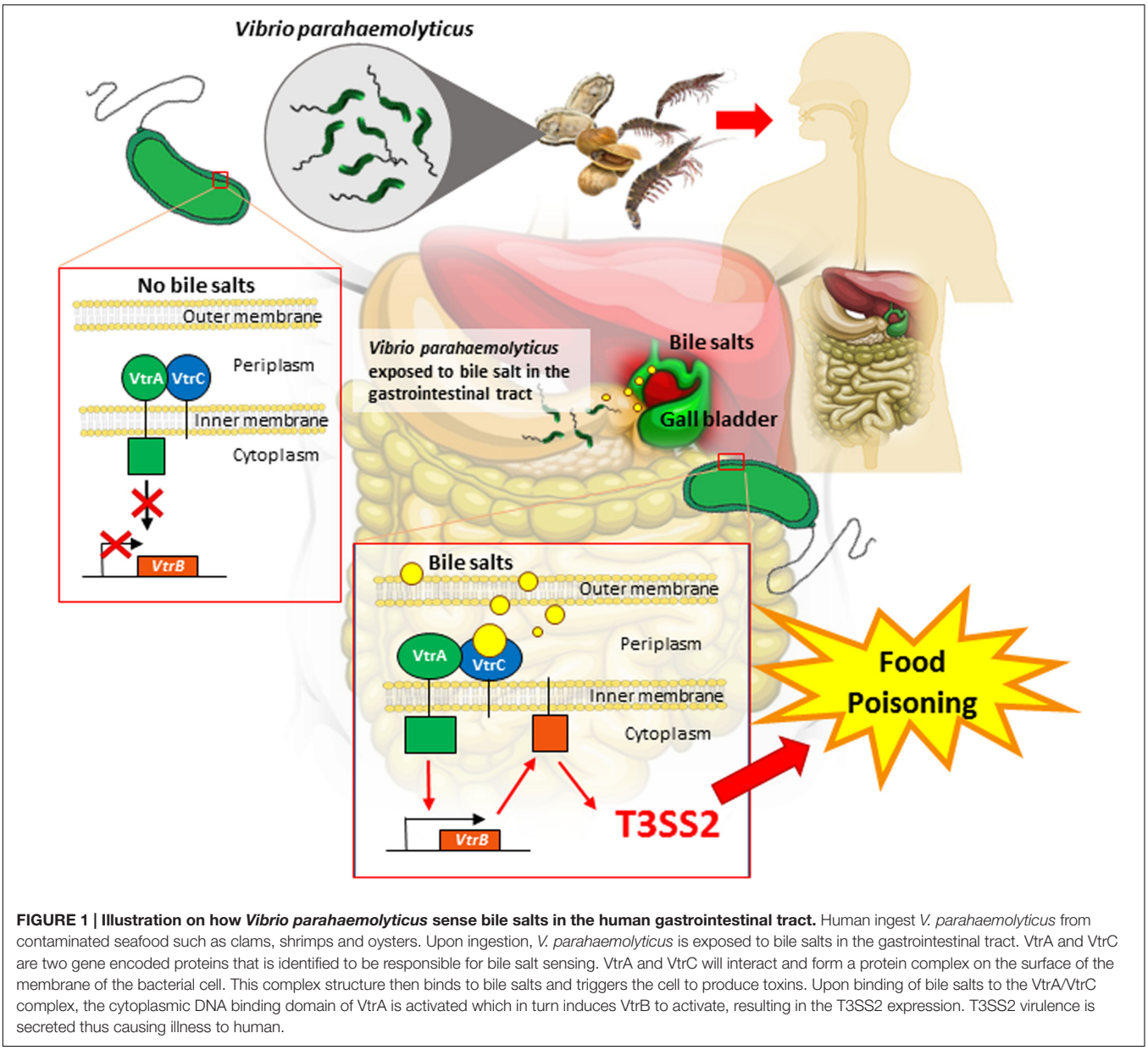

FIGURE 1 | Illustration on how *Vibrio parahaemolyticus* sense bile salts in the human gastrointestinal tract. Human ingest *V. parahaemolyticus* from contaminated seafood such as clams, shrimps and oysters. Upon ingestion, *V. parahaemolyticus* is exposed to bile salts in the gastrointestinal tract. VtrA and VtrC are two gene encoded proteins that is identified to be responsible for bile salt sensing. VtrA and VtrC will interact and form a protein complex on the surface of the membrane of the bacterial cell. This complex structure then binds to bile salts and triggers the cell to produce toxins. Upon binding of bile salts to the VtrA/VtrC complex, the cytoplasmic DNA binding domain of VtrA is activated which in turn induces VtrB to activate, resulting in the T3SS2 expression. T3SS2 virulence is secreted thus causing illness to human.

infection as a role of adhesion where else T3SS2 exports effectors by inducing enterocytotoxicity (Park et al., 2004; Yu et al., 2012).

THE SENSING OF BILE

V. parahaemolyticus with the virulence factors described are able to launch an attack on and cause illness to humans. Even with its arsenal of virulence factors, this bacterium still has to first survive the harsh conditions in the human gastrointestinal tract. It is suggested that exposure to harsh environmental conditions enables bacteria to be able to withstand the effects of bile in humans. The various pH conditions, temperatures and growth harden the bacteria toward the antimicrobial effects of bile in human. This will eventually increase their tolerance toward bile and the bacteria is able to survive in the human gastrointestinal tract. In addition, the bile levels in the human intestine are not constant and particularly in the presence of food, the bacteria would be less affected by the bile (Begley et al., 2005). Therefore, with these added advantages, it could be suggested that *V. parahaemolyticus* can indeed survive in the human gastrointestinal tract and regulate virulence during infections.

Bile is a bactericidal agent that are made up from various proteins, ions, pigments, cholesterol and bile salts. In an infection, the bile salts is believe to provide protection against bacteria (Merritt and Donaldson, 2009). When there is high amount of bile acids in the small intestine, the bacterial growth is inhibited (Inagaki et al., 2006). Where else, the growth of bacteria increases in the small intestine when bile is secreted in low amount, such seen in liver cirrhosis patients (Slocum et al., 1992). However,

enteric pathogens including *Vibrio* species have now developed a mechanism to resist the action off bile.

Gotoh et al. (2010) discovered the production of T3SS2 proteins are induced by bile under osmotic conditions similarly to the environments in gastrointestinal tract. They identified that the T3SS2 system is encoded in the pathogenicity island (Vp-PAI) and causes enterotoxicity effects to host cell. The VtrA and VtrB are the two transcriptional regulators that regulates encoded genes. Based on the study, *V. parahaemolyticus* initially recognizes its location in the human gastrointestinal tract by detecting bile acids. The transcription of Vp-PAI will be induced by bile acids via two main proteins, the VtrA and VtrB. The virulence genes then are regulated by the transduction of signals in the human intestinal tract (Gotoh et al., 2010). It was revealed that crude bile is a potent host derived inducer of *tdh* gene and T3SS2 under osmotic conditions corresponding to those in the intestinal tract.

Recently, Li et al. (2016) reported how *V. parahaemolyticus* has the ability to sense bile as an environmental cue to regulate its virulence mainly the T3SS2 during an infection. The study utilized bioinformatics tools to identify the proteins that are responsible for bile salt sensing and T3SS2 activation. **Figure 1** illustrates how the bacteria-bile sensing mechanism happens in the human body. VtrA and VtrC are two gene encoded proteins that is identified to be responsible for bile salt sensing. These two genes interact to form a protein complex on the surface of the membrane that surrounds the bacterial cell. The two proteins then create a barrel like structure that binds to bile salts and triggers the cell to produce toxins. Upon binding of bile salts to the hydrophobic chamber in the VtrA/VtrC complex, the cytoplasmic DNA binding domain of VtrA is activated which in turn induces VtrB to activate the T3SS2 virulence system. The VtrA/VtrC complex is described to be highly conserved in a group of diverse Vibrionaceae family (Li et al., 2016). Additionally, the study also found a family of monomeric lipid binding calycin domain proteins that has expanded to include an obligate heterodimer which binds to bile salts and can be utilized to transmit a signal. This increases the ability of *V. parahaemolyticus* to sense bile salt as an environmental cue to regulate virulence.

It is well understood that enteric bacteria including *V. parahaemolyticus* has the ability to sense bile which helps them identify their immediate environments and virulence factors can be expressed. *V. parahaemolyticus* releases toxins and type III secretion systems (T3SS2) in order to trigger virulence during an infection. This mode of mechanism ensures the survival of pathogenic *V. parahaemolyticus* in the environments and increase in the bacterial infections. However, this mechanism will cause more harm to we humans in future. Our own body defense fails to protect us against bacterial infections and on the other hand helps bacteria to release virulence. This situation will be worsened by the emergence of antimicrobial resistant strains in the environment which has become a major therapeutic challenge. As the effectiveness of treating bacterial infections declines, interest has been renewed toward using bacteriophages as a non-antibiotic approach to control the spreading of evolutionary *V. parahaemolyticus* strains worldwide (Wittebole et al., 2014; Letchumanan et al., 2016). Bacteriophage belonging to the *Siphoviridae* family is suitable in controlling *Vibrio* species (Letchumanan et al., 2016). This bacteriophage is highly specific to the bacterial host cell, do not affect or alter the gut microbiota (Hagens and Offerhaus, 2008), and safe to be consumed by humans. The phages are able to perform as a bio-control agent to control and inhibit virulence of pathogenic *Vibrio* species from clinical and environmental samples (Jassim and Limoges, 2014). In addition, the application of bacteriophage in the aquaculture industry can reduce the dependency of antibiotics and control the spreading of antimicrobial resistant bacteria in the environment (Letchumanan et al., 2016). The listed advantages make bacteriophage therapy a promising tool to control bacterial infections.

CONCLUSION AND FUTURE PERSPECTIVE

In summary, bile salts in human not only aid during digestion of food but possess antimicrobial activities as they have the ability to inhibit the survival of bacteria in the human gastrointestinal tract. However, certain conditions enable *V. parahaemolyticus* to develop resistance toward bile and eventually use bile as an environmental cue to regulate virulence. In order to treat infections, it is important to understand how *V. parahaemolyticus* senses bile salts and how this relates to their ability to regulate their virulence in the host during an infection. Given that there have been increasing numbers of multidrug resistant *Vibrio* strains from both clinical and environmental studies worldwide, drugs targeting suppression of bacterial virulence mechanisms should be designed instead of focusing on killing or inhibiting the growth of bacteria. Seen in this light, researchers will be able to design new drugs that may prevent the production of bacterial toxins and alleviate food poisoning symptoms. Future studies could focus on how other disease causing bacteria sense environmental cues to produce virulence during an infection.

AUTHOR CONTRIBUTIONS

VL performed the literature review and manuscript writing. KG-C, TK, SB, N-SAM, B-HG, and L-HL provided vital guidance and insight to the writing. The project was conceptualized by L-HL.

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Streptomyces Bacteria as Potential Probiotics in Aquaculture

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In response to the increased seafood demand from the ever-going human population, aquaculture has become the fastest growing animal food-producing sector. However, the indiscriminate use of antibiotics as a biological control agents for fish pathogens has led to the emergence of antibiotic resistance bacteria. Probiotics are defined as living microbial supplement that exert beneficial effects on hosts as well as improvement of environmental parameters. Probiotics have been proven to be effective in improving the growth, survival and health status of the aquatic livestock. This review aims to highlight the genus *Streptomyces* can be a good candidate for probiotics in aquaculture. Studies showed that the feed supplemented with *Streptomyces* could protect fish and shrimp from pathogens as well as increase the growth of the aquatic organisms. Furthermore, the limitations of *Streptomyces* as probiotics in aquaculture is also highlighted and solutions are discussed to these limitations.

Keywords: *Streptomyces*, probiotic, aquaculture, fish pathogens, antibiotic resistance

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INTRODUCTION

Statistics have revealed that the global aquaculture production continue to increase rapidly without the sign of reaching its peak. Meanwhile, the production from global capture fisheries has stabilized around 90 million tons since the mid-nineties (Mathieson, 2012). According to the United Nations Food and Agriculture Organization report (FAO, 2014), the global aquaculture production achieved another all-time high of 90.4 million tons including the 66.6 million tons of food fish and 23.8 million tons of aquatic algae in 2012 in response to the rising domestic and international seafood demand. Currently, it has been reported that food fish provides an average of one-fifth of total animal protein intake for the world population estimated at 7.3 billion people (Moffitt and Cajas-Cano, 2014). However, major disease outbreaks have been reported within the aquaculture sector in many part of the world due to the increased fish stocking density, over-crowding and lack of sanitary management with the rapid growth of aquaculture. The rapid spread of infections have led to global estimate of disease losses ranges about a quarter billion US$ annually (Bondad-Reantaso et al., 2005). For instance, the viral infections (white-spot syndromes, yellow head disease and taura syndrome) in shrimp industry has cost billions of dollars worldwide (Flegel, 2012; Lightner et al., 2012). Also, bacterial pathogens such as *Vibrio* sp. (*Vibrio harveyi, V. parahaemolyticus, V. campbellii*) caused luminous vibriosis in shrimp farms resulted in 50–100% mortality and vibrio infections in human (Shruti, 2012; Letchumanan et al., 2014; Wang et al., 2015).

Ever since the discovery of penicillin by Fleming in 1928 (Fleming, 1944), antibiotics have played unparalleled roles in disease prevention and treatment for human and animal

health and welfare. In addition to the use in human medicine, antibiotics are widely utilized in food animals and aquaculture either as prophylactic or for growth enhancement (Marshall and Levy, 2011). Therefore, antibiotics are extensively used to ensure the development of the intensive and large-scale aquaculture industry. However, the uncontrolled and indiscriminate use of antibiotics has given rise to the emergence of antibiotic resistant bacteria in the aquaculture (Huang et al., 2015; Letchumanan et al., 2015a,b,c) and aquaculture ponds also have been evidenced as reservoirs for antibiotic resistance genes (Tomova et al., 2015; Xiong et al., 2015). These antibiotic resistance genes can be acquired by human and animal pathogens via horizontal gene transfer (Tomova et al., 2015), hence leading to difficulty in the treatment of infectious diseases. Moreover, the recent evidences of residual antibiotics in the cultured organisms could pose a potential health risk to human consumers (Chen et al., 2015; Pereira et al., 2015; Pham et al., 2015).

In order to overcome the continuous emergence of antibiotic resistance pathogens due to abuse of antibiotics in aquaculture, an alternative to antibiotics is urgently needed for disease prevention and treatment and also improvement of quality and sustainability of aquaculture production. Extensive reviews have done indicating that probiotics could be a promising alternative for antibiotics in aquaculture, demonstrating beneficial effects to host by combating diseases, improving growth and also stimulating immune responses of host toward infections (Newaj-Fyzul et al., 2014; Hai, 2015). Therefore, the aim of this review is to provide an insight on the use of the genus *Streptomyces* bacteria as an alternative to antibiotics, being a probiotic in controlling diseases and improving the health and quality of aquaculture production. Furthermore, this review also discusses the prospects and limitations of *Streptomyces* species as a probiotic in aquaculture.

PROBIOTICS

The term 'probiotic' was initially defined as 'organisms and substances which contribute to intestinal microbial balance' (Parker, 1974). It was then revised as 'live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance' (Fuller, 1989). Meanwhile, in the case of aquatic animals which have much closer interactions with the external environment as compared to the terrestrial organisms, the external environment and feeding have substantial impacts on the microbial status of the aquatic animals. Hence, Verschuere et al. (2000) suggested that a probiotic for aquatic environments should be known as a live microbial adjunct exhibiting beneficial effect on the host by modulating the host-associated or ambient microbial community. Lately, probiotic was described as live, dead or component of a microbial cell that exerts beneficial effect on host by improving disease resistance, growth performance, feed utilization and health status, through the achievement of microbial balance in both host and ambient environments (Hai, 2015). Literatures have showed the possible mode of action of probiotics in aquaculture include (i) growth promoter, (ii) production of inhibitory compounds, (iii) improvement in nutrient digestion, (iv) water quality improvement, (v) enhancement of immune response, and (vi) competition for nutrient (Defoirdt et al., 2007; Martínez Cruz et al., 2012). In order to achieve a probiotic status, the microbes have to fulfill a number of criteria in term of their biosafety and functionality. The desirable characteristics of a potential probiotic include; (i) not harmful toward the host; (ii) ability to survive during transport to the active site; (iii) capability of colonizing and proliferating within the host; (iv) no virulence genes or antibiotic resistance genes (Hai, 2015). The common microorganisms used as probiotics are *Lactobacillus acidophilus, Lactobacillus casei, Bacillus* sp., *Bifidobacterium bifidum*, *Lactococcus lactis* and also the yeast, *Saccharomyces cerevisiae* (Ouwehand et al., 2002; Salamoura et al., 2014). However, less attention has been put on the use of *Actinobacteria* as probiotics in aquaculture despite being widely known as prolific producer for secondary metabolites, particularly the genus (Butler, 2008). The genus *Streptomyces* demonstrated promising results as probiotics (Das et al., 2010; Augustine et al., 2015). This review aim to discuss the prospects of using *Streptomyces* as a probiotic candidate in aquaculture. **Table 1** summarizes all the features and mechanism of actions of the probiotic effects evidenced in the genus *Streptomyces*.

STREPTOMYCES SP. AS PROBIOTICS IN AQUACULTURE

The genus *Streptomyces* (phylum: *Actinobacteria*) are Gram-positive, high G + C (70%) genome content, soil-living bacteria with characterized branching filamentous morphology. *Streptomyces* sp. has been widely recognized as industrially important microorganism due to its potential in producing diverse range of secondary metabolites (Lee et al., 2014b; Ser et al., 2015a,b; Tan et al., 2015) including antibiotics (Lee et al., 2014a), antitumor agents, antiparasitic, immunosuppressive agents, and enzymes (Manivasagan et al., 2013). The production of a variety of wide-spectrum chemical compounds as demonstrated by *Streptomyces* has the advantage of producing potential antagonistic and antimicrobial compounds that can be valuable as probiotics in aquaculture. The ability of producing antagonistic compounds may help the probiotics to compete for nutrients and attachment sites in the host. For instance, the production of bacteriocins (Desriac et al., 2010), siderophores (Lalloo et al., 2010), enzymes (protease, amylase, lipase; Augustine et al., 2015), hydrogen peroxide (Sugita et al., 2007) and organic acids (Sugita et al., 1997) have been documented from the probiotics used in aquaculture. You et al. (2005) reported a *Streptomyces* sp. with siderophores producing activities and suggested that the use of this *Streptomyces* sp. can influence the growth of pathogenic *Vibrio* sp. by competition for iron in the aquatic environment. Siderophores are ferric ion-specific chelating agents with low molecular masses that are usually produced by microorganisms and plants under Fe-limiting conditions (Ahmed and Holmstrom, 2014). Probiotics with the capability of producing siderophores are believed to outcompete the pathogens by limiting the bioavailability of iron

TABLE 1 | The probiotic effects demonstrated by *Streptomyces* bacteria through different mechanism of actions.

Features/Mechanism of actions	Probiotic *Streptomyces* bacteria	Outcomes	References
Antagonistic compounds production • Siderophore production	*Streptomyces cinerogriseus* A03 and A05 *Streptomyces griseorubroviolaceus* A26 and A42 *Streptomyces lavendulae* A41 *Streptomyces roseosporus* A45 *Streptomyces griseofuscus* B15	• All the strains positive for siderophore production, detected using CAS-agar • Displayed antagonistic activity toward *Vibrio* species tested (*V. harveyi*, *V. nereis*, *V. fluvialis*, *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus* and *V. anguillarum*) ranging from <10 mm to >30 mm inhibition zones • Suggested the ability of the siderophore-producing *Streptomyces* strains controlled the *Vibrio* pathogens by competing for iron in the marine environment	You et al., 2005
• Anti-biofilm and anti-quorum sensing activity	*Streptomyces albus* A66	• Attenuated the biofilm formation of *V. harveyi* with inhibition rate of 99.3% at 2.5% (v/v) • Dispersed the mature biofilm of *V. harveyi* with degradation rate of 75.6% at 2.5% (v/v) • Suggested the anti-biofilm activity demonstrated by *Streptomyces* A66 through the degradation of the quorum-sensing factor *N*-AHSL (*N*-acylated homoserine lactone)	You et al., 2007
• Anti-virulence activity	*Streptomyces* sp. K01-0509	• Produced guadinomine B, a type III secretion system inhibitor of Gram-negative bacteria, including *Vibrio* sp., with IC_{50} at 14 nM	Iwatsuki et al., 2008
• Anti-viral activity	*Streptomyces* sp. AJ8	• Administrated intramuscularly ethyl acetate extract of the secondary metabolite reduced the white spot syndrome virus load significantly (85%) in the *Fenneropenaeus indicus* after third day of injection	Jenifer et al., 2015
Exoenzyme secretion	*Streptomyces* CLS-28 *Streptomyces* CLS-39 *Streptomyces* CLS-45	• All strains showed good proteolytic activity and variable amylolytic and lipolytic activities • Suggested to facilitate the feed utilization and digestion of the host, resulting in increased weight of *Penaeus monodon* when incorporated in the feed	Das et al., 2010
Growth enhancing effect	*Streptomyces fradiae* and *Streptomyces* sp.	• Improved growth of post-larval shrimp *P. monodon* and ornamental fish, *Xiphophorus helleri* • Produced growth-promoting hormone, indoleacetic acid which enhanced growth of *X. helleri*	Dharmaraj and Dhevendaran, 2010; Aftabuddin et al., 2013
Low pH tolerance and intestinal enzymes resistance	*Streptomyces* sp. JD9	• Showed excellent viability at pH 2 • Displayed resistance to pepsin at 3 mg/mL, bile at 0.3% and pancreatin at 1 mg/mL • Demonstrated good survivability in gastrointestinal conditions	Latha et al., 2015
Water quality amelioration	*Streptomyces fradiae* *Streptomyces* sp. *Streptomyces* CLS-28	• Reduced the ammonia level in the water • Increased the total heterotrophic bacterial populations in the water which helped to accelerate the decomposition of waste materials	Das et al., 2006, 2010; Aftabuddin et al., 2013
Single cell protein	*Streptomyces* sp.	• Used as a protein source for host, increased food conversion rate and food conversion efficiency, enhanced growth performance	Dharmaraj and Dhevendaran, 2010; Suguna, 2012; Selvakumar et al., 2013
In vivo protection/challenge experiment	*Streptomyces* CLS-28 *Streptomyces* CLS-39 *Streptomyces* CLS-45	Protection of *Artemia* against *V. harveyi* • *V. harveyi* at 10^6 CFU/mL killed all *Artemia nauplii* in 72 h • Addition of *Streptomyces* strains [at 1% (v/v)] increased the survival of *Artemia nauplii* by 67% and adults by 61% after 72 h exposure to *V. harveyi* at 10^6 CFU/mL Protection of *P. monodon* against *V. harveyi* • *V. harveyi* at 10^7 CFU/mL killed 55% of *P. monodon* after 5 days exposure • *Streptomyces* CLS-28 incorporated in the feed (after feeding for 15 days) increased the survival of *P. monodon* by 67% compared to control (without *Streptomyces*) in 5 days exposure	Das et al., 2010

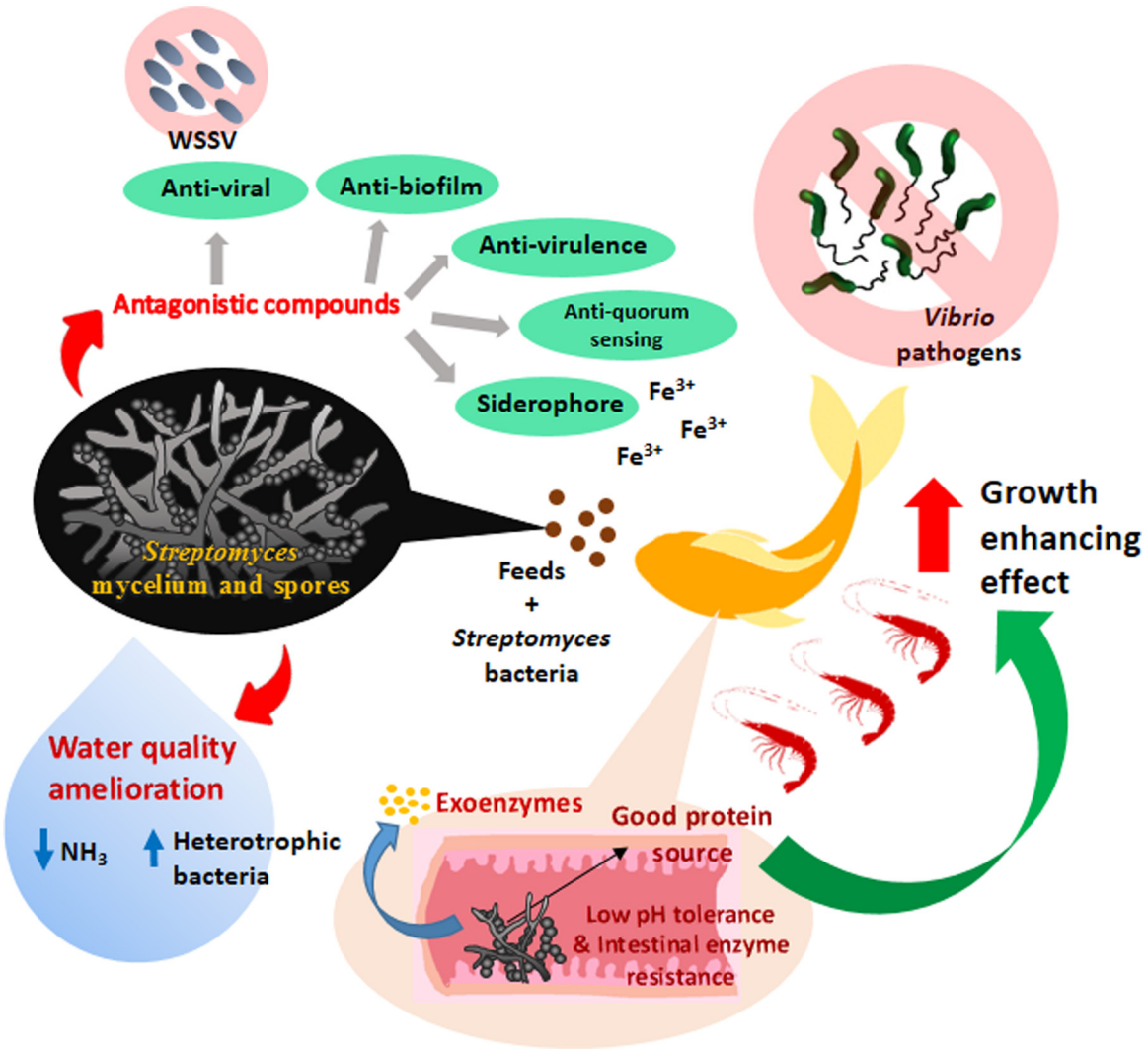

FIGURE 1 | The mechanism of action of probiotic effects of *Streptomyces* bacteria and their applications in aquaculture. The *Streptomyces* as probiotic in aquaculture could protect the livestock from pathogens through the production of various antagonistic compounds (e.g., anti-biofilm, anti-quorum sensing and anti-virulence) against *Vibrio* pathogens. *Streptomyces* probiotics attenuate the growth of pathogens by producing siderophores which reduce the bioavailability of iron (Fe^{3+}) for the pathogens in the aquatic environment. Anti-viral compounds are also produced by *Streptomyces* probiotic to prevent viral infection caused by white spot syndrome virus (WSSV) in aquaculture. Besides, the *Streptomyces* probiotics also play a role in ameliorating the water quality of aquaculture. *Streptomyces* probiotics help to regulate the microflora, especially to increase the protein mineralizing and ammonifying bacterial populations which accelerate the decomposition process of wastes materials and also ameliorate the water quality by reducing the ammonia level (NH_3). The consumption of feeds incorporated with the low pH tolerance and intestinal enzymes resistance *Streptomyces* probiotics could enhance the growth performance of the livestock by providing good protein sources. *Streptomyces* probiotics exhibit the ability to secrete hydrolytic exoenzymes which improve the amylolytic and proteolytic activity in the digestive tract of the livestock for more efficient use of the feed; eventually contribute to better growth performance of the livestock.

and resulting in growth attenuation of the pathogens as iron is essential for growth as well as biofilm formation (Weinberg, 2004). In addition, *Streptomyces* was also evidenced in the production of inhibitory compounds and metabolites involved in the attenuation of biofilm formation, anti-quorum sensing activity (You et al., 2007) and anti-virulence activity in *Vibrio* sp. (Iwatsuki et al., 2008). Besides displaying inhibitory effect on bacterial pathogens in aquaculture, *Streptomyces* also has been report to exhibit anti-viral activity, specifically against the white-spot syndrome virus (WSSV; Jenifer et al., 2015).

Streptomyces is primarily saprophytic, living in diverse soil habitats with the development of branching hyphal filaments under conducive environment (Flardh and Buttner, 2009). This unique growth adaptation allows *Streptomyces* in colonization of the solid substrates by adhering and penetrating to gain access on insoluble organic materials in the soil (Flardh and Buttner, 2009). Different hydrolytic enzymes such as amylase, protease and lipase can be produced by *Streptomyces* to break down the insoluble organic materials to provide nutrients for the formation of densely packed substrate mycelium which

is reused to fuel the reproductive phase of aerial growth in producing chains of spores (Chater et al., 2010). These unique physiological adaptations of *Streptomyces* are believed to make them as potential probiotics such as the secretion of exoenzymes which may be helpful in facilitating the feed utilization and digestion once they colonize the host intestine in aquaculture. Das et al. (2010) demonstrated that the feed incorporated with *Streptomyces* increased the weight of *Penaeus monodon* shrimp, suggesting that these *Streptomyces* sp. secreted hydrolytic exoenzymes to improve the amylolytic and proteolytic activity in the shrimp digestive tract for more efficient use of the feed. The feed supplemented with *Streptomyces fradiae* isolated from mangrove sediment was also shown to enhance the growth of the post-larval *P. monodon* (Aftabuddin et al., 2013). Besides showing good growth promoting effects in shrimp, all the feeds supplemented with *Streptomyces* was also shown to improve growth performance of the ornamental fish, *Xiphophorus helleri* (red swordtail fish) after 50 days of feeding trial when compared to control without the *Streptomyces* sp. (Dharmaraj and Dhevendaran, 2010). Furthermore, the similar study also showed that the production of growth-promoting hormone, indoleacetic acid by the *Streptomyces* sp. could be contributed to the better growth rate as demonstrated by *Xiphophorus helleri* fed with *Streptomyces* supplemented feeds (Dharmaraj and Dhevendaran, 2010).

The formation of enzymatic digestion, sonic vibration and desiccation-resistant spores demonstrated by *Streptomyces* are also some of the attractive features for this genus of bacteria to resist the harsh environment conditions (McBride and Ensign, 1987), thereby allowing them to retain longer shelf life in the aquaculture ponds before being taken up or to resist the low pH in the gastrointestinal tracts of the animals. However, it should be noted that *Streptomyces* spore is only resistant to moderately high temperature (McBride and Ensign, 1987) as compared to the highly heat resistant endospores of *Bacillus* sp. which is compositionally and physiologically different from the *Streptomyces* spore. Nevertheless, Latha et al. (2015) reported that the *Streptomyces* sp. isolated from fecal sample of chicken showed excellent viability at pH 2, exhibited strong pepsin resistance (at 3 mg/mL) as well as the resistance toward both bile (at 0.3%) and pancreatin (at 1 mg/mL), suggesting that strains from the animal internal cavities would be better in adapting and colonizing the gastrointestinal of the animals. This is also demonstrated by Das et al. (2010) which isolated *Streptomyces* sp. from the sediment of the shrimp culture system able to reach the digestive system of the shrimp, hence allow easier establishment and growth of the probionts in the host. These findings indicate that the spore-forming capacity of *Streptomyces* with high acidity and bile acids tolerance makes them a more practical alternative than those bacteria with non-spore forming capability and further ascertain the potential of *Streptomyces* as probiotic in aquaculture (Das et al., 2010).

The *in vivo* challenge experiment conducted further proved that *Streptomyces* should be spotlighted as probiotics in aquaculture (Das et al., 2010). This study successfully demonstrated the protection effect of *Streptomyces* on both juvenile and adult *Artemia* (15 days old) from *Vibrio* pathogens. The study showed that the *Streptomyces* at 1% concentration (v/v) resulted in higher survival rates than the untreated control group of *Artemia* after challenged with *V. harveyi* or *V. proteolyticus* at 10^6 CFU/mL. The protective response shown by the study suggests that *Streptomyces* could be administrated to target organisms through bioencapsulation in *Artemia* as a vector for supplementing the beneficial *Streptomyces* probiotics in aquaculture. Bioencapsulation of probiotics in live food such as *Artemia* and rotifers was demonstrated to be more effective in delivery of the probiotics to the digestive tract of the target aquaculture organisms by previous studies (Gatesoupe, 2002; Suzer et al., 2008). The study also further evaluated the efficacy of the *Streptomyces* in protecting the shrimp *P. monodon* from the *Vibrio* pathogens. The feed supplemented with *Streptomyces* sp. CLS-28 for 15 days was found to be exerting protection effect on shrimp *P. monodon* against the 12 h challenge of *V. harveyi* (LD_{50} at $10^{6.5}$ CFU/mL; Das et al., 2010). A more recent study reported a marine *S. rubrolavendulae* M56 (accession number KJ403746) was shown to exhibit antagonistic activity against all four *Vibrio* sp. including *V. harveyi, V. alginolyticus, V. parahaemolyticus* and *V. fluvialis* in an *in vitro* co-culture experiment (Augustine et al., 2015). In order to confirm the *in vitro* findings, Augustine et al. (2015) demonstrated that the biogranules *S. rubrolavendulae* M56 resulted in lower percentage of mortality of *P. monodon* post-larvae with the reduction of viable *Vibrio* sp. in the culture system after 28 days.

The build-up of ammonia and nitrite level is a major water quality problem which has considerable effects on the health status of the aquaculture livestock due the accumulation of metabolic waste of cultured organisms and the decomposition of the residual feed. The probiotic *Streptomyces* was also found to regulate the microflora of the aquaculture water besides controlling the pathogenic microorganisms and resulted in a better pond conditions. Literature showed that the application of probiotic product did not adversely affect the microflora of aquaculture in turn increased the protein mineralizing and ammonifying bacterial population which help to accelerate the decomposition process of the accumulated wastes materials (Devaraja et al., 2002). Several studies also demonstrated similar results indicating the reduction of ammonia level and increased in the total heterotrophic bacteria in the ponds/tanks treated with the probiotic *Streptomyces* as compared to the control ponds/tanks (Das et al., 2006, 2010; Aftabuddin et al., 2013). These findings suggested that *Streptomyces* could be applied as probiotics which ameliorate the water quality of aquaculture indirectly improve the growth performance and yield of the cultured organisms.

Traditionally, fish meal has been an indispensable ingredient in commercial aquaculture feeds due to its high protein content with excellent amino acid profile and is highly digestible (Gatlin et al., 2007). However, current feed formulations have shifted to other alternative protein source due to the high cost and limited availability of fish meal. Microbial single cell protein of

Streptomyces is one of the alternative sources of protein and has been utilized and evaluated for better food conversion efficiency and growth for fish (Suguna, 2012; Selvakumar et al., 2013) and shrimp (Manju and Dhevendaran, 1997). Dharmaraj and Dhevendaran (2010) suggested that the use of *Streptomyces* not only showing beneficial effects as probiotic in aquaculture, the incorporation of *Streptomyces* in the feed is also a cost effective approach as the probiotic bacteria replaced around 30–40% of the fish meal used in a feed. The study demonstrated that *Streptomyces* can be a cheaper alternative protein source in the aquaculture feed (Dharmaraj and Dhevendaran, 2010).

LIMITATIONS OF *STREPTOMYCES* AS PROBIOTIC IN AQUACULTURE

Geosmin and MIB (2-methylisoborneol) are two common semivolatile terpenoid compounds that exhibit earthy/musty taste and odor produced by *Streptomyces* have been known to reduce the palatability of the cultured livestock and negative impact for aquaculture industries (Auffret et al., 2011). These off-flavor compounds are known to be absorbed and bio-accumulated in the gills, skin and flesh of fish up to 200- to 400-folds as compared to the ambient concentration, resulting in lower commercial value of the fish (Howgate, 2004). Many efforts have been shown in literatures for the removal of these earthy odor compounds from water involving the use of powdered activated carbon, ozonation and biofiltration (Elhadi et al., 2004). Among these technologies, ozonation is suggested to effective in this case with the use of *Streptomyces* as the probiotics in aquaculture. Ozone has been known to remove odorants such as geosmin and MIB from water via oxidation (Gonçalves and Gagnon, 2011). A study has demonstrated that the combined effect of ozonation (at 0.3 mg O_3/L ROC) and probiotic diets (*Bacillus* sp. S11) was able to protect shrimp *P. monodon* from *Vibrio* challenge test without harming shrimp and the probiotic bacteria in the internal system of shrimp (Meunpol et al., 2003).

Furthermore, the risk of lateral gene transfer of antibiotic resistance genes could be an argument against the use of *Streptomyces* as probiotic in aquaculture. Despite that, there are increasing reports on the antibiotic resistance developed by most of the commonly used probiotics such as *Lactobacillus* sp. (Sharma et al., 2015), *Bifidobacterium* sp. and *Bacillus* sp. (Gueimonde et al., 2013). Furthermore, studies also reported that the antibiotic resistance phenotypes displayed by the probiotic *Streptomyces* strains were generally conferred by their intrinsic resistance properties (Das et al., 2010; Latha et al., 2015). Hence, systematic screening for potential antibiotic resistance gene determinants in potential probiotics genome has to be conducted to assess the potential risks and mobility. Furthermore, curative strategies can be valuable tool to remove the genetic element that harbor antibiotic resistance from the relevant probiotic strains (Morelli and Campominosi, 2002; Rosander et al., 2008). For instance, Rosander et al. (2008) successfully demonstrated the protoplast formation curing method able to remove two resistant plasmids from the parent *Lactobacillus reuteri* (ATCC 55730) and without affecting the probiotic properties of the strain. All in all, *Streptomyces* can be one of the interesting probiotics to be further exploited as an alternative to antibiotics in maintaining a sustainable aquaculture.

CONCLUSION AND FUTURE WORK

To date, the number of study employs *Streptomyces* as probiotics in aquaculture is still limited although promising results have been represented by previous studies. A schematic figure is also illustrated to show the mechanism of action of the probiotic effects demonstrated by the *Streptomyces* in aquaculture (**Figure 1**). In order for *Streptomyces* being included among the commonly used biological control agents in aquaculture, further extensive trials are still required to establish the probiotic nature of *Streptomyces* in disease prevention and growth enhancement of aquaculture animals. Furthermore, a better understanding is needed on the exact mode of action of *Streptomyces* involved in probiotic effects. Hence, further research could focus more on molecular techniques to elucidate the possible underlying mechanism portrayed by *Streptomyces* probiotic in aquaculture settings.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Insights into Bacteriophage Application in Controlling *Vibrio* Species

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Bacterial infections from various organisms including *Vibrio* sp. pose a serious hazard to humans in many forms from clinical infection to affecting the yield of agriculture and aquaculture via infection of livestock. *Vibrio* sp. is one of the main foodborne pathogens causing human infection and is also a common cause of losses in the aquaculture industry. Prophylactic and therapeutic usage of antibiotics has become the mainstay of managing this problem, however, this in turn led to the emergence of multidrug resistant strains of bacteria in the environment; which has raised awareness of the critical need for alternative non-antibiotic based methods of preventing and treating bacterial infections. Bacteriophages – viruses that infect and result in the death of bacteria – are currently of great interest as a highly viable alternative to antibiotics. This article provides an insight into bacteriophage application in controlling *Vibrio* species as well underlining the advantages and drawbacks of phage therapy.

Keywords: bacterial, *Vibrio* sp., antibiotics, bacteriophages, multidrug resistant strains

INTRODUCTION

The increased occurrence of foodborne disease has led to substantial morbidity and mortality around the world yearly, frequently associated with outbreaks or food contamination. Foodborne illness is known to be a ubiquitous, costly, yet preventable public health concern (Centers for Disease Control and Prevention [CDC], 2014). World Health Organization has stated that food safety remains an endless challenge to everyone particularly in the management of infectious and non-infectious foodborne pathogens (Rocourt et al., 2003). Despite the current effective technologies and the good manufacturing practices, the food safety is constantly threatened by the factors related to changes in lifestyle, consumer eating habits, food and agriculture manufacturing processes and also the increased international trade (Newell et al., 2010; Law et al., 2015).

There is no doubt that bacterial infection is a significant threat to mankind in many forms – human illness as a result of bacterial infection is common, with *Vibrio* species including *Vibrio cholerae*-associated from food contamination or transmission of infection from person

to person, *Vibrio parahaemolyticus*- associated with food contamination and *Vibrio vulnificus*- associated with wound infection. *Vibrio* species are gram-negative curved rod shaped bacteria that belong to the *Vibrionaceae* family. They naturally inhabit the estuarine, coastal and marine environment worldwide (Letchumanan et al., 2014; Raghunath, 2015). The presence of this bacterium in the marine environment raises the concern of human on food safety due to the latter potential in causing disease outbreaks depending on the environmental conditions (Ceccarelli et al., 2013). There are many clinically used antibiotics as a choice of treatment for *Vibrio* species infections including cephalothin (first generation cephalosporins), cefuroxime (second generation cephalosporin), cefotaxime and ceftazidime (third generation cephalosporins), tetracycline, doxycycline, or fluoroquinolone (Tang et al., 2002; Han et al., 2007; Al-Othrubi et al., 2014).

Aside from this, these organisms have also been responsible for large scale losses in the aquaculture industry due to infection of the aquatic livestock leading to prophylactic as well as therapeutic use of antimicrobials (Devi et al., 2009; Manjusha and Sarita, 2011; Letchumanan et al., 2014, 2015b). In the Asian aquaculture industry, oxytetracycline, tetracycline, quinolones, sulphonamides, and trimethoprim are among the antimicrobials permitted and utilized to control bacterial infections (Rico et al., 2012; Yano et al., 2014). Our dependence on antibiotics to control bacterial infections in humans, aquaculture, agriculture, and veterinary medicine resulted to indiscriminate use which in turn led to the emergence of multidrug resistant strains in the biosphere (Rao and Lalitha, 2015).

Studies have reported the isolation of multidrug resistant *Vibrio* strains from both clinical and environmental samples (Letchumanan et al., 2015a; Shrestha et al., 2015; Zavala-Norzagaray et al., 2015). In Iran, *Vibrio cholerae* isolated from clinical samples has been reported to be resistant toward erythromycin, sulfamethoxazole-trimethoprim, and ampicillin (Tabatabaei and Khorashad, 2015). Antibiotic resistance was also observed in a study done in India which reported serogroups O1 of *Vibrio cholerae* classical biotype and sub serotype, Ogawa isolated from clinical strains were resistant to ampicillin, nalidixic acid, and cotrimoxazole (Shrestha et al., 2015). This bacterium is the causative agent of cholera and appears to be emerging as the etiological agent of disease outbreaks in many developing countries such as India (Garg et al., 2000), Bangladesh, Haiti (Sjölund-Karlsson et al., 2011), Vietnam (Tran et al., 2012), and Africa (Dalsgaard et al., 2001). *Vibrio cholerae* from clinical samples is reported to be resistant to many clinically used antibiotics including tetracycline (Roychowdhury et al., 2008), ampicillin (Petroni et al., 2002), nalidixic acid (Khan et al., 2015), streptomycin, sulfonamide, trimethoprim, gentamicin (Dalsgaard et al., 1999), and ciprofloxacin (Khan et al., 2015).

A study in Thailand has revealed that shrimp farmers were highly dependent on various antibiotics as a preventive measure against shrimp bacterial infections with 14% of farmers using antibiotics on a daily basis in their farms (Holmstrom et al., 2003). In Malaysia, *Vibrio parahaemolyticus* strains isolated from seafood and environmental sources were reported to be resistant toward cefalexin and ciprofloxacin (Al-Othrubi et al., 2011). Besides, antibiotic resistant *Vibrio parahaemolyticus* strains have been isolated from both clinical and environmental samples in India (Pazhani et al., 2014; Reyhanath and Kutty, 2014; Sudha et al., 2014). A study assessed the diversity of antibiotic resistant bacteria and their resistance genes from mariculture environments of China. It was reported that the strains exhibited multidrug resistance profile toward oxytetracycline, chloramphenicol, and ampicillin (Dang et al., 2007). Frequent use of antibiotics is also widely apparent in other regions such as Mexico (Roque et al., 2001), Philippines (Tendencia and De La Pena, 2001), Italy (Lalumera et al., 2004), Malaysia (Al-Othrubi et al., 2011; Sani et al., 2013; Letchumanan et al., 2015b), Thailand (Yano et al., 2014), and China (Peng et al., 2010; Zou et al., 2011; Xu et al., 2014). The various antibiotics used in aquaculture has led to the occurrence of antibiotic resistant genes (ARGs) in bacteria. Many different ARGs can be found in bacteria in the environment. For example, β-lactam and penicillin resistant genes *penA* and *blaTEM-1* (Srinivasan et al., 2005; Zhang et al., 2009), chloramphenicol resistant genes *cat*I, *cat*II, *cat*III, *cat*IV, and *floR* (Dang et al., 2007, 2008), tetracycline resistant genes *tatA, tatB, tatC, tatD, tatE, tatG, tatH, tatJ, tatY, tatZ*, and many more (Macauley et al., 2007; Zhang et al., 2009, 2012; Kim et al., 2013). It is reported that ARGs could be transferred among bacteria via conjugation, transduction, or transformation (Manjusha and Sarita, 2011).

The widespread of emergence of antimicrobial resistant bacteria worldwide has become a major therapeutic challenge (Giamarellou, 2010). There is need for development of novel non-antibiotic approach to fight against bacterial infections due to the shortage of new antibiotics in developmental pipeline (Rice, 2008; Freire-Moran et al., 2011). Recently, there has been renewed interest in the application of bacteriophage as a non-antibiotic approach to control bacterial infections in various fields including human infections, food safety, agriculture, and veterinary applications (Wittebole et al., 2014). This article provides an insight into bacteriophage application in controlling *Vibrio* species as well underlining the advantages and drawbacks of phage therapy.

BACTERIOPHAGES

Historical Background

Early discovery of bacteriophage was reported by M. E. Hankin in 1896 after observing antibacterial properties of this viral-like agent against *Vibrio cholerae* in Ganges River, India (Adhya and Merril, 2006). The phage's nature was clearly defined following the observation of its capability of lysing bacterial cultures by Frederick Twort and Felix d'Herrelle, in 1915 and 1917, respectively (Adhya and Merril, 2006). It was Felix d'Herrelle who named this viral-like agents as bacteriophage and implemented in the treatment of human diseases almost instantly after their discovery. Bacteriophage therapy appeared as the frontline therapeutics against infectious disease before the discovery of the broad spectrum antibiotic and were used in various countries until The Second World War (Enderson et al., 2014). Unfortunately, the use of phages as therapeutic agents and

phage research declined due to the limited knowledge of phage properties, contradictory results from various published studies and discovery of antibiotics (Wittebole et al., 2014). However, over the last decade, the therapeutic value of bacteriophage has been reconsidered due to the occurrence of multi-drug resistant bacteria. Bacteriophages are regarded as an alternative non-antimicrobial tool to treat bacterial infections while controlling the emergence of antibiotic resistance (Meaden and Koskella, 2013; Payet and Suttle, 2014). The research into phage therapy has been further encouraged given that regulatory bodies in charge of food safety have approved the utilization of certain phages for use in food products such as ListShield^{TM}, and Listex P100 (Bren, 2007; Coffey et al., 2010). ListShield^{TM}, (a phage which targets *Listeria*) from Intralytix is approved by the USFDA for the treatment of food products, and the phages are classified as Generally Recognized As Safe (GRAS; FDA, 2013).

Morphology

Phages are bacterial viruses that are able to infect bacterial host cells with high host specificity of strain or species level (Hagens and Loessner, 2010) and subsequently multiply, eventually resulting in death of the host cell. While high host specificity is typical, a few phages do exhibit wide host ranges and are able to infect a large subset of a given species or even multiple species (Chen and Novick, 2009). Bacteriophages species can be differentiated as they vary both in size 24–400 mm in length and genome length. All bacteriophages have a head that stores genetic materials and form a part of the overall feature of a bacteriophage (Orlova, 2012). Structurally, a phage consist of a core nucleic acid encapsulated with a protein or lipoprotein capsid which is connected with a tail that interacts with various bacterial surface receptors via the tip of the tail fibers. This interaction shows an affinity that is specific to a certain group of bacteria or even to a particular strain (Deresinski, 2009; Tan et al., 2014). The capsid is icosahedral in shape and has the main function to protect the genetic material from the environment. A bacteriophage head is attached to a tail through a connector that functions as adaptor between these two structures of the phage. The tail is a hollow tube which acts as a passage way for genetic materials to pass thru from capsid to host bacteria (Lurz et al., 2001). Tail fibers and base plate which are located at the end structure of the phage are involved in the binding process of the phage to the bacterial outer membrane (Sao-Jose et al., 2006).

Nature of Bacteriophages

Bacteriophages are the most abundant organisms in the environment, with the total number of phages on Earth estimated to be between 10^{30} and 10^{31}, an approximately 10 times more than their bacterial hosts (Abedon et al., 2011; Burrowes et al., 2011). Phages are natural predators of bacteria, self-limiting and self-replicating in their host cell, and can adapt to resistant bacteria (Carvalho et al., 2010; Jaiswal et al., 2014). They are commonly found in large numbers wherever their hosts live; in sewage, in soil, in hatchery, in deep thermal vents, or in natural bodies of water (Karunasagar et al., 2007; Kim et al., 2012; Rong et al., 2014). To date, most of the marine viruses reported are bacteriophages that belong to order *Caudovirales*, which is divided into three families: *Siphoviridae* (icosahedral capsid with filamentous non-contractile tail), *Myoviridae* (icosahedral symmetrical head with a helical contractile tail separated by neck) and *Podoviridae* (icosahedral symmetrical head with very short non-contractile tail; Suttle, 2005; Rao and Lalitha, 2015).

Genetic and Genome of Phages

Bacteriophages are viruses with either DNA or RNA as their genetic material. They appear in both single and double stranded forms. The structure is similar to the living organisms found in the environments; with a polynucleotide chain consisting of a deoxyribose (or ribose) phosphate backbone to which are attached to a specific sequence of the four nucleotides – adenine, thymine (or uracil), guanine, and cytosine. It is exceptional in single stranded phages where two complementary chain are paired together in a double helix (Benett and Howe, 1998).

The complete genome sequence of the T4-like phage, vibriophage KVP40 has been studied in Japan. This vibriophage belong to *Myoviridae* family has a double-stranded DNA genome sequence in length of 244,835 bp, a prolate icosahedral capsid, and a contractile tail with associated baseplate and extended tail fibers. KVP40 has a very broad host range covering several species of *Vibrio* including *Vibrio cholera, Vibrio anguillarum, Vibrio parahaemolyticus*, and the non-pathogenic species *Vibrio natriegens*, and *Photobacterium leiognathi*. The presence of several copies of genes encoding proteins linked with phage tail or tail fibers in the KVP40 genome suggest an increased flexibility in host range adaptation (Miller et al., 2003). Another genome study reported on Phage vB_VpaM_MAR isolated from fresh non-treated seawater samples in Mexico. Phage vB_VpaM_MAR belongs to *Myoviridae* family has a high specificity to host, able to lyse 76% of the *Vibrio parahaemolyticus* strains tested. Sequence analysis shows the genome of phage MAR is 41,351 bp double-stranded DNA with a G+C content of 51.3% and encodes 62 open reading frames (ORFs; Villa et al., 2012). A novel *Vibrio vulnificus*-infecting bacteriophage, SSP002, belonging to the *Siphoviridae* family, was isolated from the coastal area of the Yellow Sea of South Korea. Host range analysis revealed that the growth inhibition of phage SSP002 is relatively specific to *Vibrio vulnificus* strains from both clinical and environmental samples. A comparative genomic analysis of phage SSP002 and *Vibrio parahaemolyticus* phage vB_VpaS_MAR10 showed differences among their tail-related genes, supporting different host ranges at the species level, even though their genome sequences are highly similar (Lee et al., 2014). Recently, two broad-host range phage (H1 and H7) were isolated from Danish fish farms. Both these phages belong to the *Myoviridae* family and had large genome size (194 kb; Demeng et al., 2014). Interestingly, vibriophage genome provides a detailed characterization on the phage properties as well as understanding of the phage host range and interaction. This information is essential in order to overcome the drawbacks of phage therapy and ensure successful phage application.

Life Cycle of Phages

As natural viruses of bacteria, phages their infection in their bacterial host by reversible adsorption to the specific host cell via specific cell-surface proteins. They then eject their genetic material into the cytoplasm of the bacterial host (Burrowes et al., 2011; Molineux and Panja, 2013). Bacteriophages have two apparent lifecycles; the lytic cycle and lysogenic cycle. The lytic cycle is a form of infection which results in direct damage to the bacterial host. It involves a series of events that occur between attachment of phage particle to a bacterial cell and release of daughter phage particles. There are four phases in the lytic cycle; the adsorption of phage to host cell by binding to specific host, penetration of phage nucleic acid, intracellular development and finally destruction of the cell wall, releasing the newly assembled phages into the environment. In detail, after binding and injection of phage genome into the host cell, the virulent bacteriophages will control the host cell's protein machinery via the expression of specific enzyme encoded by phage genome. It redirects the bacterial synthesis machinery to reproduction of the new phage particles. The production of phage's enzyme in the later stage such as lysins and holins induce destruction of the cell membrane allowing the newly formed phages burst out from the lysed host cell into the environment (Young, 1992). This entire process takes about 20 min to 2 h (Rao and Lalitha, 2015).

The lysogenic cycle, by contrast, involves the replication of phage nucleic acid along with host genes for several generations without major destruction to the host cell. It is a latent mode of infection which happens in a very low frequency (Cochran et al., 1998). The phage genome remains in a repressed state in the host genome and is replicated as part of the bacterial chromosome until lytic cycle is induced. Hence, temperate phages are not suitable for direct therapeutic use as it may mediate transduction by transferring genetic material of one bacterium to another. This process may lead to the development of antibiotic resistance or even increased virulence of the host by acquiring genes from the prophage. Lytic bacteriophages which replicate exponentially and destroy the bacterial host regardless of their antibiotic resistance profile, are more suitable for the biotherapy purposes (Sillankorva et al., 2012).

PHAGE THERAPY

Bacteriophages have been used in many countries since 1929 – before the discovery of broad spectrum antibiotics – as a therapeutic agent against infectious disease (Tan et al., 2014). The first bacterium tested against bacteriophage therapy was *Vibrio cholerae* but the phage activity was reported to be higher *in vitro* compared to *in vivo* (Adams, 1959). The clinical use of phages as therapeutic agents and phage research started to decline and eventually ceased due to the limited knowledge of phage properties and contradictory results from various published studies. The therapeutic use of bacteriophages was further reduced after the emergence of antibiotics (Tan et al., 2014) although phage research and development still remained active in former Soviet Union and Poland (Sulakvelidze et al., 2001). Interestingly, the therapeutic value of bacteriophages has been reevaluated over the most recent decade because of the rise of multidrug resistant bacteria.

Vibrio sp. such as *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio campbellii* are known to be the causative agent of luminous vibriosis disease in shrimp farm. This has resulted in 50–100% mortality rate among shrimps and cause of *Vibrio* infection in human (Shruti, 2012; Letchumanan et al., 2014; Wang et al., 2015; Tan et al., 2016). Bacteriophages isolated from hatchery water have proven to be effective in controlling luminous vibriosis disease, suggesting the phage's potential as a biocontrol agent for luminous vibriosis (**Table 1**). Vinod et al. (2006) reported the isolation and trial of a phage that has potential to control population of pathogenic *Vibrio harveyi* in a hatchery setting. The study isolated a double stranded DNA bacteriophage of *Vibrio harveyi* belonging to the family *Siphoviridae* from shrimp water farm from the west coast of India. The application of phage to control luminescent vibriosis of shrimp larvae was tested in laboratory and hatchery trial. In the laboratory microcosm, a set up containing post larvae of *Penaeus monodon* was exposed to *Vibrio harveyi* and the level of the pathogen was around 10^6 cfu ml^{-1}. The treatment with 100 ppm phage twice has led to two log reduction of *Vibrio harveyi* counts. Larval survival without treatment was only 25% at 48 h but 80% with treatment with two doses of bacteriophage. In the hatchery trial setting, three sets of 500 L tanks containing 35,000 nauplii of *Penaeus monodon* was reared for 17 days. The antibiotic treated tanks (treated with oxytetracycline 5 ppm and kanamycin 100 pm daily) resulted to initial reduction of luminous bacterial counts but after 48 h, the disease appeared again and proliferated to a level of about 10^6 ml^{-1}. While in the tanks treated with bacteriophage, luminous bacteria were not detected throughout the 17-day study period. The luminous bacteria proliferating in control tanks appeared to be virulent, causing mortalities in the larvae. Overall, the survival rate in control tank was only 17%, while in antibiotic treated tanks it was 40% and in the bacteriophage treated tank, it was 86%. Vinod et al. (2006) concluded that since there is a ban on the use of most antibiotics in aquaculture, bacteriophages have the potential to manage luminous vibriosis in the aquaculture setting.

An *in vivo* study utilizing bacteriophage to control luminous vibriosis has further proven phage's potential as a biocontrol agent of luminous vibriosis in aquaculture (Karunasagar et al., 2007). The study isolated four bacteriophages; Viha9, Viha10, Viha11 from oyster, and Viha8 from hatchery water. The morphological characteristic of both phage Viha8 and Viha10 had a non-contractile tail and contained double stranded DNA, hence both the phages were confirmed as members of *Siphoviridae*. Both Viha8 and Viha10 were subjected to laboratory trial and hatchery trials. The results from the laboratory trials revealed that phage Viha10 was able to lyses 70% of *Vibrio harveyi* strains tested while Viha8 had the ability to lyses 68% of the *Vibrio harveyi* strains. The *Vibrio harveyi* strains that were not able to be lysed by Viha10 were lysed by Viha8, and thus using this combination of Viha8 and Viha10, 94% of the *Vibrio harveyi* strains tested were lysed. Hence, Karunasagar et al. (2007) suggested the use of Viha8

TABLE 1 | Potential application of bacteriophage therapy for *Vibrio* species in aquaculture.

Aquaculture product	Etiologic agent	Bacteriophage	Bacteriophage source	Bacteriophage Administration	Results	Reference
Shrimp larvae *Penaeus monodon*	*Vibrio harveyi*	*Myoviridae* (VHLM)	Extracted from a toxin-producing strain of *Vibrio harveyi* isolated from moribund prawn larvae		VHML showed a narrow host range and an apparent preference for *Vibrio harveyi* rather than other 63 *Vibrio* isolates and 10 other genera.	Oakey and Owens, 2000; Oakey et al., 2002
Shrimp larvae *Penaeus monodon*	*Vibrio harveyi*	*Siphoviridae*	Isolated from shrimp farm water from West coast of India	Eighteen day old shrimp were challenged with the bacteria (10^5 cells ml^{-1}). Laboratory trials: (1) Bacteriophage suspension 10^9 pfu ml^{-1} were added initially and another 0.1 ml after 24 h. (2) 0.1 ml of phage suspension. (3) No addition. Hatchery trial: in triplicates (1) Treatment with 10^9 pfu ml^{-1} bacteriophage at the rate of 200 ppm daily so the phage concentration in the water is 2×10^5 pfu ml^{-1} (2) Treatment daily with 5 ppm oxytetracycline and 10 ppm kanamycin. (3) No treatment.	The laboratory trial showed that survival of *Penaeus monodon* larvae was enhanced 80% with treatment with two doses of bacteriophage as compared with the survival rate in control was only 25%. In the hatchery trial, survival in control tank was only 17% while in antibiotic treated tanks was 40%. In the bacteriophage treated tank, the survival rate was 86%. Conclusion: The study concluded that bacteriophage has the potential in management of luminous vibriosis in aquaculture.	Vinod et al., 2006
Shrimp larvae *Penaeus monodon*	*Vibrio harveyi*	Lytic bacteriophages against *Vibrio harveyi*, two from *Siphoviridae*	Three isolated from oyster tissue and one from shrimp hatchery water.	Tanks with post larval five stage larvae, showing luminescence and mortality were used. Two tanks were treated with bacteriophage: one suspension (2×10^6 pfu ml^{-1}) was added by day following the order: Viha10, Viha8, Viha10, and Viha8. Two tanks were treated with 5 mg L^{-1} of oxytetracycline and 10 mg L^{-1} of kanamycin	Over 85% survival of *Penaeus monodon* larvae after bacteriophage treatment. The normal hatchery practice of antibiotic treatment could only result 65–68% of survival. Conclusion: The study showed that bacteriophages could be used for biocontrol of *Vibrio harveyi*.	Karunasagar et al., 2007
Penaeid shrimp	*Vibrio harveyi*	Bacteriophage specific to *Vibrio harveyi* (Viha1 to Viha7), six from *Siphoviridae*, and one from *Myoviridae* (Viha4)	Isolated from coastal aquaculture systems like shrimp farms, hatcheries, and tidal creeks along the East and West coast of India		All the phage were found to be highly lytic for *Vibrio harveyi* and had different lytic spectrum for the large number of isolates tested. Three of the phages (Viha1, Viha3, and Viha7) caused 65% of the strains to lyse while Viha2, Viha4, and Viha6 caused 40% of the host strains to lyse. Viha5 had a narrow spectrum (14%). Conclusion: Six of the seven phages isolated had a broad lytic spectrum and could be potential candidates for biocontrol of *Vibrio harveyi* in aquaculture systems.	Shivu et al., 2007

(Continued)

TABLE 1 | Continued

Aquaculture product	Etiologic agent	Bacteriophage	Bacteriophage source	Bacteriophage Administration	Results	Reference
Shrimp	*Vibrio harveyi*	*Siphoviridae* (VH1 to VH8)	Isolated from shrimp farm	*In vitro* experiments	All the isolates of bacteriophage caused lysis of the host bacterial cells within 2 h. The propagation curve for each phage shows a burst time started from 1 to 10 h. Conclusion: Bacteriophage of *Vibrio* species could be effectively used *in vivo* as biological agents to control these pathogenic bacteria in aquaculture systems.	Srinivasan et al., 2007
Shrimp	*Vibrio harveyi* CS101	*Siphoviridae* (Phage PW2)	Isolated from shrimp pond water		The phage adsorption rate increased rapidly in the 15 min of infection to 80% and continued to increase to 90% within 30 min of infection. The stability of phage PW2 was dependent on temperature and pH. It was inactivated by heating at 90°C for 30 min and by treating at pH 2, 3, 11, and 12. From its one step growth curve, latent, and burst periods were 30 and 120 min, respectively, with a burst size of about 78 pfu per infected center. Six structural proteins were detected.	Phumkhachorn and Rattanachaikunsopon, 2010
Phyllosoma larvae of the tropical rock lobster *Panulirus ornatus*	*Vibrio harveyi*	Six bacteriophages from *Siphoviridae* (VhCCS-01, VhCCS-02, VhCCS-04, VhCCS-06, VhCCS-17, and VhCCS-20) and two from *Myoviridae* (VhCCS-19, VhCCS-21)	Isolated from water samples of discharge channels and grow-out ponds of a prawn farm	Bacteriophage treatments in triplicate: (1) Addition of 1 ml of VhCCS-06 phage at 2 h after inoculation. (2) Addition of 1 ml of VhCCS-06 phage at 6 h after innoculation.	The *Myoviridae* (VhCCS-19 and VhCCS-21) were lysogenic and appeared in a limited number of host bacteria. The *Siphoviridae* phage (VhCCS-06) delays the entry of a broth culture of *Vibrio harveyi* strain 12 into exponential growth but could not prevent the overall growth of the bacterial strain. This effect was most likely due to multiplication of phage-resistant cells.	Crothers-Stomps et al., 2010

(Continued)

TABLE 1 | Continued

Aquaculture product	Etiologic agent	Bacteriophage	Bacteriophage source	Bacteriophage Administration	Results	Reference
Oysters	*Vibrio parahaemolyticus*	*Siphoviridae* pVp-1	Isolated from coastal water of the Yellow Sea, Korea	Oysters infected with *Vibrio parahaemolyticus* were treated with bacteriophage by bath immersion and surface application.	After 72 h of phage application with bath immersion, bacterial growth reduction was observed at 8.9×10^6 CFU/ml (control group) to 1.4×10 CFU/ml (treatment group). Bacterial growth was properly inhibited in the surface-applied group. After 12 h of phage application on surface of oysters, bacterial growth inhibition was revealed to be 1.44×10^6 CFU/ml (control group) to 1.94 CFU/ml (treatment group).	Jun et al., 2014a
Oysters	*Vibrio parahaemolyticus*	Lytic phage VPp1	Isolated from sewage samples	Oysters were infected with 10^5, 10^6, 10^7 CFU/ml of *Vibrio parahaemolyticus* and each infected group was treated with three different MOI values: 10, 1, and 0.1. at 22, 20, 16, and 12°C for 36 h.	The temperatures <20°C were safe for oyster rearing. Depuration at 16°C with 0.1 MOI was the best condition for reducing *Vibrio parahaemolyticus* in oysters, which decreased by 2.35–2.76 log CFU/g within 36 h.	Rong et al., 2014
Fish	*Vibrio anguillarum*	Lytic phage	Isolated from bivalves	**(a) In laboratory:** Three tank conditions, one tank for each condition, as follows: Groups of 15 *S. salar* of 8–25 g per tank (100 L) were maintained in aerated dechlorinated freshwater and a water recirculation system. A fresh culture of the *Vibrio anguillarum* strain PF4 was added directly to the water to a final concentration of 5×10^5 CFU/mL, while an equal volume of fresh medium was added to the control tank. The phage was added directly to the water immediately after the addition of the bacteria **(b) In fish farm facilities:** Performed with groups of 100 *S. salar* of 20–25 g per tank (250 L). The fish were maintained at 12–15°C, in fresh water that was adjusted to 1% of salinity with seawater and in the presence of a water recirculation system that exchanged 50% of the water every day. The tank conditions were set up as the conditions as previous with the addition of one tank condition to test the effect of the phage alone on the fish. In this tank, the phages were added at the same concentration as in the tank with bacteria and phage.	The presence of the phage increased the survival of fish to 100% when it was used with a MOI of 1 and 20, versus less than 10% of survival in the absence of the phage.	Higuera et al., 2013

and Viha10 combination as the biocontrol for *Vibrio harveyi* in hatchery trials. In the hatchery trials, four tanks (A, B, C, and D) of *Penaeus monodon* larvae were infected with *Vibrio harveyi* as evidenced by luminescence. Tank A and B were treated with both phage Viha8 and Viha10 alternately; first day with bacteriophage Viha10 at a level of 2×10^6 pfu ml^{-1} and the following day, phage Viha8 was used at the same concentration. This treatment regimen was repeated on the third day with Viha10 and fourth day with Viha8. On the other hand, tank C was treated with oxytetracycline and tank D with kanamycin. The results showed that the survival rate of *Penaeus monodon* larvae in bacteriophage treated tank was 86–88% while the antibiotic treated tanks was 65–68% survival rate. The study concluded bacteriophages were effective in controlling luminous vibriosis in hatchery settings (Karunasagar et al., 2007).

Further interest on bacteriophage's potential as a biocontrol agent has led to the discovery and isolation of a novel phage in Korea. Phage pVp-1 was isolated from the coastal water of Yellow Sea in Korea demonstrated efficiency in controlling *Vibrio* species (Kim et al., 2012). In addition, this novel marine siphovirus was also reported to be effective infecting *Vibrio parahaemolyticus* ATCC33844, a clinical strain isolated from patient with food poising in Japan (Kim et al., 2012). Jun et al. (2014b) demonstrated how phage pVp-1 was utilized against a multiple-antibiotic resistant *Vibrio parahaemolyticus* pandemic strain, CRS 09-17. In the study oysters infected with CRS09-17 strain was treated with pVp-1 by bath immersion and surface application. The two different method of phage treatment was applied considering the oysters processing; oysters infected model of *Vibrio parahaemolyticus* encountered during aquaculture or fishery markets; and second, the oysters surface contamination model of *Vibrio parahaemolyticus*, which are commonly encountered at restaurants. After 72 h of phage application with bath immersion, bacterial growth reduction was observed to be 8.9×10^6 CFU/ml (control group) to 1.4×10 CFU/ml (treatment group). When pVp-1 was surface-applied on the flesh of oysters after CRS 09-17 inoculation, bacterial growth was properly inhibited. After 12 h of phage application on the surface of oysters, bacterial growth inhibition was revealed to be 1.44×10^6 CFU/ml (control group) to 1.94 CFU/ml (treatment group). Overall, the phage application to various aquaculture situation emphasizes the potential use of the phage to avoid *Vibrio parahaemolyticus* infection from aquaculture to consumption (Jun et al., 2014b).

The *Siphoviridae* phage pVp-1 was used in another *in vivo* study by Jun et al. (2014a) involving mice infected with *Vibrio parahaemolyticus*. The efficacy of phage therapy was evaluated in two experiments using the *Vibrio parahaemolyticus* CRS 09-17 infection mouse model. In the first experiment, two groups of mice (control/treatment; five mice in each group) were challenged by an IP injection of an LD_{50} of CRS 09-17. Each mouse was treated with a single IP injection of phage pVp-1 (2.0×10^8 PFU per mouse) or PBS 1 h after the bacterial challenge (2.0×10^7 CFU per mouse). In the second experiment, all conditions were similar to those of the first study except that the bacterial challenge (2.0×10^7 CFU per mouse) and phage treatment (2.0×10^8 PFU per mouse) were administered orally. Both experiments were repeated five times, and the health of the mice was monitored for 72 h. In an additional study, two groups (five mice per group) were not challenged with bacteria and received only phage (2.0×10^{11} PFU per mouse) by IP and oral routes. The health of these mice was monitored for 28 days. The study concluded that phage-treated mice exhibited from a *Vibrio parahaemolyticus* infection and survived lethal oral and intraperitoneal bacterial challenges (Jun et al., 2014a).

Rong et al. (2014) reported the effectiveness of phage VPp1 application to reduce the population of *Vibrio parahaemolyticus* in the oyster depuration. VPp1, a lytic phage that was isolated from sewage was capable of reducing *Vibrio parahaemolyticus* infection on oysters by 2.35–2.76 log cfu/g within 36 h (Rong et al., 2014). Another study isolated a lytic phage named as PW2 from shrimp pond water in Songkhla Province, Thailand. The morphological characteristics showed that this phage has an icosahedral head and a long non-contractile tail, which can be categorized under the order Caudovirales and family of *Siphoviridae*. This phage PW2 showed lytic properties against *Vibrio harveyi*. Based on previous studies, most of the *Vibrio harveyi* phages were found to be siphophages with double stranded DNA (Pasharawipas et al., 2005; Vinod et al., 2006; Karunasagar et al., 2007; Jun et al., 2014a,b). However, *Vibrio harveyi* phages from other families such as Myoviridae and Podoviridae were also reported (Oakey and Owens, 2000; Oakey et al., 2002; Shivu et al., 2007).

Cholera, a water borne disease continues to be a major public health concern in developing countries and re-emerging in countries where it disappeared long time ago (World Health Organization [WHO], 2008). The occurrence of multidrug antibiotic resistant strains of *Vibrio cholerae* in the environment has prompted the search of alternative source of treatment such as bacteriophage therapy. The usefulness of lytic cholera phage as a prophylactic agent has been studied in many countries (Monsur et al., 1970; Marcuk et al., 1971). Jaiswal et al. (2013) studied the efficacy of five lytic vibriophage cocktail in treating *Vibrio cholerae 01* biotype El Tor serotype Ogawa MAK 757 (ATCC 51352) infection in rabbit model. It was observed that oral administration of phage cocktail after oral bacterial administration reduced the shedding of bacteria significantly ($p < 0.01$). The rabbits appeared normal without any toxicity evidence. The study concluded that phage cocktail was more potent as a lytic agent compared to as individual phages. An oral administration of suitable phage cocktail would be suitable as an alternative to antibiotic treatment in case of cholera infection (Jaiswal et al., 2013).

An oral phage cocktail (ATCC- B1, B2, B3, B4, B5) was administrated in adult mice model in a study by Jaiswal et al. (2014). The study performed a comparative analysis between phage cocktail, antibiotic, and oral rehydration treatment for orally developed *Vibrio cholerae* infection. It was reported that the genome size of vibriophage B1, B2, B3, B4 was around 40 kb and phage B5 had a genome size of around 100 kb. *In vitro* characteristic of vibriophages showed theses phages could withstand variety of pH level (pH 2–pH 12) as well as temperature range of 25–60°C. The study reported that combination of five vibriophages cocktail reduced the number of *Vibrio cholerae*

cells in the orally infected mice compared to antibiotic and oral rehydration treatment (Jaiswal et al., 2014). A previous study analyzed the usefulness of phage cocktail in a *Vibrio cholerae 01* infected RITARD (removable intestinal tie-adult rabbit diarrhea) model experiment. The study concluded that cocktail of phage could provide significant protection and act as prophylaxis against *Vibrio cholerae* infection (Bhowmick et al., 2009).

In general, the selection of appropriate bacteriophage is a key factor in the success of phage therapy of *Vibrio* species (Mateus et al., 2014). Based on the studies discussed, bacteriophage belonging to *Siphoviridae* family is selected to control *Vibrio* species. *Siphoviridae* phage is reported to have a specific host range and closely related to species of *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Vibrio campbellii* (Crothers-Stomps et al., 2010). In addition phage cocktails have been demonstrated to be more effective than individual phages in treatment of *Vibrio cholerae* infection (Bhowmick et al., 2009; Jaiswal et al., 2014). By making a phage cocktail, it would become easier to treat a wide range of drug-resistant bacterial infections (Golkar et al., 2014). Although bacteriophages are isolated from different environmental source such as shrimp hatchery, sewage, ponds or from aquatic animals, they still show the same bacteriolytic activity and possess advantages over conventional antibiotics (Gutierrez et al., 2010).

ADVANTAGES OF PHAGE THERAPY

Bacteriophages are natural antibacterial agents that are able to regulate bacterial populations by inducing bacterial lysis. Phages are reported to be active against both Gram-negative and Gram-positive bacteria including multidrug resistant pathogens in the environment (Biswas et al., 2002; Matsuzaki et al., 2003; Wang et al., 2006; Vinodkumar et al., 2008; Wittebole et al., 2014). Bacteriophages have a number of desirable properties that make them compelling candidates for tackling antibiotic resistance in bacteria. A bacteria is unable to regain its viability after been lysed by lytic phage; by contrast antibiotic therapy may not kill the targeted bacteria, facilitating the development of antibiotic resistance (Stratton, 2003).

The high specificity for their host cell is another advantage of bacteriophages relative to antibiotics. Phages are very specific to their host thus reducing the chances of secondary infections. They do not affect or alter the gut microbiota nor change the organoleptic properties of food products (Hagens and Offerhaus, 2008). Phages are specific bacterial host killers and do not affect normal microbiota compared to antibiotics which affect bacterial cells non-selectively (Rao and Lalitha, 2015). Additionally, there is no adverse effects reported during or after the phage treatment whereas allergies, secondary infections and bacterial resistance are common side effects seen after antibiotic treatments (Sulakvelidze et al., 2001).

Phages have the capability to replicate selectively at the site of infection where they are needed to lyse their bacterial hosts in contrast to antibiotics which distribute throughout the body fluids and tissues based on their inherent pharmacokinetic properties rather than becoming concentrated at the site of infection (Golkar et al., 2014). Phages are very environmentally friendly and evolved based on natural selection. Isolating and identifying suitable phages for therapy is a relatively simple, rapid process compared to development of new antibiotics which takes several years and require costly clinical trials prior to use (Weber-D et al., 2000). Furthermore, owing to the abundant and ubiquitous nature of bacteriophages, phages against the major pathogenic bacteria are readily discovered and isolated from environments that are habitats for host bacteria, especially from sewage, soil, water, and waste materials which contain high bacterial concentration, hence aiding in lowering the cost of production (Vinod et al., 2006; Skurnik et al., 2007). Phages are considered to have low environmental impact as they consist of nucleic acids and proteins only and have narrow host ranges (Loc-Carrillo and Abedon, 2011). In addition, phages can easily be applied as sprays or by directly mixing with water. In 2006, the Food and Drug Administration (FDA) approved a bacteriophage mixture, called a "lytic cocktail," in a spray-on form designed to reduce the presence of *Listeria monocytogenes* bacteria in meat and deli products (Zach, 2010). For example, ListShield^{TM} (Intralytix, Inc.) is a commercial product marketed in a concentrated aqueous phage that is stored in 2–6°C. For direct food applications, the diluted working solution is typically applied directly on food surfaces by spraying at a concentration of approximately 1–2 mL per 250 square cm of food product surface. The recommended application rate for foods with complex surfaces is usually 1–4 mL of the diluted working solution per pounds of food. While, for environmental applications, the diluted working solution is typically applied onto the surfaces by spraying, or with a cloth, mop, or sponge, so that the targeted surface is thoroughly covered. About 50 mL of the diluted working solution is able to treat approximately 4 ft^2 of surface.

Currently, Biologix, an Australian biotechnology company is developing phage therapy for *Vibrio* sp. associated with mortalities in the aquaculture. Jafral, an independent contract manufacturing organization (CMO) and contract research organization (CRO) located in Slovenia has been manufacturing bacteriophages. Here, bacteriophages has been successfully manufactured using manufacturing processes that have up to 10-time higher productivity. The end product can be used either in food industry or for animal and human treatments where it is desirable that phage titres are high and impurities levels are low.

The usage of antibiotics in the aquaculture industry has led to the increase of antibiotic resistant bacteria and development of ARGs in the environment which shade health risks to humans and animals (Kemper, 2008; Letchumanan et al., 2015a). Bacteriophages have the potential to reduce the dependency of aquaculture industry on use of antibiotics. The phages could be utilized instead of antibiotics to control bacterial infections that occur in aquaculture industry. Hence, plasmid mediated ARGs profile among bacteria would reduce when there is no antibiotics present in the environment. This eventually will preserve the ecosystem and reduce the effects on humans and animals.

In addition, bacteriophages have ability to disrupt bacterial biofilms (Azeredo and Sutherland, 2008). Bacteriophages have the capabilities to produce depolymerases which could hydrolyze extracellular polymers in bacterial biofilms. The use of phages

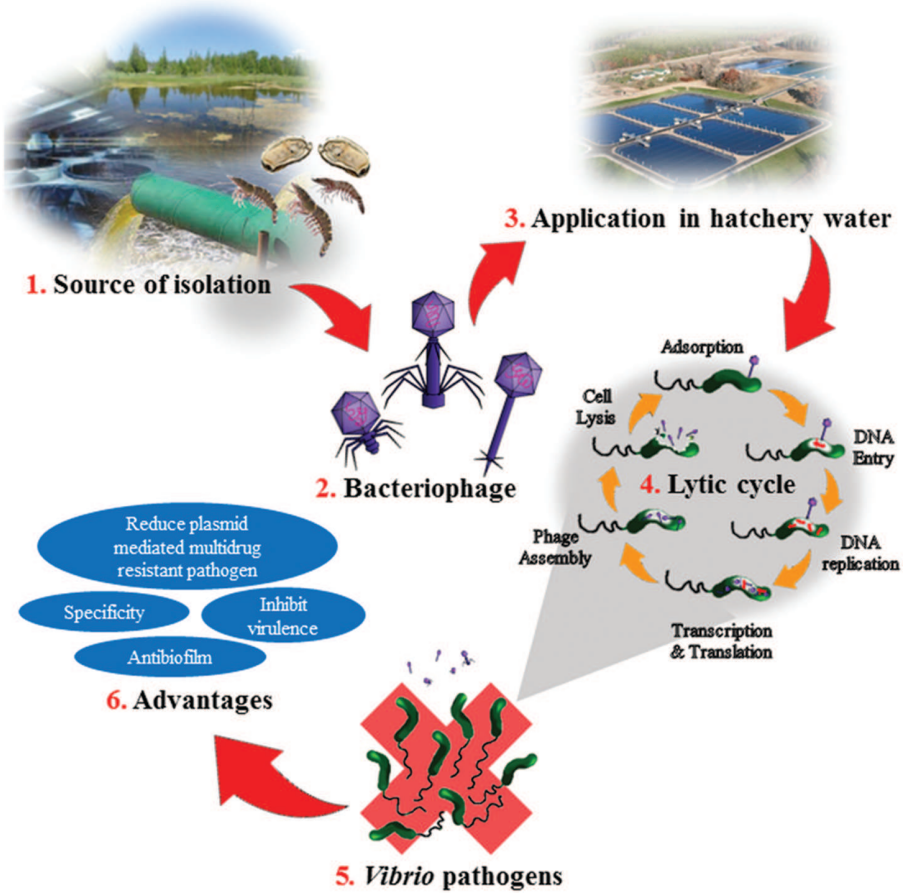

FIGURE 1 | Illustration on bacteriophage application in the aquaculture and the advantages. (1) Bacteriophages are isolated from environmental sources such as hatcheries, sewage, ponds, or aquatic animals. (2) The isolated bacteriophage are purified and identified. The three common phages used as biocontrol agent for *Vibrio* sp; *Siphoviridae*, *Myoviridae*, and *Podoviridae*. (3) In the hatchery, the bacteriophage is added to the hatchery water. The amount of phage need to be added depends on size of the pond and amount of shrimps or cockles or fish in the pond. (4) Once added, the bacteria on host cell will undergo lytic cycle. Adsorption step would take place when the phages come in contact with the infected host cell. Then the phage's DNA would penetrate into the host cell and replicate. It is followed by transcription and translation of the phage and DNA. Then the phage would assemble, host will lysis and phages will be released out from the host cell. (5) Bacteria such as *Vibrio* sp. would be eliminated from the hatchery. (6) Advantages of bacteriophages applications in a bacterial infection.

were reported useful in the treatment of biofilm forming pathogens such as *Pseudomonas aeruginosa* (Fu et al., 2010), *Escherichia coli* (Doolittle et al., 1995), and *Staphylococcus aureus* (Sass and Bierbaum, 2007). It has been reported that around 80% of bacterial cases in the United States are associated with biofilms (Janssens et al., 2008). Biofilm has been a problematic disease in many food industries including seafood processing (Shikongo-Nambabi, 2011), dairy processing (Chmielewski and Frank, 2003), poultry processing (Harvey et al., 2007), and meat processing (Sofos and Geornaras, 2010). A study in USA has offered an insight into the potential use of phages to treat biofilm diseases by using an *in vitro* catheter model that was treated with phages. The results demonstrated that while phage treatment never fully prevented biofilm formation, biofilm biomass and cell density was significantly reduced (Curtin and Donlan, 2006; Fu et al., 2010). Luo et al. (2015) isolated two phages P4A and P4F which belong to the *Siphoviridae* family from seawater of an abalone farm and applied the phages to reduce *Vibrio* biofilm. In the study, the both phages were able to bring about 2 logs reduction in a *Vibrio harveyi* biofilm cell density after 24 h of phage treatment (Luo et al., 2015).

DRAWBACKS OF PHAGE THERAPY

Despite all the listed advantages above, bacteriophage therapy does have its drawbacks. One of those that caused much concern is the potential emergence of phage-resistant bacteria, similar to that seen with antibiotic treatments. Typically, resistance would develop toward a particular phage when the bacterial surface proteins facilitating phage attachment are lost or lack of adsorption, thus preventing the phage from infecting its host. However, from the literature, the rate of developing resistance to phages is approximately 10-fold lower than to antibiotics (Tanji et al., 2004). Besides, other concern of *Vibrio* phage therapy

is that some bacteriophage may be involved in the transfer of virulence genes to the bacteria. It was reported that toxicity of *Vibrio harveyi* to *Penaeus monodon* is induced by bacteriophage (Munro et al., 2003). Therefore, before using a bacteriophage for therapy it is important to test if they carry any virulence genes and would it be safe to use the bacteriophage (Vinod et al., 2006).

Another potential drawback on phage therapy is the bacterial defense system called CRISPR/Cas (clustered regularly interspaced short palindromic repeats). This CRISPR base immunity acts by integrating short virus sequences in the bacteria's CRISPR locus, allowing the bacteria to recognize and clear infections. However, it has been demonstrated that this system can be utilized by the bacteriophages to promote infection. *Vibrio cholerae* ICP1 phages carry a Type I-F CRISPR–Cas system that targets a host locus, PLE, containing an anti-phage system. *Vibrio cholerae* ICP1 phage uses the CRISPR/Cas system to target the PLE for host cell destruction and successfully replicate. Due to bacteria cell death and DNA damage by lytic phage infection, CRISPR-mediated DNA cleavage of the PLE does not affect *Vibrio cholerae* ICP1 infection (Seed et al., 2013).

In addition, there is also a need to overcome the understandable stigma among consumers regarding safety of intentional consumption of viruses in spite of certification by the regulatory bodies. Additional work should be carried out in order to assess consumer knowledge and acceptance of phage therapy followed by targeted educational campaigns to raise awareness and acceptance.

CONCLUSION

Vibrio species infection poses a threat in many fields, the treatment and control of which is currently dependent on antibiotic therapy; however, the use of antibiotics needs to be restricted due to the increase of antibiotic resistant bacteria (World Health Organization [WHO], 2006). Bacteriophage therapy is regarded as a highly viable alternative to prevent and control bacterial infections and in some conditions it has been proven to be superior to antibiotics. A schematic figure is been represented to illustrate the application of bacteriophage in the aquaculture and the advantages (**Figure 1**). The phages poses great advantages such as having host specificity, environmental friendly, readily discovered and isolated from the environment, and cost effective compared to antibiotics. Bacteriophages have the ability to control luminous vibriosis among *Vibrio* species (Vinod et al., 2006; Karunasagar et al., 2007). The phages have great potential as a bio-control agent to control and inhibit virulence of *Vibrio* species isolated from both clinical and environmental samples (Jassim and Limoges, 2014). In addition, it can be utilized in the agriculture and aquaculture industries instead of antibiotics to control bacterial infections that occur in aquaculture industry. This eventually will reduce the dependency toward antibiotics that leads to resistant genes profile in the environment (Golkar et al., 2014). Bacteriophages – being natural products are also generating less adverse effects compared to antibiotics. In 2006, US Food and Drug Administraion (FDA) approved the use of commercial phage cocktail ListShield^{TM} targeting *Listeria monocytogenes*. This is a confirmation that FDA has viewed phages are safe for human application and opens the doors for phage commercialization for human application and consumption (Housby and Mann, 2009). In March 2016, Intralytix, a biotechnology company received USDA, NIFA Phase II SBIR Grant to develop a phage based application to protect hatchery raised oysters from *Vibrio tubiashii* and *Vibrio coralliilyticus*. Moreover, there are many vibriophages that has been patented including phage patent number CN 103992990 A, CN 102524131 B, and US 20140105866 A1. The phage (US 20140105866 A1) is specific against *Vibrio anguillarum* was identified belonging to *Siphoviridae* family with a genome size of 48 kb. It possess prophylaxis properties, control and/or treatment of infection caused by *Vibrio anguillarum* in all types of species of fish, mollusks and crustaceans (Espejo et al., 2014). A lytic phage VP4B was reported to cause a significant growth inhibition effect of pathogenic *Vibrio harveyi*, and this patented phage can be used for biological prevention or control of vibrio diseases in mariculture (CN 103555671 A; Zhuhua et al., 2014). Qiu et al. (2012) reported a technique that utilize aquatic invertebrate larvae and adults to harmlessly carry *Vibrio* phages. The phages obtained through this technique are not virulent and can retain the lysis activity for host bacteria during a long period of time (CN 102550458 A). Jinyong et al. (2012) patent a phage BPH-VP-1 (CN 101798568 B) that exhibited broad lysis properties against *Vibrio parahaemolyticus*. It was reported that phage BPH-VP-I could be used alone or in combination, and as fungicides sprayed on food production plants in order to control *Vibrio parahaemolyticus* contamination. In summary, all the above listed advantages make bacteriophage therapy an attractive and promising tool as a biological control of bacterial infections.

AUTHOR CONTRIBUTIONS

VL and L-HL contributed to the literature database search, data collection, data extraction and writing of the manuscript. N-SA, PP, SS, AD, B-HG, K-GC and L-HL contributed vital insight and proofread on the writing. The research topic was conceptualized by L-HL.

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The Inhibition and Resistance Mechanisms of Actinonin, Isolated from Marine *Streptomyces* sp. NHF165, against *Vibrio anguillarum*

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Vibrio sp. is the most serious pathogen in marine aquaculture, and the development of anti-*Vibrio* agents is urgently needed. However, it is extreme lack of high-throughput screening (HTS) model for searching anti-*Vibrio* compounds. Here, we established a protein-based HTS screening model to identify agents targeting peptide deformylase (PDF) of *Vibrio anguillarum*. To find potential anti-*Vibrio* compounds, crude extracts derived from marine actinomycetes were applied for screening with this model. Notably, crude extract of strain *Streptomyces* sp. NHF165 inhibited dramatically both on *V. anguillarum* PDF (VaPDF) activity and *V. anguillarum* cell growth. And actinonin was further identified as the functional component. Anti-VaPDF and anti-*V. anguillarum* activities of actinonin were dose-dependent, and the IC_{50} values were 6.94 and 2.85 μM, respectively. To understand the resistance of *V. anguillarum* against actinonin, spontaneous *V. anguillarum* mutants with resistance against actinonin were isolated. Surprisingly, for the resistant strains, the region between 774 and 852 base pairs was found to be absent in the gene *folD* which produces 10-formyl-tetrahydrofolate, a donor of *N*-formyl to Met-$tRNA^{fmet}$. When compared to the wild type strain, Δ*folD* mutant showed eight times of minimum inhibition concentration on actinonin, however, the *folD* complementary strain could not grow on the medium supplemented with actinonin, which suggested that *folD* gene mutation was mainly responsible for the actinonin resistance. To our knowledge, this is the first report showing that marine derived *Streptomyces* sp. could produce actinonin with anti-VaPDF activity and the resistance against actinonin by *V. anguillarum* is mediated by mutation in *folD* gene.

Keywords: peptide deformylase, high-throughput screening assay, *Vibrio anguillarum*, marine *Streptomyces* sp. NHF165, resistance mechanism, actinonin

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INTRODUCTION

Sudden outbreak of diseases is a major setback in aquaculture, and it leads to high mortality and severe economic loss in all producing countries. Marine *Vibrio* species are associated with large-scale losses of penaeids and also cause diseases to fish (Letchumanan et al., 2015b). *Vibrio anguillarum* is the causative agent of vibriosis, a deadly haemorrhagic septicaemic disease affecting

various marine and fresh/brackish water fish, bivalves and crustaceans. In both aquaculture and larviculture, this disease is responsible for severe economic losses worldwide (Frans et al., 2011). *Vibrio* species inhabit aquatic environments at temperatures ranging from 10 to 30°C and are highly susceptible to antibiotics (Shaw et al., 2014). Therefore, antibiotics is one of the main choices for controlling the proliferation of *Vibrio* sp. in aquaculture. Oxytetracycline, tetracycline, quinolones, sulphonamides and trimethoprim are antimicrobial agents permitted and utilized in the Asian aquaculture industry (Letchumanan et al., 2015a). However, extensive use of antibiotics has been postulated to be a major contributing factor in the rising incidence of antimicrobial resistance in pathogenic bacteria. Three fundamental mechanisms of antimicrobial resistance have been summarized: (1) prevention of access to target, (2) changes in antibiotic targets by mutation, and (3) modification (and protection) of targets (Blair et al., 2015). New resistance mechanisms are constantly being described, such as combined novel gene *mph*(G) coding macrolide phosphotransferase and gene *mef* (C) coding efflux pump were found to be responsible for high-level macrolide resistance *Vibrio* sp. (Nonaka et al., 2015).

To find novel anti-*Vibrio* sp. agents, screening models targeting *Vibrio* sp. whole cells or proteins involved in quorum sensing have been widely used (Zhang et al., 2016; Zhao et al., 2016). Because of serious antibiotics resistance, screening models with new targets are always needed. Peptide deformylase (PDF) is a class of metalloprotease responsible for catalyzing the removal of *N*-formyl group from *N*-terminal methionine following translation in prokaryotes. The widespread occurrence, conservation, and essential nature of deformylase in bacteria make it an attractive target for antibacterial drug discovery (Giglione et al., 2000; Sangshetti et al., 2015). PDF is widely used in human bacteria infection treatment caused by *Staphylococcus aureus, Streptococcus pneumonia, Helicobacter pylori*, *Haemophilus influenza* and *Mycobacterium tuberculosis*, etc (Sharma et al., 2009; Peyrusson et al., 2015). PDF inhibitors, GSK-1322322, BB-83698 and LBM-415, have entered into clinical developments (Sangshetti et al., 2015).

However, very little was investigated about PDF of aquaculture pathogen *V. anguillarum*. Actually, like other gram-negative organisms, *V. anguillarum* has one chromosomal copy of *pdf* gene, and no results have been published regarding PDF as an anti-*Vibrio* sp. target in marine aquaculture. Actinonin was reported in 1962 (Gordon et al., 1962) and was the first characterized PDF inhibitor (Chen et al., 2000). Up to now, resistance to actinonin has been reported in *Staphylococcus aureus, Streptococcus pneumonia, Bacillus subtilis, Haemophilus influenza, Streptococcus pyogenes* and *Escherichia coli*. Mechanisms causing actinonin resistance were also investigated in these strains. Genes *pdf*, *folD*, *fmt,* and *glyA* involved in translation initiation were the most frequency mutation sites (Margolis et al., 2000, 2001; Duroc et al., 2009).

Natural products are essential for the novel antibiotics screening. A lot of compounds had been developed to efficient antibiotics and applied in diseases treatment of human and aquaculture (Varoglu et al., 1997; Vinothkumar and Parameswaran, 2013). It is well known that the biodiversity of the marine environment and the associated chemical diversity constitute a practically unlimited resource of new bioactive substance, and the bioactive compounds from marine microorganisms have been exploited for decades (Varoglu et al., 1997). Marine actinomycete is one of the most efficient organisms of natural bioactive metabolite producers. The genus *Streptomyces* is considered as the most prolific producer of bioactive agents amongst actinomycete (Miao and Davies, 2010). Interestingly, *Streptomyces* sp. isolated from arctic were found to have biofilm inhibitory activity against *Vibrio* sp. by attenuating the signal molecules *N*-acylated homoserine lactones' activity (You et al., 2007), and *Streptomyces* producing siderophores derived from nearshore marine sediments were found to inhibit the growth of *Vibrio* sp. by competition for iron in the aquatic environment (You et al., 2005).

In this study, we established an high-throughput screening (HTS) model targeting PDF of pathogenic bacterium *V. anguillarum* YN isolated from infected *Scophthalmus maximus* samples. Actinomycetes from eight different South China Sea sediments were isolated and corresponding crude extracts were prepared and subjected to anti-*V. anguillarum* agents screening. Actinonin produced by marine *Streptomyces* sp. NHF165 exhibited high inhibitory both on *V. anguillarum* PDF (VaPDF) activity and *V. anguillarum* cell growth. Furthermore, actinonin-resistant *V. anguillarum* mutants were obtained and the mechanism of resistance was also elucidated.

MATERIALS AND METHODS

V. anguillarum PDF (VaPDF) Expression and Purification

The *pdf* gene was amplified from *V. anguillarum* YN genome DNA by PCR using the following primers: For: 5′-CGCGGATCCATGTCTGTATTACAAG-3′ (the underlined region indicates *Bam*H I site) and Rev: 5′-CCGCTCGAGTTA GTTTTTTTCGTTATAG-3′ (the underlined region indicates *Xho* I site). PCR products were cloned into pMD18-T vector (TaKaRa). After sequence confirmation, PCR products were inserted in the multiple cloning site of vector pET30a(+) (Novagen) and the resulting plasmid was designated as pET30a(+)::*pdf*. Plasmid pET30a(+)::*pdf* was transformed into *E. coli* BL21(DE3) cells. Recombinant PDF was expressed and purified as follows. Briefly, cells harboring plasmids pET30a(+)::*pdf* were grown to an absorbance at 600 nm (A_{600}) of 0.6 and induced with 0.5 mM isopropyl-D-thiogalactopyranoside at 16°C overnight. Cells were harvested by centrifugation, washed in HEPES buffer (25 mM, pH 7.4) and resuspended in HEPES (pH 7.4)-75 mM KCl-10% glycerol (buffer A). Then cells were lysed by sonication and centrifugated at 25,000 × *g*. The supernatant was loaded onto a 5 ml HisTrap FF column (GE healthcare) and equilibrated in buffer A. The column was further washed and eluted with a gradient of imidazole from 0 to 300 mM using ÄKTA protein purification system (GE healthcare).

Anti-VaPDF Screening Assay

Peptide deformylase catalyzes the removal of the *N*-formyl group from formyl-Met-Ala-Ser. The free amino group reacts with fluorescamine to form highly fluorescent products which can be monitored with a TECAN Infinite M1000 PRO multi-mode microplate reader by exciting at 390 nm and emission at 470 nm. For screening, assays were performed in black flat-bottom 96-well microplates (Corning). First, 49.5 μl reaction solution (20 nM VaPDF, 1 mM formyl-Met-Ala-Ser and 25 mM HEPES, pH 7.4) was dispensed in each well and then 0.5 μl dimethylsulfoxide (DMSO) or samples dissolved in DMSO (4 mg/ml) was dispensed. Plates were incubated at 37°C for 30 min. Then fluorescamine was added to a final concentration of 60 μg/ml. The fluorescence intensity (FI) of each well was detected. The inhibitory values were calculated as $(FI_{sample} - FI_{negative\ control})/(FI_{positive\ control} - FI_{negative\ control}) \times 100\%$.

Dimethylsulfoxide was chosen as negative control and heat-inactivated VaPDF as positive control during measurements. The Z' factor and CV values were calculated as follows:

$Z' = 1–3(SD_{FImax} - SD_{FImin})/(Mean_{FImax} - Mean_{FImin})$, SD: standard deviation. The theoretical value is between 0.5 and 1. $CV(\%) = SD_{FImax}/Mean_{FImax}$ or $CV(\%) = SD_{FImin}/Mean_{FImin}$. The acceptable value of CV for HTS assay is less than 10%.

Anti-*V. anguillarum* Cell Based Assay

The anti-*V. anguillarum* assay utilized strain *V. anguillarum* YN which was isolated from infected *Scophthatmus maximus* sample. The activities of crude extracts or compounds against *V. anguillarum* were determined in a clear flat-bottom 96-well plate. *V. anguillarum* YN was grown at 28°C to mid-log phase in Luria Bertani (LB) medium (peptone 10 g, yeast extract 5 g, NaCl 10 g, in 1000 ml distilled water, pH 7.0). Then the culture was diluted to $A_{600} = 0.025$ with LB medium. 80 μl bacterial suspension was added to each well, followed by adding 0.8 μl of sample solution (4 mg/ml). DMSO served as the negative control and chloramphenicol as the positive control. The plate was incubated at 28°C for 15 h and the growth of *V. anguillarum* YN was measured by detecting A_{600} of each well.

Marine Actinomycetes Isolation and Crude Extracts Preparation

Sediment samples were collected using the mud sampler in the South China Sea during 26th April to 23th May 2010 (Supplementary Table S1). The samples were transported to laboratory in an insulated container at 4°C and then stored at −80°C. All samples were pretreated using dispersion and differential centrifugation (DDC) method (Hopkins et al., 1991) to enrich for spore-forming actinomycetes. Five different agar media were selected for spreading sediment samples: (1) M1 agar: raffinose 10.0 g, L-histidine 1.0 g, K_2HPO_4 1.0 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.01 g, agar 15.0 g; (2) M2 agar: trehalose 5.0 g, proline1.0 g, $(NH_4)_2SO_4$ 1.0 g, NaCl 1.0 g, $CaCl_2$ 2.0 g, K_2HPO_4 1.0 g, $MgSO_4.7H_2O$ 1.0 g, agar 20.0 g; (3) M3 agar: humic acid 1.0 g, KCl 1.7 g, NaH_2PO_4 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.01 g, $CaCO_3$ 0.02 g, agar 15.0 g; (4) M4 agar: glycerol 12.5 g, arginine 1.0 g, K_2PO_4 1.0 g, NaCl 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $CuSO_4.5H_2O$ 0.001 g, trace salt solution 1.0 ml, agar 15.0 g, trace salt solution contains $FeSO_4.7H_2O$ 0.001 g, $MgCl_2.4H_2O$ 0.001 g, $ZnSO_4.7H_2O$ 0.001 g, distilled water 1000 ml; (5) M5 agar: soluble starch 10.0 g, hydrolyzed casein 0.3 g, NaCl 5.0 g, KNO_3 2.0 g, K_2HPO_4 2.0 g, $MgSO_4.7H_2O$ 0.5 g, $CaCO_3$ 0.02 g, $FeSO_4.7H_2O$ 0.01 g, agar 15.0 g. All media were prepared using the artificial seawater and adjusted to pH 7.5 and were supplemented with nalidixic acid (20 μg/ml) and nystatin (100 μg/ml) or cycloheximide (100 μg/ml) to inhibit the growth of fungi and Gram-negative bacteria. Spreaded plates were incubated at 28°C for 1 month. Actinomycetes were selected and transferred to GT agar medium until pure cultures were obtained for further study (GT agar medium: soluble starch 20 g, L-asparagine 0.5 g, KNO_3 1.0 g, $K_2HPO_4.H_2O$ 0.5 g, NaCl 0.5 g, $MgSO_4.7H_2O$ 0.5 g, distilled water 1000 ml, pH 7.5). Pure actinomycetes were maintained on GT slants at 4°C and 25% (v/v) glycerol suspensions at −80°C. Morphological features of spores and mycelia were observed by light microscopy (model BH2; Olympus) and scanning electron microscopy (Quanta 200). For crude extracts preparation, all the selected strains were cultured in 250 ml flask containing 40 ml fermentation medium (MPG medium consisting of glucose 10.0 g, millet meal 20.0 g, cotton seed gluten meal 20.0 g, MOPS 20.0 g, distilled water 1000 ml, pH 7.2). The liquid cultures were grown for 7 days at 28°C with shaking at 160 rpm. An equal volume of ethyl acetate was added to the liquid cultures for extraction and evaporated to give crude extracts.

16S rRNA Gene Amplification and Phylogenetic Analysis

The 16S rRNA genes were amplified by using universal bacterial primers: 27F and 1492R (Lane, 1991). PCR products were sent to Sangon Biotech (Shanghai, China) Co. Ltd. for DNA sequencing and deposited in GenBank (accession numbers: KU500358-KU500370, KU312336- KU312339, KU529470- KU529472, KU550963, JQ911670). The 16S rRNA gene sequences were compared with available 16S rRNA gene sequences from GenBank database by using BLAST program[1] to determine an approximate phylogenetic affiliation. Neighbour-joining (NJ) tree was constructed using software package Mega version 6.0 (Tamura et al., 2013). Bootstrap re-sampling method with 1000 replicates was used in evaluating the topology of the phylogenetic trees (Felsenstein, 1985).

Compound Separation and Identification

The fermentation of active strain *Streptomyces* sp. NHF165 was carried out in 1000 ml flask containing 250 ml MPG medium that inoculated 3 ml seed culture of strain *Streptomyces* sp. NHF165. The fermentation broth was cultured at 28°C for 7 days on a rotary shaker at 160 rpm. After fermentation, total broth (10 L) was fractionated by centrifugation. Supernatant was extracted with the same volume ethyl acetate thrice. The evaporated ethyl acetate phase crude extract was applied on a Sephadex LH-20 column [elution reagent,

[1]http://blast.ncbi.nlm.nih.gov/Blast.cgi

dichloromethane:methanol = 2:1 (v/v)] and separated into 10 fractions. The sixth fraction with anti-VaPDF activity was subjected to a preparative HPLC C18 column (9.4 mm × 250 mm, 5 μm, Agilent) using acetonitrile and water as mobile phase at 3 ml/min to give pure compound 1 (5.2 mg) and 2 (3.5 mg). And the compounds were identified by checking NMR data.

Resistant Mechanism Study of Actinonin against *V. anguillarum*

To isolate *V. anguillarum* resistant to actinonin, exponential-phase cells were inoculated into Mueller-Hinton (MH) broth supplemented with 25 μM of actinonin and incubated for 1 day at 28°C. Then 100 μl culture was plated onto MH agar containing 25 μM of actinonin. Resistant colonies were picked and restreaked for single-cell colonies on the same plate. Purified resistant mutants were frozen at −80°C in LB with 10% DMSO. Growth curves for wild type and mutant strains were tested using MH broth without actinonin at 28°C for 25 h. The growth was monitored at different time points by reading A_{600}. Cells were also plated on minimal medium (MM) agar (Duroc et al., 2009) to test the growth. For MICs (minimum inhibition concentration) determination, actinonin was serially diluted twofold from 1000 to 0.49 μM in each column using a clear flat-bottom 96-well plate. The plate was incubated at 28°C for 15 h, and after incubation, the plate was read under absorbance at 600 nm. In this study, the MIC was defined as the lowest actinonin concentration which prevented *V. anguillarum* growth (an A_{600} value < 0.05).

The PCR primers used for DNA amplification of the *pdf*, *folD*, *fmt,* and *glyA* genes were designed from the appropriate sequences of the corresponding public genome sequences from NCBI website[2]. PCR amplification was performed with both wild type and mutants genome DNAs of *V. anguillarum*. PCR products were confirmed by sequencing in Sangon Biotech (Shanghai, China) Co. Ltd. Alignment of the DNA sequences of the *pdf*, *folD*, *fmt,* and *glyA* genes from wild type and mutant strains was carried out using software package Mega version 6.0. To confirm whether mutation of gene *folD* leads to resistance, complementary experiment was taken out. Briefly, full length of *folD* was amplified from wild *V. anguillarum* genome DNA by PCR and ligated into vector pACYC184 (Milton et al., 1992), which was transformed conjugately into mutant *V. anguillarum* by a donor strain *E. coli* 17-1. The positive clones were selected on LB agar containing tetracycline.

Expression changes in transcription level between wild type and Δ*folD* strain were compared by performing RT-PCR. RNA was extracted from 2 ml culture broth of bacterial samples using an Ultrapure RNA Kit (CWBio) as described by the manufacturer. 1 μg total RNA of each sample was subjected to reverse transcription using random hexamers to prepare cDNAs. RT-PCR was optimized with a SYBR Premix Ex Taq kit (TaKaRa) for each primer pair (**Table 1**). Each cDNA sample was independently quantified three times, with two technical replicates of each. Relative mRNA levels were calculated.

[2]http://www.ncbi.nlm.nih.gov

RESULTS

Establishment and Validation of Screening Model Targeting VaPDF

The genome sequence of *V. anguillarum* on NCBI web was used as a major reference to clone the *pdf* gene. The sequencing result showed that the length of *pdf* gene of *V. anguillarum* YN was 510 bp (including stop codon) which encodes a 19.21 kDa "Class I" PDF (Giglione et al., 2000) (**Figure 1A**), and the GenBank accession number of this gene is KU214433. BLAST result showed that its encoding protein VaPDF had 98.0% identity to other types of *Vibrio* sp. PDFs in amino acid sequence. VaPDF shared three highly conserved characteristic stretches (Baldwin et al., 2002): motif 1 (GIGLAATQ), motif 2 (EGCLS), and motif 3 (HELDH) (Supplementary Figure S1) with other types of PDFs.

Activity of targeting protein is essential for the establishment of screening model. Based on previous data, PDFs purified from *Leptospira interrogans* etc. catalyzed the removal of a formyl group from the N-termini of nascent polypeptides (Li et al., 2002). Consistently, the purified VaPDF catalyzed the removal of the *N*-formyl group from formyl-Met-Ala-Ser (**Figure 1B**) and the free *N*-formyl group could reacted with fluorescamine to form highly fluorescent products. The optimized reaction conditions were determined as 40 nM VaPDF, 1 mM substrate in 25 mM HEPES buffer (pH 7.4) for 30 min at 37°C. The VaPDF screening model can tolerate up to 2% DMSO (Supplementary Figure S2). Moreover, the Z′ factor was calculated in order to evaluate the PDF assay for HTS. In this model, the value of Z′ factor was 0.71(≥0.5) which is considered acceptable for HTS. The CV values were $CV_{FImax} = 6.7\%$ and $CV_{FImin} = 5.1\%$. Both values were less than the threshold value of 10% that is recognized as delineation of correct assays (**Figure 1C**).

Selective Isolation of Actinomycetes

To find potential novel compounds against *V. anguillarum* with our HTS model mentioned above, we sought to isolate marine actinomycetes derived natural products for the screening. Totally, 84 actinobacterial strains were isolated from eight marine sediment samples based on the characteristic colonial morphology. As expected, the predominant population of marine actinomycetes was similar to the previous report with marine sediment samples (Maldonado et al., 2005), which showed that *Streptomyces* was the most abundant species, then was the *Micromonospora*. Other rare actinomycetes were also recovered from sediment samples. Thereafter, 22 strains were selected and subjected to 16S rRNA gene sequence analysis. GenBank accession numbers were shown in **Table 1**. Results indicated that these 22 strains shared 99% of similarities with their closest strains. And they belonged to eight genera, which were *Micromonospora, Nocardiopsis*, *Prauserella*, *Promicromonospora, Saccharopolyspora*, *Salinispora, Streptomycetes,* and *Verrucosispora*. The phylogenetic affiliation was investigated and the results were presented in **Figure 2**.

TABLE 1 | Activity assays of marine actinomycetes crude extracts.

Strain number	Sediment sample number	Closest species	Anti-*V. anguillarum* activity (%)	Anti-PDF activity (%)
NHF7	54	*Streptomyces labedae*	39.2 ± 3.4	0
NHF15	54	*Nocardiopsis lucentensis*	18.1 ± 5.0	25.3 ± 11.3
NHF22	54	*Nocardiopsis lucentensis*	9.8 ± 6.3	28.3 ± 5.0
NHF26	54	*Nocardiopsis valliformis*	3.8 ± 3.2	28.8 ± 2.2
NHF27	76	*Nocardiopsis lucentensis*	20.8 ± 3.6	37.7 ± 4.7
NHF28	33	*Prauserella marina*	34.9 ± 2.7	0
NHF45	37	*Salinispora arenicola*	0	0
NHF48	31	*Nocardiopsis lucentensis*	12.9 ± 3.1	42.1 ± 0.9
NHF57	37	*Micromonospora humi*	10.0 ± 6.0	49.9 ± 0.12
NHF61	37	*Promicromonospora aerolata*	0	0
NHF69	76	*Micromonospora aurantiaca*	0	0
NHF86	33	*Streptomyces violascens*	18.0 ± 3.4	50.0 ± 2.5
NHF90	65	*Streptomyces praecox*	21.5 ± 3.8	0
NHF93	32	*Streptomyces griseoplanus*	4.9 ± 1.6	0
NHF97	31	*Streptomyces anulatus*	37.9 ± 5.3	22.2 ± 10.0
NHF107	54	*Prauserella marina*	0	0
NHF129	37	*Micromonospora* sp.	42.5 ± 2.7	0
NHF132	65	*Saccharopolyspora spinosa*	0	0
NHF133-2	65	*Saccharopolyspora spinosa*	0	0
NHF142-1	69	*Verrucosispora gifhornensis*	0	0
NHF148	69	*Micromonospora carbonacea*	0	0
NHF165	32	*Streptomyces cacaoi* subsp. *cacaoi*	70.0 ± 8.3	49.1 ± 20.6

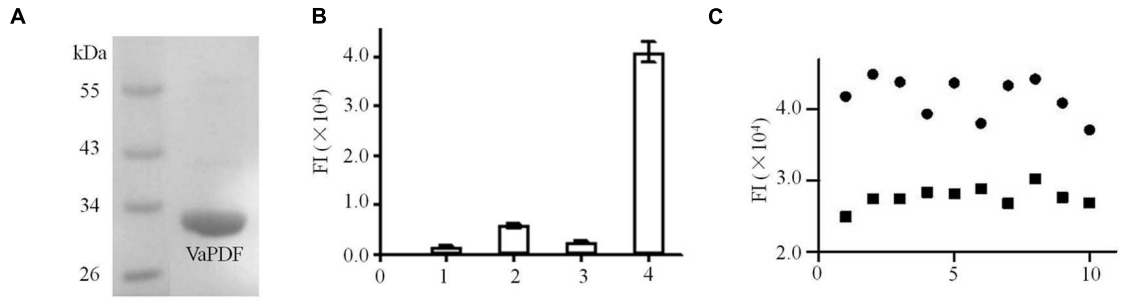

FIGURE 1 | Purification and activity assays of *Vibrio anguillarum* PDF (VaPDF). (A) The purified VaPDF with 6 × His tag. **(B)** Activity assays of purified VaPDF. 1, HEPES buffer. 2, HEPES buffer + formyl-Met-Ala-Ser. 3, HEPES buffer + VaPDF. 4, HEPES buffer + formyl-Met-Ala-Ser + VaPDF. Data are representative of three independent experiments. Error bars indicate standard deviation. **(C)** Validation of VaPDF assay. Fluroscence intensity of positive controls and negative controls were detected ($n = 10$). Solid circle, negative control; solid square, positive control.

HTS for Crude Extracts of Marine Actinomycetes

To identify the anti-VaPDF activity of different marine actinomycetes mentioned above with the present HTS model, the corresponding crude extracts were prepared with ethyl acetate extraction method. Thereafter, the crude extracts were used for screening to discover anti-VaPDF agents. For the first round screening, each crude extract was added to a final concentration of 20 μg/ml to the reaction system. Screening results showed that crude extracts isolated from strains NHF27, NHF48, NHF57, NHF69, NHF86, and NHF165 exhibited anti-VaPDF activity with minimum 30% inhibition. Active crude extracts were produced by strains affiliated to genera *Micromonospora, Nocardiopsis,* and *Streptomyces.* To confirm the anti-vibrio activities of above active crude extracts, anti-*V. anguillarum* YN cell activity results were also checked and shown in **Table 1**. Notably, crude extract isolated from strain *Streptomyces* sp. NHF165 exhibited the highest inhibitory both on VaPDF activity and *V. anguillarum* YN cell growth. Therefore, *Streptomyces* sp. NHF165 was chosen for further study. Strain NHF165 had a highest 16S rRNA gene similarity (>99%) with *Streptomyces cacaoi* subsp. *Cacaoi*, and colonies of this strain appeared to be yellow substrate mycelium and white aerial mycelium. Oval spores were produced along the long, straight and smooth aerial mycelium after 7 days of cultivation on medium GT (**Figure 3**).

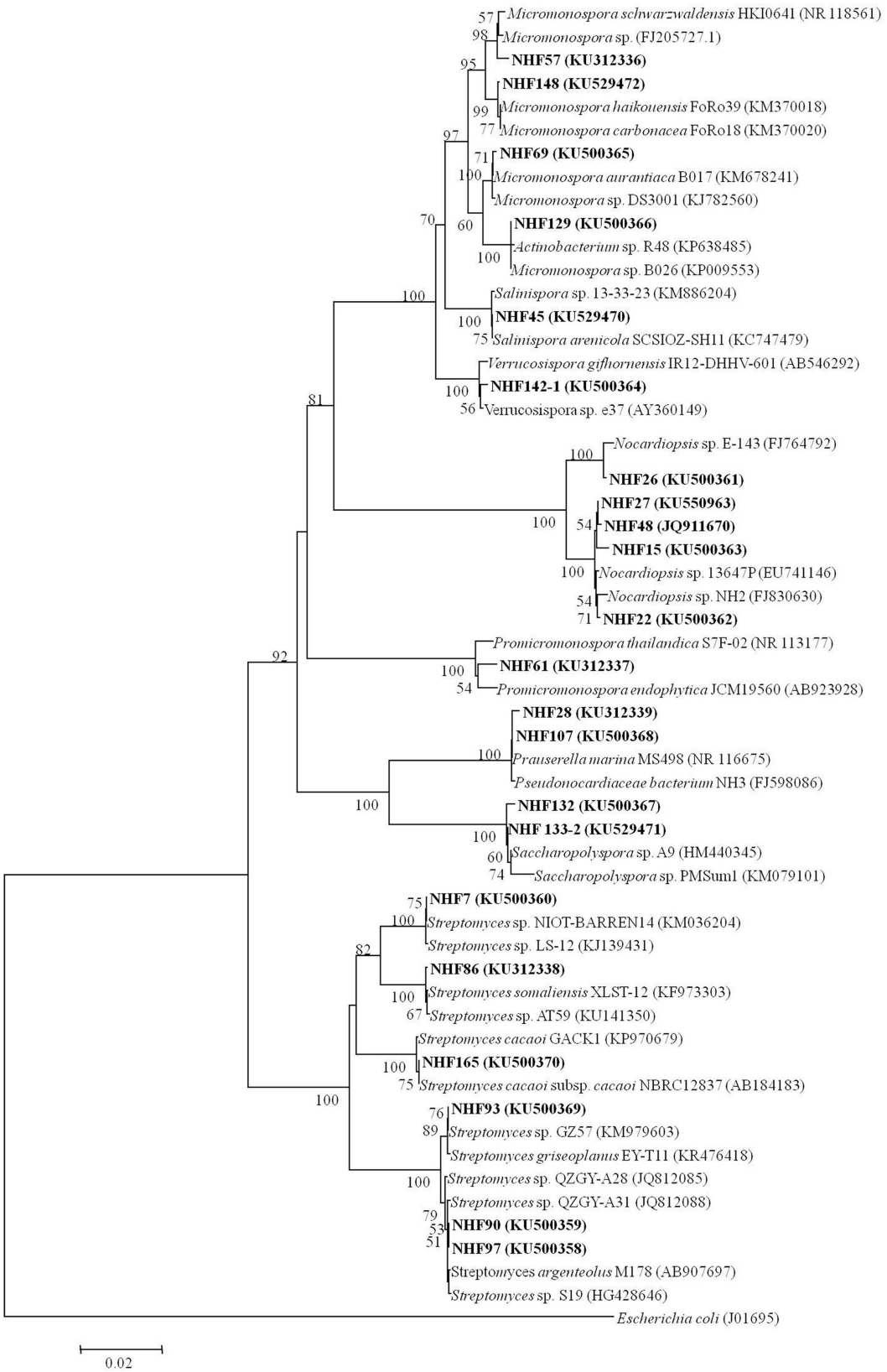

FIGURE 2 | Neighbor-joining tree showing the phylogenetic relationships of actinobacterial 16S rRNA gene sequences of obtained strains from South China Sea sediments. Bar, 0.02. Bootstrap values of >50% (for 1000 replicates) are shown.

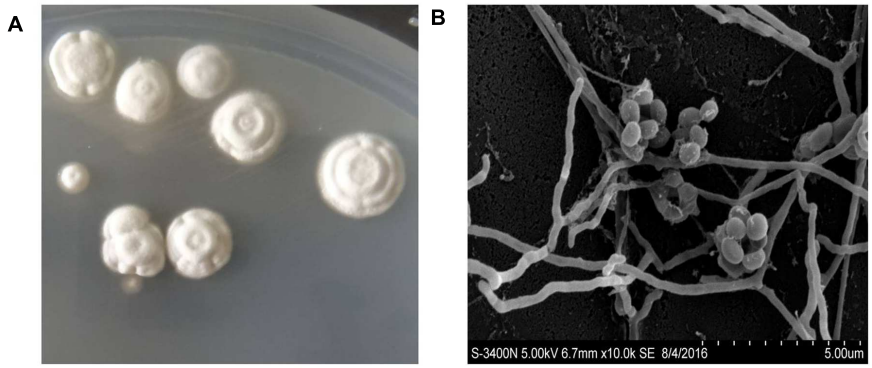

FIGURE 3 | Characteristics of *Streptomyces* sp. NHF165. (A) Colony characteristics of *Streptomyces* sp. NHF165. **(B)** Scanning electron micrograph of *Streptomyces* sp. NHF165 grown on GT agar at 28°C for 7 days. Bar = 5 μm.

Structure Elucidation of Compounds Produced by *Streptomyces* sp. NHF165

To identify the exact structure of compound with anti-VaPDF activity isolated form *Streptomyces* sp. NHF165, the corresponding crude extract was separated with sephadex LH-20. The purification results showed that fraction 6 contained the main anti-VaPDF constitute. Then fraction 6 was further separated with HPLC with C18 column and two compounds were finally obtained (**1** and **2**). Their structures were elucidated by UV, 1D NMR, 2D NMR (^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC). ESI-MS data revealed molecular ion peaks at *m/z* 386.2961 $[M+H]^+$, and 408.2498 $[M+Na]^+$ for compound **1** (Umezawa et al., 1985). The compound **1** with anti-VaPDF activity was identified by comparing the NMR data with previous published data, and it was considered to be actinonin (**Figure 4A**) (Umezawa et al., 1985). The total yield of actinonin was 5.3 mg per 10 L broth. Correspondingly, this marine derived-actinonin inhibited the VaPDF activity in a dose-dependent manner and the IC_{50} was 6.94 μM. The IC_{50} of this actinonin on *V. anguillarum* cell viability was 2.85 μM (**Figures 4B,C**).

Compound **2** was obtained as light brown amorphous powder. Its HRESIMS revealed a molecular ion peak of *m/z* 565.2565 for $C_{32}H_{33}N_6O_4$ $[M+H]^+$ (Calcd. 565.2485) and suggested 564 as the molecular weight and $C_{32}H_{32}N_6O_4$ as the molecular formula. UV spectrum with the maximal absorbance at 206, 228, and 288 nm.^{13}C NMR spectrum of compound **2** revealed signals of 32 carbons, including four amide carbonyl ^{13}C resonances were suggested by signals of δ_C 165.2, C-13; 165.6, C-35; 166.0, C-16; 169.1, C-32. The ^{1}H and ^{13}C NMR spectra in combination with ^{1}H–^{1}H COSY and ^{1}H–^{1}C HSQC NMR data indicated signals of two substituted benzene groups (1, 2- substituted benzene: δ_H 7.16, *d*, 12.0, δ_C 123.8, C-5; δ_H 6.62, *t*, overlap, δ_C 118.1, C-6; δ_H 6.98, *t*, 7.2, δ_C128.1, C-7; δ_H 6.65, *d*, 6.0, δ_C 109.5, C-8; δ_C 133.0, C-4; δ_C 149.4, C-9 and 1, 2, 4- substituted benzene: δ_H 7.21, d, 6.0, δ_C 111.5, C-20; δ_H 7.03, *d*, 12.0, δ_C 119.2, C-21; δ_H 7.62, *s*, δ_C 114.6, C-23; δ_C 134.1, C-22, δ_C 127.1, C-24; δ_C 134.8, C-25). ^{1}H–^{13}C HMBC NMR data revealed HN-26 connected with C-24, C-25, C-27 (δ_C 124.7), C-28 (δ_C 109.6), and H-27 connected with C-24 and C-25. The 1, 2, 4-substituted benzene moiety was an indole structure. Combined ^{13}C and HMBC spectrum, C-30, 32, 33, 35, 36, 37, 38 signals showed a diketopiperazine moiety. H-29 [δ_H 3.23, dd (14.4, 4.2); 3.06, dd (12.0, 6.0)] connected with C-24, C-27, C-35, and HN-31 (δ_H 7.7) connected with C-32, C-35. These data suggested this group was a condensation product of tryptophan and proline. The HMBC signals from H-2 (δ_H 5.63, *s*) to C-4, C-9 and from HN-1 (δ_H 6.61) to C-2 (δ_C 81.1), C-3 (δ_C 58.7), C-4, C-8, and C-9 demonstrated that the 1, 2- substituted benzene moiety was an indoline structure. C-11, 13, 15, 16, 17, 18, 19 signals were assigned to another diketopiperazine moiety. A methylene group contributed to establish connectivity of indoline and diketopiperazine moieties. Signal from H-2 to C-16 demonstrated the connection of C-2 to N-10. Signal from H-2 to C-22 showed the connection of C-3 to C-22. ROESY data showed signals from H2 to H-11 and H-21 which suggested H-1, H-11 and indolyl diketopiperazine structure on the same side. Thus the structure of **2** was established (Supplementary Figure S3). It was apparent that compound **2** was related to asperazine derived from a marine fungi *Aspergillus niger* (Varoglu et al., 1997). Compound **2** was shown to be a new compound of indolyl diketopiperazine analogs, and it showed no activities against *V. anguillarum* or VaPDF.

Resistance Mechanism of *V. anguillarum* against Actinonin

The resistance of *V. anguillarum* YN to actinonin was challenged on MH agar with 25 μM actinonin. The frequency of resistance in *V. anguillarum* YN was 5×10^{-6}. Notably, the mutants were stable, as re-streaking on actinonin-free MH agar did not lose resistance, and no phenotypic differences between wild type and mutant were observed for this strain. Compared with parent strains, *V. anguillarum* YN mutants grew at much slower rates when cultured in MH broth (**Figure 5A**) and showed 8 × MIC to actinonin (**Figure 5B**). Moreover, these mutants showed resistance to actinonin but still remained susceptibility to streptomycin, chloramphenicol, carbenicillin, kanamycin, and ampicillin as wild type strains do.

In order to understand the mutation details, open reading frame regions of *pdf*, *folD*, *fmt,* and *glyA* DNA sequence from

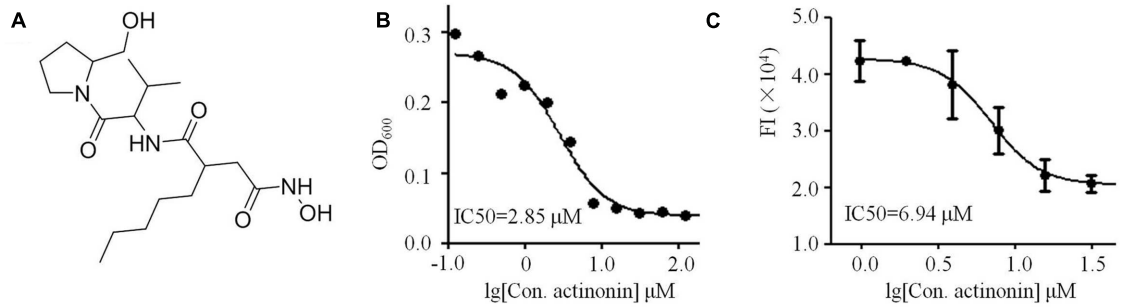

FIGURE 4 | Characterization of marine derived actinonin. (A) Structure of actinonin isolated from *Streptomyces* sp. NHF165. **(B)** Anti-*V. anguillarum* IC_{50} value of actinonin. **(C)** Anti-VaPDF IC_{50} value of actinonin.

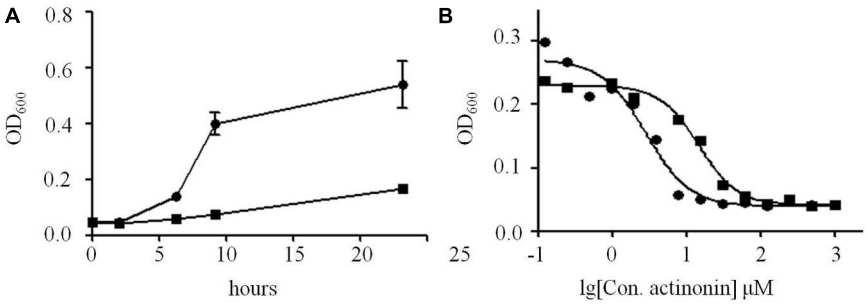

FIGURE 5 | Proliferation differences between *V. anguillarum* mutant and wild type strains. (A) Growth of *V. anguillarum* mutants and wild type strains in MH broth, measured as optical density at 600 nm. **(B)** MIC tests of actinonin on *V. anguillarum* mutants and wild type strains. Solid circle, wild type strain; solid square, mutant strain.

the mutant strains were amplified, sequenced and aligned with those from parent strains. The results showed that no mutation was retrieved in *pdf*, *fmt,* and *glyA*, and all five mutant strains harbored a mutation in *folD* gene possessing deletion of base pairs 774–852 (Supplementary Figure S4). As reported, *folD* catalyzes the formation of 10-formyl-tetrahydrofolate (THF), which supplies *N-formyl* group to Met-$tRNA^{fMet}$. To our knowledge, Δ*folD* mutants have been described only in species *Salmonella enterica* and *B. subtilis* (Duroc et al., 2009). None of the resistant strains could grow on MM medium, which consisted with the results described previously (Duroc et al., 2009). To determine whether mutation of gene *folD* is the main cause for the actinonin resistance of *V. anguillarum*, complementary experiment was performed. Plasmid pACYC184::*folD* was successfully constructed and introduced into Δ*folD* mutants to get pACYC184::*folD*/Δ*folD* strains. Complementary strains could not grow on MH agar with 25 μM actinonin in this study, which further confirmed that *folD* gene mutation was responsible for actinonin resistance in *V. anguillarum*.

To understand the expression changes between wild type and mutant strains, genes involved in translation initiation (*pdf*), amino acid biosynthesis (*gtlB*), metabolites biosynthesis (*srfAC*), ATP production (*atpH*), cell protection (*ahpF*), ABC transporter (*fhuD*), TCA cycle (*pdhA*) were checked with RT-PCR (Supplementary Table S2) and the expression of *rplL* gene was used as a reference for the determination of induction levels. Significant expression changes of *pdf*, *atpH*, and *ahpF* genes were observed for genes encoding functions of the intermediary metabolism (**Figure 6**). pdf and *atpH* genes were significantly down-regulated, which suggested that the translation initiation was hampered by less *N-formyl* group supply. However, the expression of gene *ahpF* corresponding for protecting cells was significantly up-regulated. Thus, in the tested condition, *V. anguillarum* mutants developed an adaptation mechanism to survive in high concentration of actinonin.

DISCUSSION

Vibrio anguillarum is an opportunistic fish pathogen that is common to marine and estuarine environments. It has been identified as the main cause of vibriosis, a potentially fatal septicemia that affects fish and shellfish in marine aquaculture, with consequent economic losses (Frans et al., 2011). To find novel antibiotics against *V. anguillarum* is urgently needed.

It is now widely accepted that the traditional screening methods are unlikely to generate many promising molecules. Alternative strategies must therefore be developed to find new compounds. One possible strategy is to identify a molecular target at the outset and then to screen the available libraries

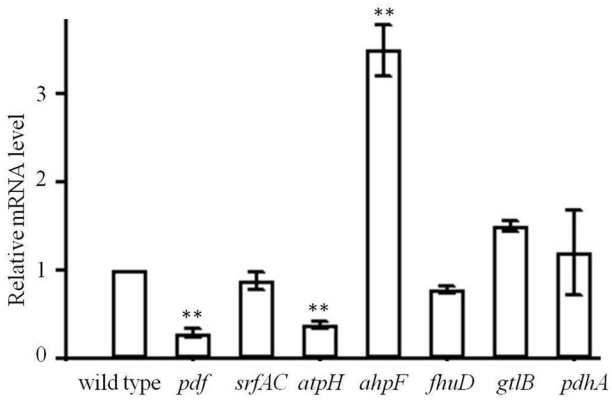

FIGURE 6 | Trancription level of *pdf, srfAC, atpH, ahpF, fhuD, gtlB,* and *pdhA* in the mutant of *V. anguillarum*. Wild type was used as a control. Values reported are the mean of three replicates. Error bars indicate the standard deviation. $^{**}p < 0.01$ was taken to indicate statistical distinct significance.

of chemical compounds looking for hits with potent inhibitory capacities *in vitro* with HTS model. However, it is extreme lack of HTS model for searching anti-*Vibrio* compounds. For this approach, the identification of a good target is vital. PDF has been suggested as a possible candidate that may fulfill all those criteria for HTS and has become a promising and attractive bacterial target to explore for the discovery of new antibacterial agents (Giglione et al., 2000). We confirmed VaPDF shared the three highly conserved characteristic stretches and was essential for *V. anguillarum* growth. Therefore, active agents against VaPDF can be potential drugs for vibriosis treatment. Due to the lack of effective anti-*Vibrio* HTS methods, we first developed a protein-based assay based on VaPDF activity and screened crude extracts derived from marine actinomycetes.

In recent years, great attention has been paid to the isolation and characterization of actinomycetes from marine environment, which provides a valuable source for discovering bioactive metabolites. South China Sea located in the southeast of China with tropical oceanic climate and was poorly studied. Therefore, we chose deep-sea sediment samples collected from South China Sea to isolate anti-*Vibrio* actinomycetes, which might be used in marine aquaculture industry. Totally, 84 actinobacterial strains belonging to eight genera were obtained. The predominant numbers of *Streptomyces* and *Micromonospora* strains is in line with the results reported previously (Maldonado et al., 2005). Representative strains isolated in the present study showed bioactivities against VaPDF and *V. anguillarum* cell. Among 22 strains, 14 strains showed anti-bacteria activity against *V. anguillarum* and 9 strains showed anti-activity against VaPDF. These strains belonged to genera *Streptomyces, Micromonospora,* and *Nocardiopsis*.

As is well known, *Streptomyces* could produce diverse range of secondary metabolites with relevant anti-inflammatory, antimicrobial, antioxidant activities (Dubert et al., 2015) and are potential probiotics in aquaculture (Tan et al., 2016). *Streptomyces rubrolavendulae* M56 isolated from the sediments of Bay of Bengal could significantly exclude the pathogenic *Vibrio* spp. in co-culture experiments (Augustine et al., 2015). Addition of 1% wet cell mass of marine *Streptomyces* strains can reduce mortality rate of nauplii and adult Artemia caused by both *V. harveyi* and *V. proteolyticus* (Das et al., 2010). Crude extract of *Streptomyces* sp. LCJ94 showed good inhibitory activities against *V. harveyi*, *V. vulnificus*, *V. alginolyticus* with the MIC values of 250, 250, and 500 μg/ml, respectively (Mohanraj and Sekar, 2013). In this study, *Streptomyces* sp. NHF165 exhibited the highest activity against *V. anguillarum*, and the functional component was finally determined as actinonin. Actinonin was isolated from soil *Streptomyces* in 1962 and was reported to be an inhibitor targeting *E. coli* PDF and *M. tuberculosis* PDF (Sharma et al., 2009). Our discovery is the first report to show that marine derived actinonin possesses anti-*Vibrio* activity via targeting VaPDF. Considering *Streptomyces* sp. NHF165 with high yield (5.3 mg/10 L) and low IC_{50} of actinonin on *V. anguillarum* (2.85 μM), it might be a good candidate for the management of vibriosis in marine aquaculture industry. On the other hand, as a natural product, actinonin shows derivative of L-prolinol and hydroxamic acid of the type R-CO-NHOH and some structural relationship to other polypeptide antibiotics. Hence, it will be very interesting to dig the conserved DNA sequence of non-ribosomal peptide synthetases (NRPS) adenylation domain (Ayuso-Sacido and Genilloud, 2005) in the genomic DNA of *Streptomyces* sp. NHF165 in the future.

Nowadays, antibiotics have been routinely applied to water to treat and prevent bacterial disease in fish and shellfish culture industries. However, extensive use of antibiotics goes with development of resistant strains, especially resistant vibrios. Characterization of antibiotic-resistant vibrios is necessary to elucidate mechanism of resistance. *Vibrio* strains with resistance to chloramphenicol, tetracycline, amoxicillin, or streptomycin were successfully isolated from hatchery larval cultures, and R-plasmids harboring resistant genes (chloramphenicol acetyltransferase, tetracycline resistance markers, etc.) were elucidated (Dubert et al., 2015). In other report, about 63% of the isolated *V. parahaemolyticus* strains were resistant to ampicillin, cephalexin, or kanamycin (Bhattacharya et al., 2000). Hence, appearance of resistance to actinonin is a predictable consequence, and it is necessary to study the resistance mechanism of *V. anguillarum* against actinonin.

It was reported that mechanisms causing PDF inhibitor resistance involve (i) mutations in the target gene, (ii) bypassing of the formylation pathway, or (iii) efflux of PDF inhibitor (Duroc et al., 2009). Notably, we could amplify genes involved in translation initiation including *pdf*, *fmt,* and *glyA* but failed to get *folD* fragment from mutants, and then we confirmed a fragment deletion happened in the gene *folD*. Interestingly, similar mutations in the gene *fold* of *S. enterica* and *B. subtilis* had been described previously (Duroc et al., 2009). The loss of function of *folD* could inactivate translation initiation pathway that uses 10-formyl-THF, which led to a dramatic decrease of growth rate of Δ*folD* mutants. It is proposed that, in

addition to *folD*, mutations in the genes involved in efflux pump, modification of actinonin or coding enzymes that degrade actinonin might also happened. Additionally, the RT-PCR results showed the expression of genes *pdf*, *atpH*, and *ahpF* were significantly regulated, which suggested that *V. anguillarum* mutants might develop an adaptation mechanism to survive in high concentration of actinonin.

Collectively, it is evident that VaPDF can be a good target for anti-*Vibrio* agents screening. And actinomycetes isolated from marine could be promising candidates for treating pathogens in marine aquaculture. It will also be very interesting to find more anti-*Vibrio* compounds with the present HTS model and develop the corresponding anti-bacteria drugs in the future.

AUTHOR CONTRIBUTIONS

NY and CS conceived and designed the experiments. NY performed all of the experiments. NY and CS analyzed the data, prepared the figures and wrote the paper. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01467

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Bovine Lactoferrin and Lactoferrin-Derived Peptides Inhibit the Growth of *Vibrio cholerae* and Other *Vibrio* species

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Vibrio is a genus of Gram-negative bacteria, some of which can cause serious infectious diseases. Vibrio infections are associated with the consumption of contaminated food and classified in *Vibrio cholera* infections and non-cholera *Vibrio* infections. In the present study, we investigate whether bovine lactoferrin (bLF) and several synthetic peptides corresponding to bLF sequences, are able to inhibit the growth or have bactericidal effect against *V. cholerae* and other *Vibrio* species. The antibacterial activity of LF and LF-peptides was assessed by kinetics of growth or determination of colony forming unit in bacteria treated with the peptides and antibiotics. To get insight in the mode of action, the interaction between bLF and bLF-peptides (coupled to FITC) and *V. cholera* was evaluated. The damage of effector-induced bacterial membrane permeability was measured by inclusion of the fluorescent dye propidium iodide using flow cytometry, whereas the bacterial ultrastructural damage in bacteria treated was observed by transmission electron microscopy. The results showed that bLF and LFchimera inhibited the growth of the *V. cholerae* strains; LFchimera permeabilized the bacteria which membranes were seriously damaged. Assays with a multidrug-resistant strain of *Vibrio* species indicated that combination of sub-lethal doses of LFchimera with ampicillin or tetracycline strongly reduced the concentration of the antibiotics to reach 95% growth inhibition. Furthermore, LFchimera were effective to inhibit the *V. cholerae* counts and damage due to this bacterium in a model mice. These data suggest that LFchimera and bLF are potential candidates to combat the *V. cholerae* and other multidrug resistant *Vibrio* species.

Keywords: lactoferrin, lactoferrin peptides, LFchimera, bactericide, *Vibrio cholerae*

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INTRODUCTION

The human innate-immune system is made up of a large variety of important components that attack or destroy any form of infection, in these components are included antibodies; white blood cells, antimicrobial proteins, and peptides, etc (Zaiou, 2007). This defense system is also found in other species of mammals, including bovines, sheep, and camels (Baveye et al., 1999). Although the development of new generation of antibiotics has rapidly progressed and gained popularity over the antimicrobial peptides, even the most powerful antibiotics have been unsuccessful to diminish morbidity and mortality due to the antimicrobial resistance showed by emergent multi-resistant strains of pathogens (Longworth, 2001; Spellberg et al., 2008). Antimicrobial peptides that are less prone to induction of resistance by bacteria are a class of substances that are now investigated to combat multi-drug resistant bacteria, with promising results (Ellison et al., 1990b; Garbacz et al., 2017; Greber and Dawgul, 2017). Such compounds include bovine lactoferrin (bLF) and LF-derived peptides (Ellison et al., 1990b). LF is an abundant iron-chelating protein present in colostrum and milk of most mammals, participating in the newborn protection against infections (Brock, 1980, 2002). LF is also present in mucosae and secreted bodily fluids such as bile, bronchioalveolar fluid, and intestinal and reproductive tract secretions, and it is produced and released by the polymorphonuclear neutrophils during inflammation (Brock, 2002). LF from bovine (bLF) and the LF derived peptides have been studied most extensively, due to exhibit antibacterial, antifungal, antiviral, and antiparasitic activities, in a direct way by a direct damage on pathogens and also by enhancing the mucosal immune function against pathogens (Brock, 2002; Orsi, 2004; Aguilar-Diaz et al., 2017; Juretic et al., 2017). bLF exerts its bactericidal action in two ways: indirectly, by limiting the amount of iron available for the growth and metabolism, and directly, by affecting the bacterial membrane (Ellison et al., 1988, 1990a; Ellison and Giehl, 1991; Orsi, 2004; Vogel, 2012). Other functions such as inhibition of bacterial adhesion or invasion to target cells, decrement of aggregation or biofilm development, have been also reported to LF and LF-peptides in bacteria (Singh et al., 2002; Orsi, 2004; Abbas et al., 2007; Juretic et al., 2017). The antimicrobial activity of LF is attributed to a region located at the N1-domain of the protein (Farnaud and Evans, 2003). In this sense, a peptide called lactoferricin B (LFcinB) is released from the N-terminus of bLF in during its passage through the intestine (Bellamy et al., 1992). Other antimicrobial peptides of the N1-domain have been identified and synthetically produced, for example, lactoferrampin (LFampin) (Van Der Kraan et al., 2004, 2005). Furthermore, a chimerical structure based in the active parts of the protein LF was designed and synthesized, this peptide contain the amino acids 17–30 of LFcinB and amino acids 265-284 of LFampin 265-284, the resulting peptides was called LF chimera (Bolscher et al., 2009). The bactericidal activity of LFchimera has been definitively stronger than that of the peptides (LFcin17-30 and LFampin265-284), as has been demonstrated in many experiments; due to lower concentrations, shorter incubation time, and salt concentrations present in the environment (needed for the growth of halophile bacteria) permit the bactericidal activity of LF chimera, compared with the peptides that conform this molecule which is not effective at these conditions (Bolscher et al., 2009; Haney et al., 2009; Leon-Sicairos et al., 2014). Otherwise, the microbicidal effect of LFchimera against *Candida* spp, *Vibrio parahaemolyticus, Staphylococcus aureus*, enterotoxigenic and enterohaemorragic *Escherichia coli,* or in the parasites *Entamoeba histolytica, Burkholderia thailandensis,* and *Leishmania pifanoi* has been established *in vitro* (Bolscher et al., 2009; Lopez-Soto et al., 2009, 2010; Flores-Villasenor et al., 2010, 2012; Kanthawong et al., 2014; Leon-Sicairos et al., 2014; Puknun et al., 2016).

Vibriosis is an infection caused by species of the *Vibrio* genus. *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* are serious human pathogens (Thompson et al., 2004). *Vibrio cholerae* is the causative agent of cholera. It has been reported that the mortality rate of untreated cholera cases is about 50 to 60% (Frost, 1976; Faruque et al., 1998). Other *Vibrios* clinically significant for humans are *V. algynoyticus*, *V. parahaemolyticus,* and *V. vulnificus*. *V. alginolyticus* is medically important since it causes otitis and wound infection (Powell, 1999; Hernandez-Robles et al., 2016). The halophilic (salt-loving) *V. parahaemolyticus* has been identified as a leading cause of human gastroenteritis, associated to the consumption of raw or improperly cooked seafood (Ellison and Giehl, 1991; Su and Liu, 2007). Another halophilic *Vibrio* has recently been identified as *V. vulnificus*. This bacterium is an opportunistic pathogen that can cause infections of humans and other animals including fish. *V. vulnificus* is extremely harmful and is responsible for the devastating majority of reported seafood-related deceases in the United States (Warnock and Macmath, 1993; Powell, 1999; Jones and Oliver, 2009). The bacteria is located as a natural flora of coastal marine environments worldwide, for that it has been isolated from a seafood, shrimp, fish, oysters and clams, water, and sediments (Do Nascimento et al., 2001; Jones and Oliver, 2009; Jones et al., 2014).

It has been reported that the number of *V. cholerae* and *V. non-cholerae* cases has augmented increasingly in recent years. However; the major health problem is the emergence and spread of *V. cholerae* and *Vibrio non-cholera* strains antibiotics-resistant (Colwell, 1996; Faruque et al., 1998; Lipp et al., 2002; Sedas, 2007). For these reasons, the search for new compounds for Vibrio infections; treatment or prevention is needed. In previous work, it was demonstrated that LF had antibacterial effect against *V. cholerae* (Ellison and Giehl, 1991). Then; we reported that LF and the LFpeptides display antibacterial activity against a *V. parahaemolyticus* multidrug resistant strain, and also in *V. cholerae* O1 Inaba and non-O1 strains (Leon-Sicairos et al., 2009). In the present study; we continue the research of the bactericidal activity of bLF and bLF-derived peptides LFcin17-30, LFampin265-284 and LFchimera against *Vibrio* species resistant to antibiotics (including *V. cholerae* strains O1 and non-O1, *in vitro* and *in vivo*). In addition, we explore the mechanism of damage of these compounds into bacteria, and their synergism with antibiotics in the bactericidal effect.

MATERIALS AND METHODS

Lactoferrins, Bacterial Strains, and Culture Conditions

Bovine LF (bLF, 20% iron saturated) was kindly donated by Abial (Santander, Spain). The purity of bLF (>98%) was confirmed by SDS-PAGE gels using silver nitrate staining. LF concentration was measured by UV spectroscopy on the basis of an extinction coefficient of 15.1 (280 nm, 1% solution) (Valenti et al., 1999). The bLF iron saturation was about 20% as detected by optical spectroscopy at 468 nm on the basis of an extinction coefficient of 0.54 (100% iron saturation). LPS contamination of bLf, estimated by Limulus Amebocyte assay (LAL Pyrochrome kit, ThermoFicherScientific, Waltham, MA, USA), was equal to 0.7 ± 0.06 ng/mg of bLF. Synthetic peptides (LFcin17-30, LFampin265-284 and LFchimera) were obtained by solid phase peptide synthesis using Fmoc chemistry, as has been reported previously (Bolscher et al., 2009; Cutone et al., 2014).

The following *Vibrio* strains obtained by us were used: *V. cholerae* O1 Inaba, *V. cholerae* non-O1 (toxigenic), *V. fluvialis*, *V. alginolyticus*, *V. vulnificus,* and *V. furnissii* (Velazquez-Roman et al., 2012; De Jesus Hernandez-Diaz et al., 2015). Bacteria were incubated in Luria-Bertani medium (LB) (Difco, Becton Dickinson, USA) with 3% NaCl, incubated in agitation (5,000 rpm) and grown at 37°C for 16–18 h. In all the experiments in the presence of bLF or peptides, to avoid saturation with iron the ion was removed from the LB medium by incubation with Chelex-100 resin (5 g/l) in constant agitation at 4°C. After 16 h, the resin was taken off by filtration and finally the medium was sterilized (iron-depleted medium). The viability of bacterial cultures grown at these conditions was not affected. Additionally, all glass materials were treated with 6 M HCl to eliminate iron traces as previously reported (Leon-Sicairos et al., 2009).

Growth Inhibition in the Presence of bLF and bLF-Peptides

To determine the antibacterial activity of bLF and bLF peptides, ∼1 × 10^7 CFU/ml of *V. cholerae* O1 Inaba or *V. cholerae* non-O1 in 96-well microplates (Corning) containing 200 µl iron-depleted LB media were incubated at 37°C with 5, 10, 20, 30, and 40 µM bLF, LFcin17-30, LFampin265-284, or LFchimera, for 1, 2, 4, and 6 h. In parallel bacterial suspensions were treated with 25 µg/ml of Gentamicin or without additions as growth control. Bacterial growth was followed by measuring the OD660 nm of cultures. Next, the percentage of viable cells was estimated in relation to untreated cultures (without peptides or antibiotics). Viable cells were also counted as colony forming units/ml (CFU/ml) from serial 10-fold dilutions incubated in Muller-Hinton-Broth (MH broth), and then plated on MH agar plates at time and conditions afore mentioned. An electronic counter (CountTM, Heathrow Scientific) was used to count colonies. All experiments were repeated at least twice in triplicate. The synergistic effect of bLf on the antibiotic MIC was evaluated using the fractional inhibitory concentration (FIC). The interpretation of results was based on the following scale: FIC > 2 indicated a synergistic effect (Luna-Castro et al., 2014). All experiments were repeated at least twice in triplicate. Statistical significance was determined using a Student's *t*-test ($p < 0.05$), or ANOVA (with Bonferroni correction).

Flow Cytometry

To see if bLF and bLFpeptides cause membrane permeabilization, we used the staining with propidium iodide (PI), a fluorescent dye (this PI-assay is a quick assay that allowed us to compare the membrane damage by inclusion of PI, in a series of peptides under different conditions and incubation times). In brief, aliquots of 10^7 CFU/ml of *V. cholerae* O1 and non-O1-strains were cultured in LB broth, harvested by centrifugation (5,000 rpm/5 min), washed three times with LB broth, and incubated with 40 µM bLF or 20 µM of either LFcin17-30, LFampin265-284, or LFchimera at 37°C for 2 h. Next, bacteria were washed and incubated with 10 mg/ml PI during 10 min at 4°C, washed five times with PBS (pH 7.4), and after fixed with 4% paraformaldehyde. Samples were washed twice with PBS and finally analyzed with a FACScan (Fluorescence-Associated Cell Scanner; Becton Dickinson, USA). Control experiments were carried out with bacteria either without the addition of bLF or bLFpeptides (membranes integrity control), or with 0.5% Triton X-100 (which permeabilizes bacterial membranes). All experiments were done at least twice in duplicate.

Electron Microscopy

V. cholerae O1 and non-O1 strains (10^8 CFU/ml) cultures were incubated in iron-depleted LB without additions (negative control of damage), with 0.5% SDS (positive control of damage), or with 40 µM of bLF, or with 20 µM LFcin17-30, LFampin265-284 or 5 µM LFchimera, at 37°C for 1.5 h. Cells were collected, placed in tubes with PBS (pH 7.4) and fixed with 4% paraformaldehyde plus 0.5% glutaraldehyde. Next, samples were washed with distilled water and deposited on bare 200-mesh copper grids. Next, phosphotungstic acid (1%, pH 5.5, 30 s) was added, replicas were then dehydrated and finally studied with a transmission electron microscope (IEM2000Ex), operated at 100 kV.

Confocal Microscopy

The interaction of *V. cholerae* with bLF and the bLFpeptides was investigated by confocal microscopy. Briefly, 10^7 CFU/ml of *V. cholerae* O1 cells were incubated in iron-depleted LB containing 2 µM FITC-labeled peptides for 30 min. Bacteria were centrifuged (5 min, 10,000 × g), resuspended and fixed (4% paraformaldehyde, pH 7.4 during 30 min at 37°C), washed twice and prepared to be examined under confocal microscopy. To find out whether bLF and bLFpeptides are recognized by the bacterial membrane of dead bacteria, *V. cholerae* O1 cells were fixed, then washed twice with PBS and incubated with 2 µM of FITC-bLF or FITC-labeled peptides for 30 min. After, samples were washed with PBS and mounted on slides and processed. All samples were analyzed under confocal microscopy by using a confocal laser-scanning microscope (Leica, Heidelberg, Germany).

Effect of bLF and Lfchimera on the Antibacterial Activity of Classic Antibiotics Used against *Vibrio* spp.

V. cholerae O1 Inaba, *V. cholerae* non-O1, *V. vulnificus* (resistant to tetracycline and ampicillin), *V. fluvialis* (resistant to ampicillin and cefotaxime), *V. alginolyticus* (resistant to ampicillin and tetracycline), and *V. furnissii* (resistant to ampicillin), were used to determine whether bLF and LFchimera potentialize the bactericidal activity of common antibiotics. First, to test the resistance level to common antibiotics, the bacterial strains were grown with or without gentamicin (2–25 μg/ml), tetracycline (2.5–20 μg/ml), chloramphenicol (2.5–30 μg/ml), or ampicillin (2.5–32 μg/ml); bactericidal activity of LFchimera (1, 5, 10, and 20 μM) was tested in parallel. Next the antibiotics ampicillin (2.5–32 μg/ml), chloramphenicol (2.5–30 μg/ml), and tetracycline (2.5–20 μg/ml) were tested in the presence of a sub-MIC concentration of bLF (10 μM) or LFchimera (1 μM). Percentage of viable cells was determined in relation to cultures without added peptides or antibiotics. All experiments were repeated at least twice in triplicate.

In Vivo Model

Bacterial Strain and Culture Conditions

The *Vibrio cholerae* O1 serotype Inaba was maintained in TCBS agar (BD, USA) at 37°C during 24 h. Bacterial cultures (used for mice inoculations) were routinely grown on LB agar plates with 100 mg/ml streptomycin for 18 h and finally were grown with shaking in LB broth with antibiotic at 37°C to mid-log phase. The OD620 nm was adjusted to 1 and this inoculum was used in the assays.

Inoculation of *Vibrio cholerae* O1 Serotype Inaba in Mice and Treatments

Six to eight-week-old female BALB/cAnNHsd mice (Harlan Laboratories, Inc., Mexico), were purchased and housed under specific-pathogen-free conditions as stipulated by the Ethical Committee for Laboratory Animals in Faculty of Medicine of UAS and were divided into five groups. Mice were given 0.1% (w/v) Streptomycin for 3 days to ablate normal flora. A day prior to inoculation, food was removed from cages to empty the stomach. Mice were injected intraperitoneally with 12.5 mg/kg xylazine. When mice were deeply sedated, 50 μl of 0.5 M NaHCO3 was administered intragastrically immediately followed by 500 μl of bacterial suspension (2.5×10^7 CFU). After inoculation, mice were kept with free access to food and sterile water without streptomycin. Then, after 4 h post-inoculation (after infection and symptoms were established) different treatments were administered into mice each 12 h for 3 days. Treatment doses administered were as follows; 65 mg/Kg of bLF, 5 μg/Kg of LFchimera, and 14 mg/Kg of Tetracycline (Sigma Inc. USA). Mice of the control group were administered 0.5 ml of PBS instead of antimicrobial agents. All of the mice were housed in groups consisting of 10 mice each and permitted food and water *ad libitum*.

Identification of *Vibrio cholerae* O1 in Infected Mice

In order to evaluate the *V. cholerae* mice infection procedure and establishment, a disposable 1 μl plastic inoculation loop (diameter 2.0 mm) was introduced into the rectum. The loop was turned around to obtain *V. cholerae* O1 from the inner surface of the rectum. The tip of the loop was subsequently clipped into a 1.5 ml tube containing 500 μl of enriched alkaline peptone water and incubated for 18 h at 37°C. The bacteria from slopes were streaked onto TCBS agar and CHROMagarTM Vibrio to confirm the infection. Once the infection was demonstrated (after 4 h) the treatments were administered.

CFU Enumeration of *Vibrio cholerae* O1 in Feces and Intestines of Mice

Briefly, fresh feces of mice were weighed and suspended in PBS. Then, samples were homogenized and serial dilutions were prepared and plated onto LB agar plates with 100 mg/ml streptomycin for 24 h at 37°C. For confirmation, developed colonies were counted and then plated onto CHROMagarTM Vibrio (CHROMagar; Paris, France). On the other hand, the intestines were dissected, homogenized in PBS and serial dilutions were prepared in PBS and plated in LB agar plates with 100 mg/ml streptomycin. Finally, colonies were counted and plated onto CHROMagarTM Vibrio.

RESULTS

bLF and bLFpeptides Inhibited the Growth of *V. Cholerae* O1 and Non-O1 Strains

Historically, *V. cholerae* O1 and non-O1 strains have caused more problems to human health that other *Vibrio* species. So, we used these strains in order to test the antibacterial activity of bLF and LFpeptides. The ability of bLF and LFpeptides (LFcin17-30, LFampin265-284, and LFchimera) to inhibit the growth of *V. cholerae* O1 and non-O1 strains of *V. cholerae* was analyzed by measuring the growth in untreated and treated cells after several incubation times. Results shown that LFchimera had the best bactericidal effect, since 5 μM inhibited the growth until 6% at 2 h of incubation (with respect to the untreated bacteria). This inhibition was better than those exerted by 25 μg/ml of Gentamicin which inhibited the culture until 22% during the first 2 h of incubation. Percentage of growth inhibition in cultures treated with 40 μM bLF was 16, 42% with 20 μM LFcin17-30 and 82% with LFampin265-284 (**Figure 1A**). In addition, only LFchimera at a concentration of 5 μM showed bactericidal activity after 4 and 6 h incubation. Gentamicin at 25 μg/ml showed low percentage of viability without any increase within 6 h, whereas in the presence of peptides LFcin17-30 and LFampin265-284 a decrease in viability was only found after 2 h and followed by a recovery of the viability after 4 and 6 h (**Figure 1A**).

On the other hand, by CFU counts after 2 h of incubation, *V. cholerae* O1 cultures treated with 5 μM LFchimera and 40 μM bLF were significantly reduced (until 2 and 6% respectively, relative to untreated bacteria) and the effect were similar to the bactericidal activity of Gentamicin (6% growth inhibition relative

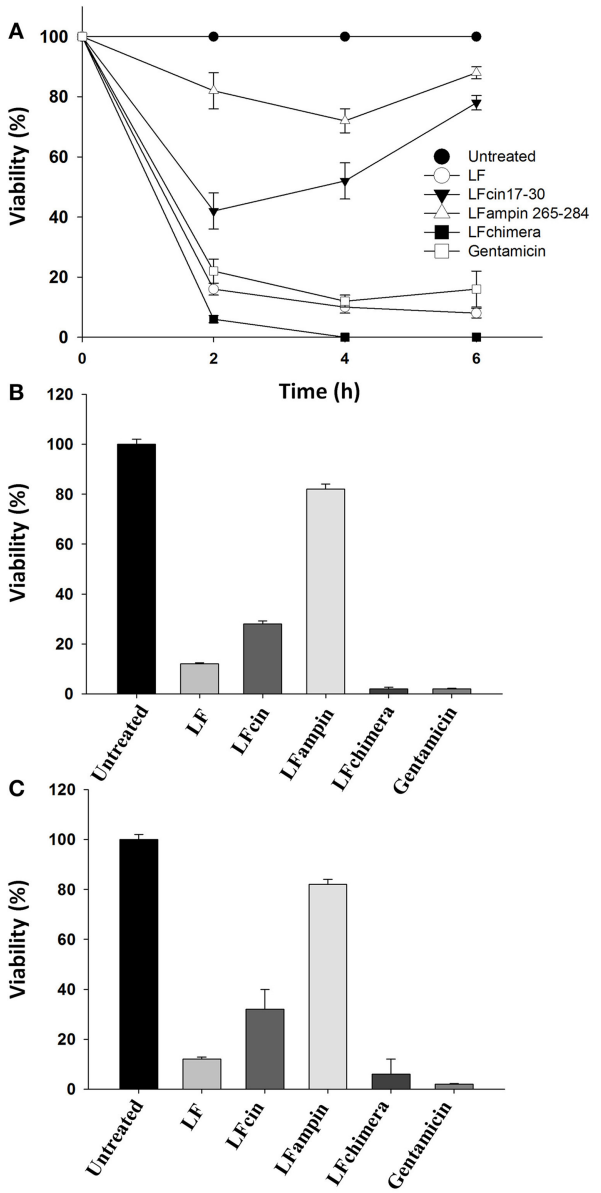

FIGURE 1 | Bactericidal effect of bLF and LF-peptides on *Vibrio cholerae* O1 and non-O1. Approximately 1×10^7 CFU/ml of *V. cholerae* O1 and non-O1 strains were incubated with bLF and LFpeptides solutions at final concentrations of 40 μM bLF or 20 μM of LFcin17-30, LFampin265-284, respectively; and 5 μM of LFchimera at 37°C with constant agitation for 1, 2, 4, or 6 h. Bacteria grown in LB broth were used as a control for optimal growth and 100 μM of Gentamicin was used as a control for growth inhibition. Bacterial growth was followed by measuring the OD660 nm of cultures. Percentage of viable cells was determined in relation to cultures Gentamicin without peptides or antibiotics **(A)**. All Experiments were repeated at least twice in triplicates. *V. cholerae* O1 **(B)** and non-O1 **(C)** strains were incubated with bLF and LFpeptides solutions at final concentrations of 40 μM bLF and 20 μM LFcin17-30, LFampin265-284, and 5 μM LFchimera; respectively. Viability was monitored by enumerating colony forming units CFU/ml (viable cells) obtained from serial 10-fold dilutions plated onto MH agar **(B,C)**. Percentage of viable cells was calculated relative to viable bacteria untreated grown in MH agar. Experiments were performed in triplicate; mean and standard deviation are indicated. Statistical significance was determined using a Student's *t*-test for *P*-values < 0.05, and ANOVA (with Bonferroni correction).

to untreated bacteria) (**Figure 1B**). However, in *V. cholerae* non-O1 strain Gentamicin and LFchimera apparently inhibited the cultures with the same efficacy (**Figure 1C**). In all treatments and incubation times (longer than 2 h) LFchimera completely inhibited the growth of *V. cholerae* O1 and *V. cholerae* non-O1 strains (**Figures 1B, C**; respectively). The bactericidal activity of LFchimera was stronger than those of bLF, LFcin17-30, and LFampin265-284 (6, 22, and 82% of growth inhibition, relative to untreated bacteria).

Lactoferrin and Lactoferrin-Derived Peptides Showed Combined Effect with Antibiotics and Inhibited the Growth of *Vibrio*

V. cholerae O1 Inaba, *V. cholerae* non-O1, *V. vulnificus* (resistant to tetracycline and ampicillin), *V. fluvialis* (resistant to ampicillin and cefotaxime), *V. alginolyticus* (resistant to amplicillin and tetracycline), and *V. furnissii* (resistant to ampicillin) were used to determine whether bLF and LFchimera, each in combination with common antibiotics increase the bactericidal effect.

In the results, the combination of 1 μM LFchimera plus 2.5 μg/ml ampicillin were able to inhibit more than 95% of growth of *V. vulnificus*, *V. fluvialis*, *V. alginolyticus,* and *V. furnissii*. Similar effects on growth inhibition (more than 95%) were found with concentrations of 5 μM LFchimera (**Table 1**), or more than 32 μg/ml ampicillin, suggesting that the combination of LFchimera and ampicillin (1 and 2.5 μg/ml) can inhibit the growth of *Vibrio* spp. resistant to ampicillin (**Table 1**). On the other hand, by using a combination of LFchimera with tetracycline, the combination of 1 μM LFchimera plus 2.5 μg/ml of tetracycline was able to inhibit more than 95% of the growth of *V. vulnificus*; this inhibition growth is only reached with concentrations of 5 μM of LFchimera or more than 20 μg/ml of tetracycline (**Table 1**). A mixture of 1 μM LFchimera and 2.5 μg/ml chloramphenicol inhibited more than 95% of the growth of *V. cholerae* O1 and non-O1 strains, whereas this growth inhibition level was only reached by using concentrations of 5 μM LFchimera or 30 μg/ml chloramphenicol (**Table 1**). Ten microliters bLF also had synergism or combined effect with antibiotics in the strains above mentioned, whereas without antibiotics the concentration needed to inhibit more than 95% was 10 μM bLF. These data suggest that LFchimera and bLF combined with low concentrations of antibiotics have bactericidal effect in multidrug resistant strains of genus *Vibrio* (**Table 1**). According to the calculation of FICs, both bLF and LFchimera have synergistic effects when they were used with the antibiotics, suggestion that they could improve the management of *Vibrio* spp multidrug resistant strains to antibiotics *in vivo*.

bLF and bLFpeptides Cause Damage on *Vibrio cholerae* O1 and Non-O1 Strains

The effect of bLF and bLFpeptides on the bacterial membrane integrity was investigated using the fluorescent dye PI (it only enters in permeabilized cells). PI was measured under flow cytometry. After the treatment, almost all *V. cholera*

TABLE 1 | Effects of LFchimera and bLF combined with antibiotics on *Vibrio* spp growth.

Concentrations (μg/ml) of antibiotics for causing more than 95% growth inhibition			Concentrations (μg/ml) of antibiotics for causing more than 95% growth inhibition		
Strain	**Ampicillin without LFchimera**	**Ampicillin with 1 μM LFchimera**	**Strain**	**Ampicillin without bLF**	**Ampicillin with 10 μM bLF**
V. vulnificus	32	2.5	*V. vulnificus*	32	2.5
V. fluvialis	32	2.5	*V. fluvialis*	32	2.5
V. alginolyticus	32	2.5	*V. alginolyticus*	32	2.5
V. furnissii	32	2.5	*V. furnissii*	32	2.5
Strain	**Tetracycline without LFChimera**	**Tetracycline with 1 μM LFChimera**	**Strain**	**Tetracycline without LF**	**Tetracycline with 10 μM LF**
V. vulnificus	20	2.5	*V. vulnificus*	20	2.5
Strain	**Cloramphenicol without LFChimera**	**Cloramphenicol with 1 μM LFChimera**	**Strain**	**Cloramphenicol without LF**	**Cloramphenicol with 10 μM LF**
V. cholerae O1	30	2.5	*V. cholerae O1*	20	2.5
V. cholerae non O1	30	2.5	*V. cholerae non O1*	20	2.5

An inoculum (OD 0.005 at 660 nm) was used in each experiment. Viability relative to the 100% growth in untreated bacteria cultures was determined by measuring the OD every 30 min during 1.5 h. Data are mean values of two experiments performed in triplicate. Standard deviations were less that 6.2% in each experiment.

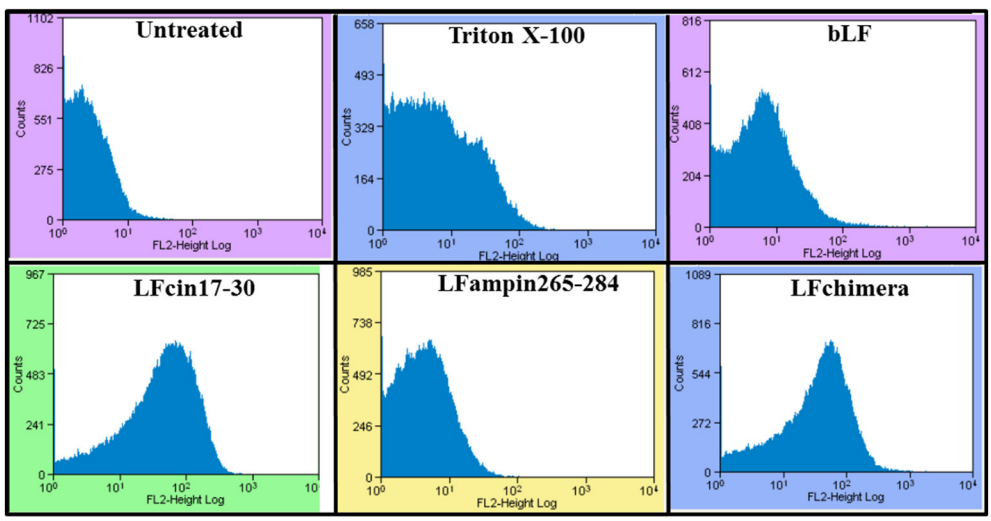

FIGURE 2 | Determination of membrane permeabilization in *Vibrio cholerae* O1 treated with bLF and LF peptides. *V. cholerae* O1 was incubated with 40 μM LF, or with 20 μM LFcin17-30, or LFampin265-284, respectively; or 5 μM LFchimera, at 37°C with constant agitation for 2 h. Then, samples were processed and stained with the fluorescent dye Propidium iodide. Untreated bacteria were used as control of membrane integrity and 0.5% Triton X-100 treated bacteria were used as control of permeabilized membranes. Experiments were performed at least twice in duplicate. Samples were processed to be analyzed by Flow Cytometry.

cells had taken up the dye fluorescence upon incubation with 5 μM LFchimera, 20 μM bLF LFcin17-30, and LFampin265-284; indicating that the bacterial membrane was permeabilized by the peptides (**Figure 2**). The treatment with Triton X-100 used as a positive control of bacterial permeabilization also stained bacterial cells, corroborating that this treatment damaged the bacteria (**Figure 2**).

Similar incubations of *V. cholerae* O1 analyzed by SEM after negative staining showed severe membrane damage such as vesicularization, the occurrence of protrusions and filamentation (**Figure 3**, arrows). The same damage was found in *V. cholerae* non-O1 cells treated with bLF and LFpeptides (**Figure 4**). These results demonstrate that LFchimera and peptides destabilize the bacterial membrane integrity and

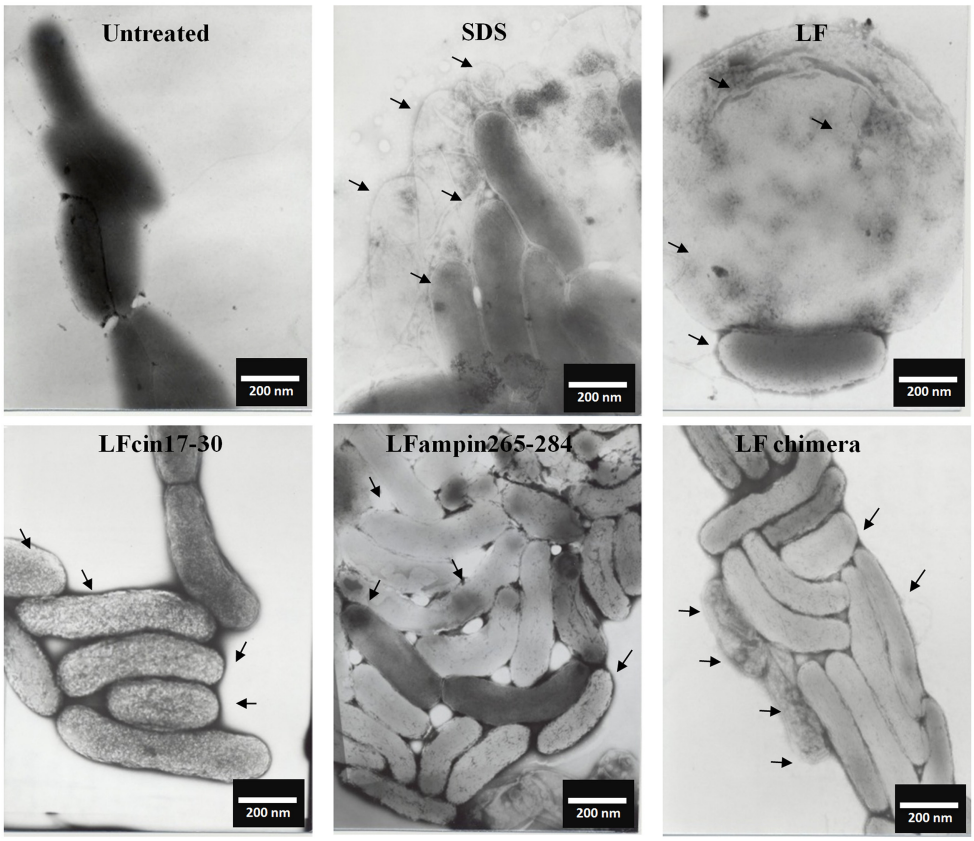

FIGURE 3 | LF and LFpeptides cause ultrastructural damage to *Vibrio cholerae* O1 cells. *V. cholerae* O1 cells (1.0×10^8 cells/ml) were incubated in LB alone (negative control for damage) or with 0.5% SDS (positive control for damage), or with 40 μM of bLF, 20 μM LFcin 17-30 or LFampin265-284 respectively, or 5 μM LFchimera for 1.5 h at 37°C. Cells were harvested, resuspended in PBS and fixed with 4% *para*-formaldehyde plus 0.5% glutaraldehyde. Next, bacterial samples were placed on 200-mesh Formvar-coated copper grids (3%), post-stained with phosphotungstic acid and examined with a JEOL electron microscope JEM1400 at 40 kv.

also that LFchimera has a higher activity than bLF and LF peptides.

bLF and bLFpeptides Interact with *Vibrio cholerae* Strains

The interaction of bLF and LFpeptides was investigated by confocal microscopy. In the results, we observed that the peptides LFcin17-30, LFampin265-284, and LFchimera interact with *V. cholerae* bacteria (**Figure 5A**). On the other hand, in fixed bacteria the fluorescent compounds were found interacting with the bacteria, indicating that the membrane of *V. cholerae* contains components which are recognized by the peptides (**Figure 5B**).

Bovine Lactoferrin and Lactoferrin Chimera Reduce Damage on Intestine and Cecum of Mice Infected with *Vibrio cholerae*

Mice infected with *V. cholerae* developed symptoms such as diarrhea, weakness, and abdominal tremor after 4 h of infection. Additionally; the infection was confirmed by counting *V. cholerae* obtained from rectal swabs (data nor shown), Once infection was confirmed, mice were treated with bLF, LFchimera, and tetracycline. Twenty-four post-infection three mice were sacrificed in order to see the effectiveness of the treatments. In the results, representative macroscopic images are shown for the gross morphological alterations of the small intestine and caecum from mice at 24 h of treatment (**Figure 6**). In a mouse of the uninfected group black arrows indicate the typical appearance of a normal small intestine, and blue arrowheads indicate normal caecum (**Figure 6A**). In a mouse from the infected and untreated group, white arrows point to injury in small intestine and blue arrow shows caecum is swelling (**Figure 6B**). A mouse from the group treated with tetracycline black arrows indicate injury in small intestine and blue arrows shows caecum is swelling and enlarged (**Figure 6C**). Interestingly, a mouse from the group treated with bLF Black arrows indicate normal small intestine as in **Figure 6A** and blue arrows indicates normal caecum (similar to macroscopic findings of uninfected mice). A mouse treated with LFchimera shows black arrows indicating a normal small intestine and blue arrow indicates a normal caecum. Similar results were observed in other mice sacrificed. These results indicated that bLF and LFchimera have the capacity

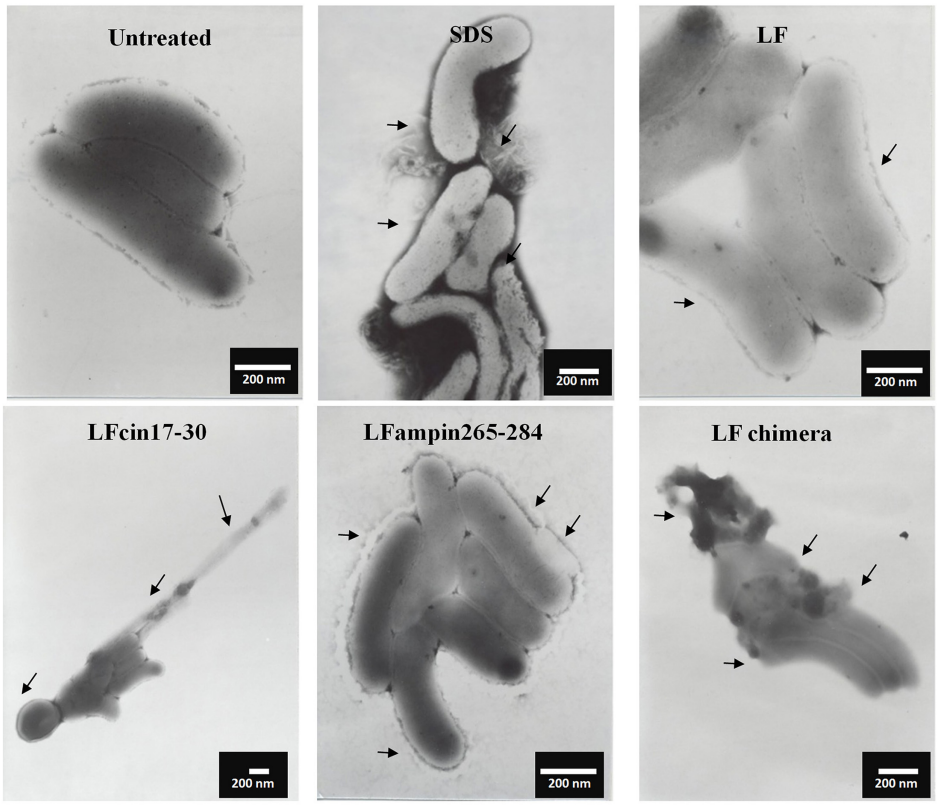

FIGURE 4 | LF and LFpeptides cause ultrastructural damage to *Vibrio cholerae* non-O1 cells. *V. cholerae* non-O1 cells (1.0×10^8 cells/ml) were incubated in LB alone (negative control for damage) or with 0.5% SDS (positive control for damage) or with 40 μM of bLF, or 20 μM LFcin 17-30 and LFampin265-284 respectively, or 5 μM LFchimera, for 1.5 h at 37°C. Cells were harvested, resuspended in PBS and fixed with 4% *para*-formaldehyde plus 0.5% glutaraldehyde. Next, bacterial samples were placed on 200-mesh Formvar-coated copper grids (3%), post-stained with phosphotungstic acid and examined with a JEOL electron microscope JEM1400 at 40 kv.

to diminishing macroscopic damage induced by *V. cholerae* in intestines and cecum in Mice.

Bovine Lactoferrin and Lactoferrin Chimera Reduce *Vibrio cholerae* Counts in Feces and Intestines

V. cholerae counts diminished in feces (**Figure 7A**) and intestines (**Figure 7B**) from mice infected and treated with bLF and LFchimera after the first dose administered (**Figure 7**), compared with infected and untreated animals (Positive control of infection). In the group treated with LFchimera, *V. cholerae* was undetected in feces and intestines after 12 h of treatment, in the group treated with bLF *V. cholerae* was undetected until 24 h of treatment and with tetracycline the minimal bacterial count was done during 12–16 h of treatment, and then the bacteria recovered its growth in feces and intestines. The results show that LFchimera and bLF kill *V. cholerae* in *in vivo* model.

DISCUSSION

Antimicrobial resistance is a global health concern because the infections can be more severe and difficult to treat (Bonomo, 2000). This is a consequence in part by overuse and misuse of antibiotics and represents a serious health concern throughout the world (Longworth, 2001). In this sense, in this problem is included the development of antibiotic resistance by *Vibrio* species (Rahmani et al., 2012; Sperling et al., 2015). The genus *Vibrio* includes at least 12 species pathogenic to humans. In these species are included *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. furnissii*, *V. fluvialis*, *V. damsela*, *V. hollisae*, *V. metschnikovii*, and *V. mimicus* (Altekruse et al., 2000). Pathogenic *Vibrio* can cause both intestinal and extra-intestinal illnesses. *Vibrio* infections potentially requiring antimicrobial therapy fall into three distinct syndromes; (1) cholera; caused by either *V. cholerae* O1 and other serogroups such as non-o1 or other species (*V. parahaemolyticus*), (2) Soft tissue infections; due to *V. vulnificus*, and (3) sepsis due to *V. vulnificus* and other *Vibrio* (Powell, 1999). Acquired multidrug resistant in *V. cholerae* O1 and other pathogenic *Vibrio* is now common and firmly established wherever infections occur (Dhar et al., 1996; Elmahdi et al., 2016).

In this work, we demonstrated that bLF and LFchimera have bactericidal activity against *V. cholerae* O1 Inaba, *V. cholerae* non-O1 (toxigenic), *V. vulnificus* (resistant to tetracycline and

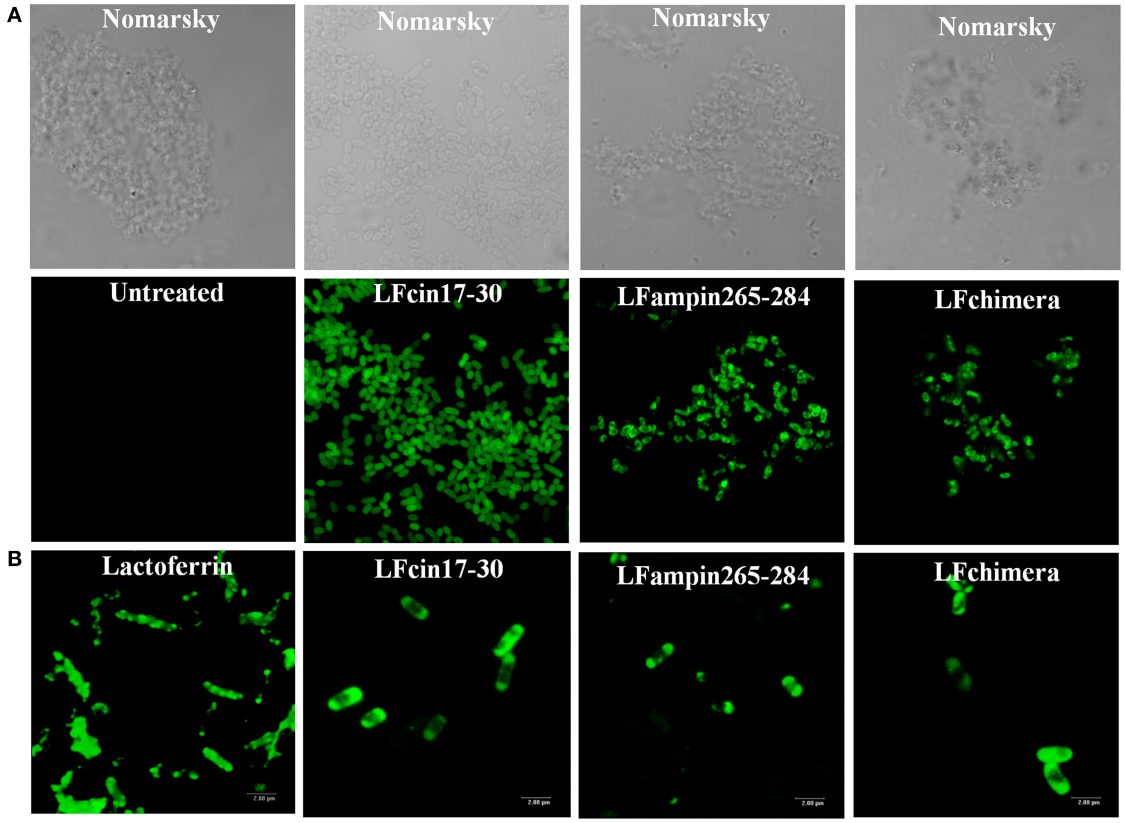

FIGURE 5 | Interaction of Lactoferrin derived peptides with *Vibrio cholerae* O1 and non-O1 strains. *Vibrio cholerae* O1 cells (10^7 CFU/ml) were incubated with 2 μM FITC-labeled peptides for 30 min. Bacteria were centrifuged (5 min, 10,000 × g), resuspended and incubated with 2 μM of FITC-bLF or FITC-labeled peptides for 30 min **(A)**, or fixed (4% paraformaldehyde, pH 7.4 during 30 min at 37°C) **(B)**, washed and then incubated with 2 μM bLF and LFpeptides as before was described. In both cases samples were washed twice with PBS mounted on slides and processed. All samples were analyzed under confocal microscopy by using a confocal laser-scanning microscope (Leica, Heidelberg, Germany). Bar 20 nm.

ampicillin), *V. fluvialis* (resistant to ampicillin and cefotaxime), *V. alginolyticus* (resistant to amplicillin and tetracycline), and *V. furnissii* (resistant to ampicillin). Previous works have reported the bactericidal activity of bLF in *V. cholerae* (Arnold et al., 1980; Ellison and Giehl, 1991); however, the mechanism of action or damage was not investigated in detail. In regards with the bactericidal activity of bLF and LFpeptides on *V. cholerae,* the peptide LFchimera and the protein bLF had the best bactericidal activity (**Figure 1**). LFchimera was more effective to inhibit the growth of *V. cholerae* strains and other *Vibrio* spp., compared with its peptides of origin (LFcin17-30 and LFampin265-284). *V. cholerae* strains were also susceptible to both peptides; however, this antibacterial activity remained curiously lower when was compared to the antibacterial action of Gentamicin (drug used as a negative control for growth), or when was compared with the action of bLF and LFchimera (**Figure 1**). Nonetheless, LFcin17-30 and LFampin265-284 may be antibacterial, due they were able to damage membranes and cause disruption on *V. cholerae* cells (**Figures 2–4**). The more effective antibacterial ability of LFchimera compared to the effect reached with native bLF, LFcin17-30, and LFampin265-284 peptides has been reported for other bacteria, as well as parasites or fungi (Bolscher et al., 2009; Kanthawong et al., 2014; Leon-Sicairos et al., 2014). These differences on the effect could be due to the LFchimera structure (Haney et al., 2012a,b). An obvious question is why human or bovine LF doesn't prevent the infection by *Vibrio* species? We speculate that human LF present in mucosae and fluids, or released by neutrophils, or bLF ingested from dairy milk products is not enough to combat *Vibrio* spp infections.

As we found that LFchimera and bLF had the best bactericidal activity, we investigate the effects of them combined with low concentrations of antibiotics on multidrug resistant *Vibrio* strains. In the results, apparently a combined effect was found when antibiotics were mixed with bLF and LFchimera (**Table 1**). It is interesting that LFchimera mixed with ampicillin inhibited the growth of *V. vulnificus*, *V. fluvialis*, *V. alginolyticus,* and *V. furnissii* and also the growth of some *Vibrio* spp resistant to ampicillin (**Table 1**). On the other hand, the combination of LFchimera tetracycline inhibited the growth of *V. vulnificus* (**Table 1**). A mixture of LFchimera and chloramphenicol inhibited the growth of *V. cholerae* O1 and non-O1 strains, whereas this growth inhibition level was only reached by

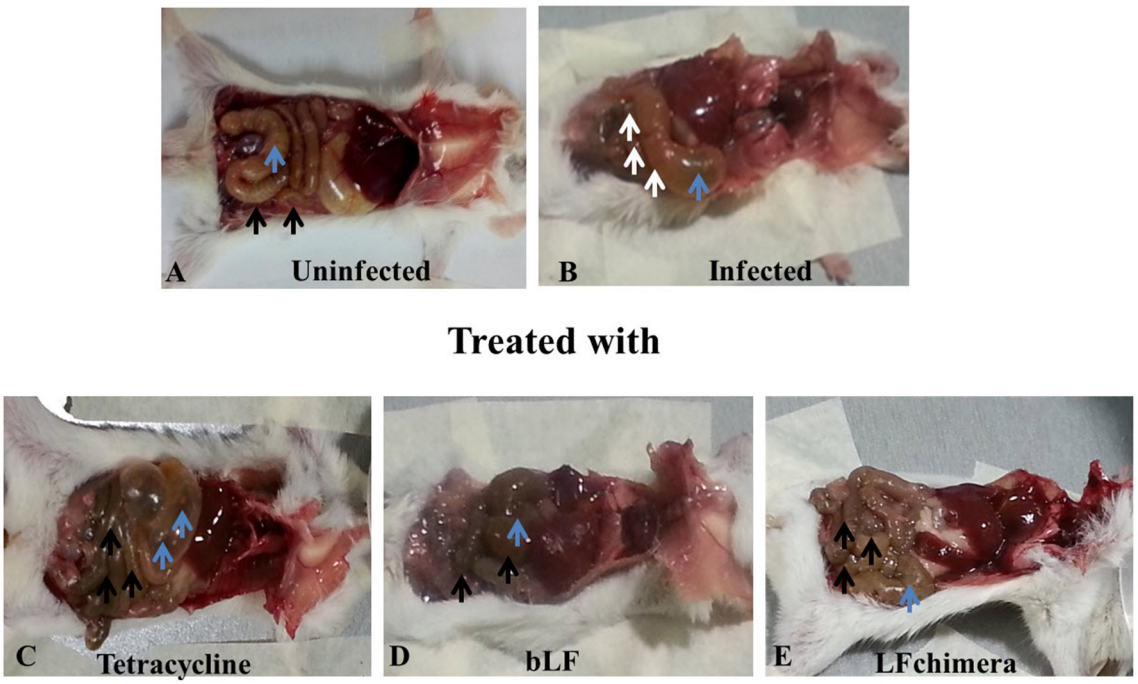

FIGURE 6 | Alterations in gut morphology of mice. Representative macroscopic images for the gross morphological alterations of the small intestine and caecum from mice at 24 h of treatment. **(A)** Small intestine Black arrows indicate normal small intestine in the uninfected group (blue arrowhead) indicate normal caecum. **(B)** White arrows point to injury in small intestine in the infected and untreated group (blue arrow) shows caecum is swelling. **(C)** Black arrows indicate injury in small intestine (blue arrows) caecum is swelling and enlarged in the tetracycline group. **(D)** Black arrows indicate normal small intestine as in A (blue arrowhead) indicates normal caecum. **(E)** Black arrows indicate normal small intestine in the LFchimera group as in D (blue arrow) indicate caecum.

using higher concentrations of LFchimera or chloramphenicol (**Table 1**). bLF also had synergism or combined effect with antibiotics in the strains above mentioned, whereas without antibiotics the concentration needed to inhibit more than 95% was higher. These data suggest that LFchimera and bLF combined with low concentrations of antibiotics have bactericidal effect in multidrug resistant strains of genus *Vibrio* (**Table 1**). According to the calculation of FICs, both bLF and LFchimera had synergistic effects when they were used with the antibiotics, suggesting that they could improve the management of *Vibrio* spp multidrug resistant strains *in vivo*. In regards with this data, we speculate that the magnified effect of LFchimera plus antibiotics in *Vibrio* resistant strains could be due to the damage exerted by bLF and LFchimera on outer membrane of *Vibrio* spp plus the effect of the antibiotics.

The marked effect of LFchimera could be explained by its composition. LFchimera is formed by the peptides LFcin17-30 and LFampin265-284 (linked by a lysine). In consequence, this new peptide presents the following characteristics; an artificial conformation that mimics the spatial arrangement of the LF native, and a net charge of 12+ at neutral pH (compared with 6+ from LFcin17-30 and 4+ from LFampin265-84; respectively) (Bolscher et al., 2009). In this sense, it has been reported that the negatively charged membrane molecules present in pathogens are the main target of cationic antimicrobial peptides, so we speculate that Fchimera can act with the negatively charged microbial membrane components and destabilizes it, causing antimicrobial effect. Additionally, it has been reported that the bactericidal effect of LFchimera was not hampered by salt concentrations present in the media of bacterial growth.

Until the best of our knowledge this is the first report of the bactericidal activity of bLF and synthetic LFpeptides (LFin17-30, LFampin265-285, and LFchimera) on *Vibrio* species resistant to antibiotics.

In this study, we sought to get further insight into this mechanism by focusing our studies on *V. cholerae* O1 and non-O1 strains due to *V. cholera* is the most pathogenic specie of genus *Vibrio*, for this reason we made experiments to assess the mechanism of action. We found that bLF and LFpeptides caused membrane perturbation in both *V. cholera* strains (**Figure 2**) as well as damage on structural level (**Figures 3**, **4**, respectively). Certainly, the measurement of FITC-labeling peptides by flow cytometry and microscopy indicated interaction of them with the bacteria (**Figure 5**), this interaction could then permits the damage of *V. cholerae* membranes. The interaction was visible by confocal microscopy (**Figure 5**) and was quantified by flow cytometry (data not shown). In additional experiments, we pre-incubated bacteria with high amounts of unlabeled bLF and then

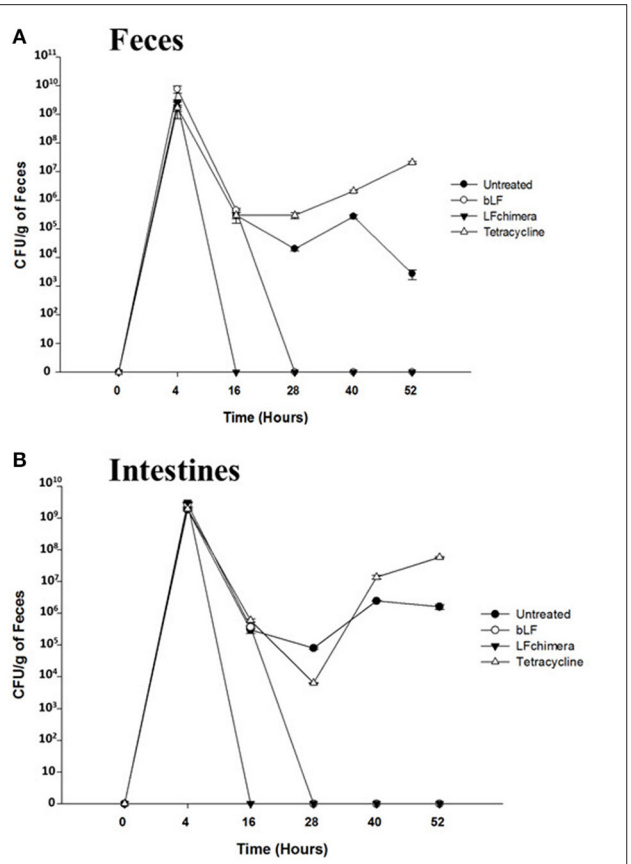

FIGURE 7 | LF chimera and bLF diminish *Vibrio cholerae* O1 counts in **(A)** feces and **(B)** intestine. Recovery feces and intestines from mice infected and treated were homogenized and serial dilutions were prepared and plated onto LB agar plates with 100 mg/ml streptomycin for 24 h at 37°C. For confirmation, developed colonies were counted and then plated onto CHROMagar™ *Vibrio* (CHROMagar; Paris, France). On the other hand, the intestines were dissected, homogenized in PBS and serial dilutions were prepared in PBS and plated in LB agar plates with 100 mg/ml streptomycin. Finally, colonies were counted and plated onto CHROMagar™ *Vibrio*.

the FICT-peptides were added. In all cases bLF did not avoid the binding of peptides to the outer membrane of *V. cholera*, however we found a significant decrease in the fluorescent exhibited by bacteria, indicating that bLF and LFpeptides uses the same sites of recognition present in *V. cholerae*, and maybe specific sites (data not shown). Together this data shown that *V. cholera* contains sites on its membrane that bind bLF and LF peptides.

It has been reported that bLF interacts in a direct manner with negative charged components present in microbial membranes; inducing alterations in its permeability through dispersion of them. For example; bLF interacts with lipopolysaccharides (LPS) from Gram-negative bacteria, or with Lipoteichoic acid (LTA) from Gram-positive bacteria. After this interaction it has been postulated that there is an alteration on membranes, leading the death of the pathogens (Ellison et al., 1988; Orsi, 2004; Leon-Sicairos et al., 2014). We speculate that lipid A from LPS is also one of the targets form LFchimera and LFpeptides. In our precious work LFchimera produced damage on *V. parahaemolyticus*, the appearance of the bacteria shown typical perturbations of a bacteria undergoing programmed cell death type II (Leon-Sicairos et al., 2009), as these kind of damage was not found in all *Vibrio* tested we think LFchimera exerts different type of damage.

Treatment for *V. cholerae* infection involves antibiotics and oral hydration for cholera since 1964. Hydration includes the drinking of fluid with electrolytes, such as sodium, potassium, calcium ions to restore the high amount of electrolytes lost due watery diarrhea (Seas et al., 1996). Regarding drugs, tetracycline has been and effective treatment for cholera with better effect compared with others antibiotics such as furazolidone, chloramphenicol, and sulfaguanidine in reducing cholera morbidity (Lewis and Sanyal, 1965; Gharagozloo et al., 1970; Finkelstein, 1996; Escobar et al., 2015). However, it has been demonstrated the resistance to the antibiotic tetracycline and others (used for *V. cholerae*) in both; endemic and epidemic cholera settings.

Concerning our model *in vivo*, in was clear that LFchimera and bLF were effective to resolve *V. cholerae* infection in mice. LFchimera and bLF had bactericidal activity against the bacteria, and this was confirmed by the resolution of macroscopic damage (**Figures 6D,E**) and by the diminution of *V. cholerae* counts in feces and intestines of mice infected and treated, compared whit those infected and untreated. It seems to be that the bactericidal effect of LFchimera and bLF was better in comparison with tetracycline. So, In our model LFchimera and bLF were effective against *V. cholerae* infection.

Antibiotic resistance can be acquired by the acquisition of selected mutations, plasmids, introns, or conjugative elements, which could confer rapid spread of resistance (Towner et al., 1980; Hassan and Teh, 1993; Weber et al., 1994; Bhattacharya et al., 2011). Furthermore, it has been demonstrated that in cholera; the mass supply of antibiotics for prophylaxis in asymptomatic persons and household contacts of cholera patients during previous epidemics, represented a risk factor for the acquisition of resistance of *V. cholera* to antibiotics employed (Kitaoka et al., 2011; Marin et al., 2014). Treatment of infections due to *Vibrio* non cholerae also has been difficult in recent times, due to the spread of multidrug resistant *Vibrio* spp strains.

These facts indicated that is necessary searching for new products and interventions that can combat *V. cholerae* and other *Vibrio* spp., because of the increasing resistance against antibiotics. LFchimera and bLF at low concentrations were antibacterial against *Vibrio* spp.; we speculate that both compounds present potential to prevent or combat infections caused by *Vibrio* spp. On the other hand, antibiotics combined with LFchimera could act together, this also represent potential of new option against infections caused by the multidrug resistant *Vibrio* species. In addition, LFchimera could be used as an antibacterial in seafood or in humans, but first its efficacy as food preservative and in *in vivo* must to be determined.

CONCLUSIONS

We performed a study in order to see if bLF and LFpeptides (LFcin17-30, LFampin 265-284, and LFchimera) are effective

as bactericides in *V. cholera* O1 and non-O1 strains and other *Vibrio* spp resistant to antibiotics. Data reported here demonstrated that LFchimera and the native bLF are bactericide peptides that damage *Vibrio* spp after a direct interaction. On the other hand, LFchimera and bLF combined with antibiotics could have a combinatory effect against *Vibrio* spp., for this reason they have potential as bactericidal agents against infections caused by *Vibrio* spp.

ETHICS STATEMENT

Mice were purchased and housed under specific-pathogen-free conditions, treated and finally killed as stipulated and approved by the Ethical Committee for Laboratory Animals in School of Medicine, University of Sinaloa.

AUTHOR CONTRIBUTIONS

EA-S, KV-J, AC-R, MR-L, JB, KN, HF-V, GA-C, MdlG, JM-G, and JV-R: Substantial contributions to the conception of the work; acquisition of data and analysis; Drafting the work; Final approval of the version to be published. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. NL-S: designed the work; analyzed and interpreted the data for the work; Revised the work for important intellectual; Final approval of the version. She is in Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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