Distribution of Putative Virulence Genes and Antimicrobial Drug Resistance in *Vibrio harveyi*

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Abstract The marine-estuarine bacterium *Vibrio harveyi* is an important pathogen of invertebrates, most significantly, the larvae of commercially important shrimp *Penaeus monodon*. In this study, we analyzed *V. harveyi* isolated from shrimp hatchery environments for understanding the distribution of putative virulence genes and antimicrobial drug resistance. The putative genes targeted for PCR detection included four reversible toxin (Rtx)/hemolysin genes, a gene encoding homologue of *Vibrio cholerae* zonula occludens toxin (Zot) and a hemolysin-coregulated protein gene (*hcp*) by polymerase chain reaction (PCR). Of the four putative reversible toxin genes, *vhh-1* was detected in 31% of the isolates, *vhh-2* in 46%, *vhh-3* in 23% and *vhh-4* was detected in 27% of the isolates. A *zot*-like sequence of bacteriophage f237 was present in 15%, while *hcp* sequence was detected in 48% of the isolates. The antimicrobial susceptibility test revealed resistance to several groups of antibiotics including β -lactams, cephalosporins, macrolides, quinolones, nitrofurantoin and tetracycline.

Keywords *Vibrio harveyi* · Genotyping · RTX · *zot* · *hcp* · Antibiotic resistance

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Introduction

The gram-negative luminous bacterium *Vibrio harveyi* is widely distributed in the marine estuarine environments and is a common pathogen of cultured fish and shell fish world wide [1, 2]. Though *V. harveyi* are considered pathogens of invertebrates, they are also known to colonize marine animals as normal flora. In India, *V. harveyi* is the dominant luminescent bacterium in *P. monodon* hatchery and grow out systems, where they can infect post larvae causing large scale mortalities [3, 4]. Infection with biofilm forming *V. harveyi* resistant to multiple antibiotics has been previously reported from India [5]. The pathogenic mechanism of *V. harveyi* is not fully understood, though several factors are known to contribute to the virulence which include proteases, cytotoxins, hemolysins, phospholipases, siderophores, proteases, cytotoxins, lipopolysaccharide and presence of a bacteriophage [6].

The whole genome sequencing of *V. harveyi* BAA-1116 accomplished recently has revealed the presence of a second chromosome, a commonly observed feature of *Vibrio* genomes [7]. The second chromosome of *V. harveyi* harbors gene sequences with potential roles in the virulence of this organism. Prominent among these are genes encoding putative reversible toxins or hemolysins, bacteriophage sequences and antimicrobial resistance genes. Thus, it would be interesting to determine the frequency of occurrence of these putative virulence genes among hatchery isolates of *V. harveyi*. Thus, the objectives of this study were to investigate the distribution of putative virulence genes in strains of *V. harveyi* and to determine their antimicrobial drug resistance patterns. We expect that identification of specific genotypes among strains of *V. harveyi* would facilitate better association of these with their pathogenicity to invertebrates and future investigations on this hypothesis.

Materials and Methods

Isolation and Identification of *V. harveyi*

Fifty-six strains of *V. harveyi* used in this study were isolated from four different *P. monodon* hatchery facilities along the south east coast of India. All the strains were identified as *V. harveyi* by performing a series of physiological tests previously outlined [8]. The strains were archived at -70°C in nutrient broth containing 30% glycerol.

Detection of *V. harveyi* by 16S rDNA PCR

All biochemically identified luminescent *V. harveyi* were further confirmed by a 16S DNA PCR using the primers 16S-F and 16S-R and the protocol previously described [9]. The PCR products obtained with 10 randomly selected isolates were sequenced and compared with the 16S rDNA sequence of *V. harveyi* in the GenBank.

Genotyping of *V. harveyi* Isolates by PCR

Genomic DNA was extracted from *V. harveyi* strains using the CTAB (cetyl trimethyl ammonium bromide) method [10]. The primers used for PCR (Table 1) were designed using online tool Primer3 (<http://frodo.wi.mit.edu/>) for conserved sequences in the target genes available from the whole genome sequence of *V. harveyi* BAA-1116 (GenBank accession number CP000790). Pure genomic DNA (100 ng) was used in all PCR reactions. The reactions were carried out in 30 μl volumes consisting of a 10 $\times$ buffer (100 mM Tris–HCl, 500 mM KCl, 15 mM MgCl_2), 200 μM concentrations of each of the four dNTPs, 30 pmol of forward and reverse primers and 1.5 U of *Taq* polymerase (MBI Fermentas). The thermocycling conditions

consisted of an initial denaturation of 5 min at 94°C followed by 30 cycles of 1 min denaturation at 94°C , 1 min annealing at 55°C and 1 min extension at 72°C . A final elongation at 72°C for 5 min was followed in all reactions. The assays were repeated at a lower annealing temperature of 50°C for isolates negative for any specific gene. The products of PCR were separated on a 2% agarose gel, stained with ethidium bromide (0.5 $\mu\text{g}/\text{ml}$) and photographed using Kodak Gel Logic 1500 (Carestream Molecular Imaging, New Haven, USA).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of *V. harveyi* to various antibiotics was determined following the Clinical and Laboratory Standards Institute (CLSI) guidelines [11]. The antibiotic used with their corresponding strengths in parenthesis were amoxicillin (25 μg), azithromycin (15 μg), bacitracin (10 U), cephalexin (30 μg), cephalothin (30 μg), ciprofloxacin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (30 μg), kanamycin (5 μg), methicillin (10 μg), Penicillin G (2 U), nalidixic acid (30 μg), norfloxacin (10 μg), novobiocin (30 μg), nitrofurantoin (100 μg), neomycin (30 μg), streptomycin (25 μg), tetracycline (10 μg), tobramycin (10 μg) and trimethoprim (10 μg).

Resistance to ethidium bromide was determined in Mueller–Hinton (MH) broth following the CLSI micro-broth dilution procedure [11]. The assay was performed in a 96-well microtitre plate with appropriate uninoculated media blanks. The test strains were grown to an O.D_{625} of 0.4, washed and resuspended in fresh MH broth to yield 10^3 – 10^4 cells/ml. A serial 10-fold dilution of the stock ethidium bromide (10 mg/ml) was performed in MH broth in microtitre plate wells and inoculated with the test strains. The plates were incubated at 37°C for 24 h, and the growth

Table 1 Oligonucleotide primers used in this study for genotyping *V. harveyi*

Primer	Target gene/putative function	Sequence (5'–3')	Coordinates ^a	Product size
Vh1-F	<i>vh-1</i> /reversible toxin	cggtgctggagacgatatt	394102–394686	340
Vh1-R		cgccatcagtaaagacagca		
Vh2-F	<i>vh-2</i> /reversible toxin	tccgtgacgagttgagctctg	1598099–1600615	440
Vh2-R		catcaatcacatccgcaaag		
Vh3-F	<i>vh-3</i> /reversible toxin	aaacgttcacgctctgctt	1604427–1607765	437
Vh3-R		tagcgcagcataaccatctg		
Vh4-F	<i>vh-4</i> /reversible toxin	aaaagtcacctcggttggtg	1612360–1614504	443
Vh4-R		gcgtaccatcgatcaggact		
Hcp-F	<i>hcp</i> /type VI secretion	tcagcctactggtcaacgtg	1061396–1061896	250
Hcp-R		ttcgattgcacggtgaagtca		
Zot-F	<i>zot</i> /phage coat protein	aattctgtcgtgggttctgg	961672–962940	279
Zot-R		agacagctggacaaggcagt		

^a Correspond to *V. harveyi* BAA-1116 genome sequence with the GenBank accession number CP000790

was examined visually. The assay was repeated thrice, and the lowest concentration of ethidium bromide that inhibited growth was recorded as the MIC for the test strains.

Bioinformatic Analysis

Signal peptide sequences were determined using SignalP 3.0 server <http://www.cbs.dtu.dk/services/SignalP/>. The sequences were analyzed for conserved motifs at <http://motif.genome.jp/>. Nucleotide and deduced amino acid sequences were analyzed for homologous sequences by nucleotide–nucleotide and protein–protein BLAST search engines of the National Centre of Biotechnology Information (NCBI) [12].

Results and Discussion

Though *V. harveyi* is a well known pathogen of invertebrates, the virulence determinants of this genetically diverse bacterium is not precisely understood. The whole genome sequence of *V. harveyi* BAA-1116 accomplished recently has revealed several genes with homologous sequences in other known human pathogens. Several of these sequences are putative hemolysins and multidrug resistance genes, distributed on the second chromosome of *V. harveyi*. The first step towards understanding the role of individual putative virulence genes will be to determine the occurrence and distribution of candidate sequences among environmental isolates. Thus, the purpose of our study was to determine the occurrence of genes encoding proteins identified as putative virulence factors in *V. harveyi* strains isolated from *P. monodon* hatchery facilities. The prominent among these are four reversible toxin (Rtx) genes of type I secretion system.

The type I secretion system (TISS) functions to secrete a variety of virulence proteins to extracellular space in gram negative bacteria, thus playing an important role in the virulence of many pathogens. TISS is composed of three proteins (i) an inner membrane ABC type transporter that binds the substrate (ii) a membrane fusion protein (MFP) and (iii) an outer membrane protein (OMP) [13]. Though TISS transports variety of proteins, all proteins secreted via TISS are characterized by the presence of repeat in toxin motifs (RTX). RTX motifs are rich in amino acids glycine and aspartate with a characteristic signature sequence GGXG(N/D)DX(L/I/F)X (where X represents any amino acid) involved in binding of Ca^{2+} ions which is essential for functioning of these proteins [14]. All the four putative Rtx/hemolysin genes of *V. harveyi* targeted in this study have sequences characteristic of hemolysins with putative Ca^{2+} -binding domains. Thus, these hemolysins are predictably secreted by type I secretion apparatus. Detailed

study of individual hemolysins will reveal their mode of secretion and precise function.

The results of PCR detection of individual genes is shown in Table 2. The gene amplified by primers Vh1-F and Vh1-R was detected in 16 of 52 strains (31%). This 585 bp nucleotide sequences encodes a putative hemolysin-like protein of 194 amino acids. A single glycine rich domain between amino acid positions 61 and 79 was identified in the deduced amino acid sequence that may be involved in calcium-binding. The homologues of this protein were found in diverse members of *Vibrio* species including *V. campbelli*, *V. alginolyticus*, *V. parahaemolyticus* and *V. vulnificus*.

Primers Vh2-F and Vh2-R were used to detect a putative reversible toxin corresponding to coordinates 1598099–1600615 in *V. harveyi* second chromosome. This 2517 nucleotide sequence encodes a protein of 838 amino acids. The gene was detectable in 24 of 52 strains (46%) by PCR. Analysis of deduced amino acid sequence revealed the presence of six putative glycine rich domains with Gly-Gly-Xaa-Gly-Asn-Asp signature sequence. A search in public databases for homologous sequences revealed the presence of similar sequence in *V. shilloni*. Homologues of this toxin were not found in completed genome sequences of other *Vibrio* species namely *V. cholerae*, *V. fischeri*, *V. parahaemolyticus* and *V. vulnificus*. This finding makes this putative repeat toxin an interesting candidate protein for future research to determine its role in the virulence of *V. harveyi*.

A putative secreted protein corresponding to coordinates 1604427–1607765 was detected in 12 strains (23%). A putative domain with the characteristic HEXXHXXGXXH signature sequence was identified between amino acids 412 and 464, characteristic of Zn^{2+} -binding metalloprotease family of proteins [15]. Secondary structure analysis of the amino acid sequence revealed three transmembrane helices. This gene was found specific to *V. harveyi*, since sequences homologous to this 1112 amino acid protein were not present in whole genome sequences of any of the *Vibrio* species available in the public data bases.

Table 2 Distribution of putative virulence genes in *V. harveyi* isolates ($n = 52$)

Target gene	No. (%) of strains positive
<i>vh-1</i>	16 (31)
<i>vh-2</i>	24 (46)
<i>vh-3</i>	12 (23)
<i>vh-4</i>	14 (27)
<i>hcp</i>	25 (48)
<i>zot</i>	8 (15)

Vh-4 is a putative ABC transporter with characteristic conserved motif LSGGQ specific to the ABC transporter family of proteins. These Transmembrane proteins hydrolyze nucleotide triphosphates and utilize the energy to transport a variety of molecules across the membrane. Homologous sequences to this protein were found in *V. cholerae* and *V. vulnificus*, but not in *V. parahemolyticus* and *V. fischeri*. The gene was detectable in 14 (27%) *V. harveyi* isolates by PCR. The first 25 amino acids of this 714 amino acid long polypeptide constitute a signal sequence. Analysis of secondary structure of this revealed the presence of five transmembrane domains that consisted of an ATP-binding domain, a Walker A/P-loop with the consensus sequence GxxGxGKST, ABC transporter motif, Walker B loop and a D loop, characteristic of ABC transporter proteins [16].

A putative hemolysin-coregulated protein gene *hcp* corresponding to coordinates 68189–69142 was detectable in 25 (48%) isolates of *V. harveyi*. This gene encodes a putative 173 amino acid polypeptide with a calculated molecular weight of 19 kDa. In *V. cholerae*, *hcp* has been reported to be co-regulated with *hlyA* by the *hlyU* regulatory system [17]. Hcp is secreted by the type VI secretion, a newly described protein transport system in gram negative bacteria [18]. Analysis of whole genome sequences of *V. harveyi* available in GenBank revealed the presence of homologues of both *V. cholerae* HlyA and HlyU, strongly suggesting that *V. harveyi* has a type VI secretion system. Thus, Hcp and its transport system will make good target proteins for future studies with respect to their machinery and their role in the physiology and the pathogenicity of *V. harveyi*.

The pathogenicity of *V. cholerae* is primarily attributed to genes found on a 7–9.5 kb genetic element. The core 4.5 kb CTX gene cassette harbors gene *ctxAB*, *ace*, *zot* and *tcp* encoding cholera toxin, accessory cholera enterotoxin, zonula occludens toxin and a toxin co-regulated pilus respectively. *ctx* gene cassette is located on a CTX ϕ lysogenic filamentous bacteriophage [19]. It is well established that virulence genes located on bacteriophages, plasmids and transposons are transferred across bacterial species by horizontal and vertical gene transfer mechanisms. The origins of these genes, however, are difficult to establish. *zot* in *V. cholerae* is restricted to only O1 and O139 strains and has not been reported in non-O1 and non-O139 serotypes. Zot protein is known to bind to tight junction of cultured intestinal epithelial cells and modulate the permeability of cells [20]. In majority of strains, *zot* co-exists with *ctx* and *ace* and the absence of one or more genes in *ctx* gene cassette has been attributed to spontaneous deletion of these genes [21]. In our study, *zot* was detected in 8 (15%) *V. harveyi* strains. The presence of *zot* homologue in *V. harveyi* raises new questions on the role of

zot in the pathogenesis of *V. harveyi* and the source of this gene.

Antimicrobial Resistance of *V. harveyi* Strains

The results of antibiotic sensitivity test are shown in Table 3. All (100%) of the strains were resistant to bacitracin, streptomycin, methicillin, kanamycin, penicillin, amoxicillin, erythromycin, cephalothin, cephalixin and azithromycin. A majority of strains (80%) were resistant to neomycin. Since kanamycin, streptomycin and neomycin belong to aminoglycoside group of antibiotics, the resistance to multiple antibiotics of the same class is expected. Similarly, resistance to macrolides erythromycin and azithromycin, β lactam antibiotics penicillin, amoxicillin and methicillin could be attributed to cross resistance. It is well known that bacteria that develop resistance to one antibiotic may become resistant to several other antibiotics of the same group. Interestingly, all strains were resistant to first generation cephalosporins cephalothin and cephalixin. A significant number of strains were also resistant to tetracycline, while two strains showed resistance to chloramphenicol and tobramycin respectively. No resistance was observed against quinolone drugs ciprofloxacin and norfloxacin, trimethoprim, gentamicin, novobiocin and nalidixic acid.

Bacteria become resistant to antibiotics through various mechanisms that include degradation of antibiotics, modification of antibiotic targets and active efflux of antibiotics [22]. The present study shows that *V. harveyi* strains are resistant to various groups of antibiotics. Two recent studies have shown that *V. harveyi* resistant to ampicillin

Table 3 Antimicrobial resistance profiles of *V. harveyi* ($n = 52$)

Antibiotic	No. (%) of strains resistant
Amoxicillin	52 (100)
Azithromycin	52 (100)
Bacitracin	52 (100)
Cephalixin	52 (100)
Cephalothin	52 (100)
Chloramphenicol	2 (3.8)
Erythromycin	52 (100)
Gentamicin	4 (7.7)
Kanamycin	52 (100)
Methicillin	52 (100)
Nitrofurantoin	12 (23)
Penicillin G	52 (100)
Streptomycin	48 (92)
Tetracycline	18 (34.6)
Tobramycin	2 (3.8)

and tetracycline harbor novel β -lactamase and tetracycline resistant genes respectively [23, 24]. The prophylactic use of antibiotics such as chloramphenicol and tetracycline in shrimp hatcheries can lead to development and transfer of resistance to antimicrobials [3, 5]. Further, being a marine-estuarine bacterium, *V. harveyi* may possess novel Na⁺-dependent efflux pumps. A preliminary analysis of the whole genome of *V. harveyi* reveals the presence of several putative multidrug resistance genes (data not shown). We further determined the resistance of isolates to ethidium bromide, a well established substrate of drug efflux pumps. Two multi-drug resistant strains had a minimal inhibitory concentration of 5000 μ g/ml ethidium bromide. Interestingly, these isolates were also resistant to chloramphenicol. Ten isolates had a MIC of 2500 μ g/ml ethidium bromide, while other isolates exhibited MICs between 156 and 625 μ g/ml (data not shown). These preliminary results suggest the presence of potent antimicrobial efflux membrane proteins in this bacterium. Since resistance phenotype to each group of antibiotics requires different physiology, it would be interesting to study the mechanism of antibiotic resistance in *V. harveyi*.

In conclusion, the present study shows that diverse genotypes of *V. harveyi* are present in the shrimp hatchery systems that are also resistant to multiple antibiotics. The type I secretion systems and the secreted hemolysins may hold vital clues towards unraveling the mechanism of *V. harveyi* pathogenicity. The presence of Zot-like sequence in *V. harveyi* needs further investigations to determine the actual role of this gene product in the physiology of *V. harveyi*. Further, the multiple drug resistance of *V. harveyi* observed in this study suggests that this organism could be used to study the physiology of drug resistance in Gram negative bacteria. Our future studies will involve determining the functions of secreted proteins and their transporters, as well as the genetic basis of multidrug resistance in *V. harveyi*.

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