

Prevalence and heterogeneity of hemolysin gene *vhh* among hatchery isolates of *Vibrio harveyi* in India

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Running title: Heterogeneity in *vhh* of *V. harveyi*

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Abstract *Vibrio harveyi* pathogenic to fish harbor a hemolysin gene *vhh*, the homologues of which are found in many species of the Genus *Vibrio*. In this study, we investigated the prevalence of *vhh* gene among *V. harveyi* isolated from *Penaeus monodon* hatcheries in India by polymerase chain reaction (PCR). *vhh* was detected in 67 of the 70 *V. harveyi* isolates tested in this study using different combinations of PCR primers. A variant *vhh* gene detected in a minority of strains was cloned, sequenced and the recombinant protein was expressed in *Escherichia coli*. The deduced amino acid sequence of the cloned gene was 86% similar to the previously reported amino acid sequences of VHH. The results of this study suggest that though *V. harveyi* strains invariably harbor *vhh*, the sequence variants of the hemolysin gene exist that may impede their detection by PCR.

Key words *Vibrio harveyi* . *vhh* . lecithinase . PCR . cloning and expression

Introduction

The Gram-negative luminous bacterium *Vibrio harveyi* is an important pathogen of cultured fish and shellfish world wide (6, 18, 28). In India, *V. harveyi* is the dominant luminescent bacterium in *Penaeus monodon* hatchery and grow out systems where they can infect post larvae causing large scale mortalities (1, 2, 16). Several factors are known to contribute to the virulence of *V. harveyi* which include proteases, hemolysins, phospholipases, siderophores, cytotoxins, lipopolysaccharide and presence of the temperate bacteriophage VHML (*V. harveyi* myovirus-like) (5, 14, 19, 20, 22). Quorum sensing and bioluminescence have also been associated with the virulence of *V. harveyi* (12, 15, 21). Scientific evidence suggests that a duplicated hemolysin gene (*vhhA/vhhB*) is responsible for the virulence of a strain of *V. harveyi* to salmonids (28). The protein product of *vhh* gene exhibited strong phospholipase and hemolytic activities and was cytotoxic to flounder gill cells (29). A highly homologous hemolysin gene *vch* has been recently cloned from *V. campbelli* (24). VHH has been proposed to be a member of GDSL (Gly-Asp-Ser-Leu) family of proteins owing to the presence of the conserved Gly-Asp-Ser-Leu motif (26).

Recent studies have described the use of *vhh* gene targeted PCR for the identification of pathogenic *V. harveyi* (9, 10, 13). Though *vhh*-targeted PCR is commonly used for detection of *V. harveyi* pathogenic to *P. monodon* larvae in India, there are no published reports on the occurrence of *vhh* gene among hatchery isolates of *V. harveyi*. In the study described here, we investigated the distribution of *vhh* gene in *V. harveyi* strains isolated from the *P. monodon* hatchery facilities in India by PCR. Our results indicate that different combinations of oligonucleotide primers were necessary to detect *vhh* gene in all the isolates, owing to nucleotide sequence heterogeneity of the gene within *V. harveyi*. In a preliminary attempt towards studying the diverse members of the VHH family of proteins in *V. harveyi*, a variant *vhh* gene was cloned, sequenced and the recombinant VHH protein was expressed in *Escherichia coli*.

Materials and methods

Bacterial strains, plasmids and media

Seventy isolates of luminescent *V. harveyi* used in this study were collected from *Penaeus monodon* hatchery facilities along the south east coast of India. Of these, 18 were isolated from

seawater samples, 30 were isolated from *P. monodon* larvae collected from hatcheries affected by luminescent vibriosis and 22 were isolated from healthy *P. monodon* larvae. The isolates were archived at -70 °C in nutrient broth containing 30% glycerol. All the isolates were identified as *V. harveyi* by performing a series of physiological tests as previously outlined (3). Genomic DNA was extracted from *V. harveyi* using the CTAB (cetyl trimethyl ammonium bromide) method (7). Expression plasmids pQE-30 and the *E. coli* host strain SG13009 [pREP4] were purchased from Qiagen, CA, USA. Plasmids from recombinant clones were prepared using a plasmid kit (Qiagen, CA, USA). Electrotransformations were followed for mobilization of recombinant plasmids into host *E. coli* strains.

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

All biochemically identified luminescent *V. harveyi* were further confirmed by a 16S rDNA PCR using the primers 16S-F and 16S-R and the protocol previously described (13).

PCR screening of *V. harveyi* isolates for hemolysin gene *vhh*

The primers used for PCR detection of *vhh* gene are listed in the Table 1. Pure genomic DNA (100 ng) was used in all PCR reactions. The reactions were carried out in 30 µl volumes consisting of a 10× buffer (100 mM Tris-HCl, 500 mM KCl, 20 mM MgCl₂), 200 µM concentrations of each of the four dNTPs, 30 picomoles of forward and reverse primers and 1.5 U of Taq polymerase (MBI Fermentas, MD, USA). The products of PCR were separated on a 2% agarose gel, stained with ethidium bromide (0.5 µg/ml) and photographed using Kodak Gel Logic 1500 (Carestream Molecular Imaging, New Haven, USA).

Southern blotting and hybridization

The 308 bp PCR product obtained using primers F1 and R2 was labeled with digoxigenin (DIG) using random primed labeling kit (Roche, Mannheim, Germany). The genomic DNA of 3 *V. harveyi* strains (VH34, VH39 and VH 56) were digested with HindIII restriction enzyme (MBI Fermentas, MD, USA), separated on a 0.7% agarose gel, transferred on Hybond-N⁺ nylon membrane (GE Healthcare, NJ, USA) and hybridized with DIG-labeled probe. The hybridized DNA was detected using alkaline phosphatase-labeled anti-DIG antibody and the chromogenic

substrates nitroblue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT) (Roche, Mannheim, Germany).

Amplification, cloning and sequencing of full length *vhh* gene

Primers LCF and LCR (Table 1) were designed to amplify the entire hemolysin gene corresponding to nucleotide sequence numbers from 138130 to 139386 of *V. harveyi* ATCC BAA-1116 chromosome II complete sequence (GenBank accession number CP000790). The PCR product obtained was cloned into pQE-30 UA cloning vector (Qiagen, CA, USA) to yield the recombinant plasmid pVhh. Sequencing of the cloned gene was done on both strands by primer walking using M13 primers and the gene specific primers (Bangalore Genei, Bangalore, India). The nucleotide sequence derived was subjected to GenBank homology search using standard nucleotide-nucleotide BLAST (4).

Expression and purification of VHH

The plasmid pVhh was mobilized into *E. coli* SG13009[pREP4] and the cells were grown in LB broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin to an OD₆₀₀ of 0.5, induced with 1 mM IPTG (Isopropyl-β-D-thiogalactoside) and further incubated for 4 h with shaking. The recombinant protein with 6×His tag was purified by nickel affinity chromatography using Ni-NTA spin kit following manufacturer's protocol (Qiagen, CA, USA).

Determination of hemolytic and phospholipase activities of *E. coli*/pVhh and the recombinant VHH

Egg yolk agar was prepared by adding 1% (v/v) of egg yolk (HiMedia, Mumbai, India) to LB agar. Sheep blood agar was prepared by adding 5% (v/v) of washed sheep blood to the blood agar base. *E. coli* SG13009 harboring pVhh was spot inoculated on sheep blood agar and egg yolk agar plates. In a separate experiment, the pure recombinant VHH was separately added at 20 µl volumes into 5 mm diameter wells made in the sheep blood and egg yolk agar plates. The plates were incubated overnight at 30 °C. *E. coli* SG13009 lacking recombinant plasmid was used as the negative control.

Nucleotide sequence accession number

The nucleotide sequence of the *vhh* gene derived in this study has been assigned GenBank accession number EU827170.

Results and discussion

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

A total of 70 isolates of *V. harveyi* were identified by 16S rDNA PCR that yielded a 967 bp amplicon with all the isolates. The PCR products obtained with 5 representative isolates were sequenced and the sequences were found to be 99-100% identical with the *V. harveyi* 16S rDNA sequences in the GenBank (data not shown).

PCR detection of *vhh* in *V. harveyi* isolates

To detect *vhh* gene in *V. harveyi* isolates, we initially used primers F1-R2 that amplify a 308 bp region of *vhh* gene (10) (Table 1). However, only a proportion of the isolates (24/70) were PCR positive with these primers, indicating the possible presence of nucleotide sequence variations within the *vhh* gene. Following this, the amplification of the entire *vhh* gene was attempted using primers F1-R1 (29) and LCF-LCR. The primers LCF-LCR were designed in this study based on the *vhh* gene of the *V. harveyi* type strain ATCC BAA-1116, the whole genome sequence of which has been deciphered recently (8). We further screened all the isolates with combinations of forward and reverse primers and the results are presented in Table 2. Using different combinations of primers, the *vhh* gene could be detected in 67 of 70 isolates tested. Overall, the majority (55/70) of the isolates were positive with the primers F2 and R1, followed by primers F1 and R1 (43/70). All seawater isolates (n=18) and 20 of 22 isolates from healthy *P. monodon* larvae were positive by F2-R1 primer combination. Compared to these, the *vhh* gene of *V. harveyi* from infected larvae were more heterogeneous, since the maximum number of isolates detectable by a single PCR was 17 of 30 isolates using primers F2-R1. These results suggest that the populations of *V. harveyi* causing infections of larval *P. monodon* are not clonal, at least with respect to the *vhh* gene. The reason for this nucleotide variation in *vhh* gene and its significance to the virulence of *V. harveyi* could not be determined in this study.

The primers LCF and LCR designed based on the *vhh* gene of BAA1116 could amplify the gene in 3 isolates VH34 and VH39 (both from infected *P. monodon* larvae), and VH56 (from healthy larvae) only, and interestingly, these were negative with the F1-R1 primers. When HindIII restricted genomic DNA of these three isolates were hybridized with DIG-labeled *vhh* probe, all the three isolates showed a single fragment of approximately 4 kb hybridizing with the probe (Fig. 1). The 1254 bp *vhh* gene amplified from one of the strains (VH34) was cloned and sequenced and the recombinant protein was expressed in *E. coli*.

Sequence analysis of *vhh* from *V. harveyi* VH34

The 1257 bp *vhh* gene sequence obtained from the isolate VH34 encoded a polypeptide of 418 amino acid precursor with a 20 amino acid signal sequence. The GDSL motif of lipolytic enzymes was identified between amino acid positions 147-158. The nucleotide sequences of *vhh* from VH34 revealed 98% identity with the *vhh* gene of *V. harveyi* BAA1116, *vch* of *V. campbellii* (GenBank accession no.DQ434996) and 80% identity with *vhhA* of *V. harveyi* strain VIB 645 (GenBank accession no. AF203430). The amino acid sequence deduced from nucleotide sequence exhibited 98% identity with VHH of ATCC BAA-1116 and VCH of *V. campbellii* and 86% identity with VhhA of VIB 645. It appears that the *vhh* gene identical to that of *V. harveyi* ATCC BAA-1116 and *vch* of *V. campbellii* is found in a small proportion of hatchery isolates of *V. harveyi* in India. Sequence heterogeneity within known hemolysin genes is observed among members of the family vibrionaceae. The human pathogenic *V. parahaemolyticus* produces either or both of the two hemolysins, thermostable direct hemolysin (TDH) and thermostable direct hemolysin-related hemolysin (TRH) encoded by *tdh* and *trh* genes respectively, that share approximately 68% sequence homology (23). *trh* genes are further classified into *trh1* and *trh2* genes, that share 84% similarity at nucleotide level (17). Several events, such as mutations during gene duplications, may lead to hemolysin genes with altered nucleotide sequences but similar biological properties.

Based on our PCR results, it also appears that the nucleotide variations in *vhh* gene are not restricted to any specific locations in the gene, as different combinations of primers were necessary to amplify the gene in majority of strains used in this study. As can be seen from the Table 2, primers F1-R1 amplified the gene in 43 isolates of *V. harveyi*, while the primers F2-R1

increased the number of positive isolates to 55. However, when primers F1/F2 were used in combination with LCR, the corresponding numbers of positive *V. harveyi* were 6 and 4 respectively. Analysis of the nucleotide sequence at 3' end of the *vhh* gene sequence derived in this study and *vhhA* (GenBank accession number AF203430) indicated 6 nucleotide mismatch in a stretch of 23 nucleotides corresponding to the sequence of the primer LCR, of which 4 were towards 3' end of the primer. The corresponding nucleotide mismatch with the *vch* was 4 of 23 nucleotides from the 3' end. These mismatches were presumably responsible for the inability to amplify *vhh* gene in majority of *V. harveyi* isolates using the primers LCF and LCR designed based on the *vhh* gene of *V. harveyi* BAA1116.

Expression of VHH in *E. coli* and purification of recombinant protein

The *vhh* gene cloned from VH34, one of the three strains which yielded 1.3 kb amplicons with primers LCF and LCR, was expressed in *E. coli* SG13009 by IPTG controlled induction. The 6×His-tagged protein was purified using Ni-NTA affinity chromatography. The purified protein showed a molecular mass of about 45 kDa (Fig. 2). The phospholipase and the hemolytic activities of the pure recombinant protein could be demonstrated on egg yolk agar and sheep blood agar respectively.

Though several virulence-related factors have been identified in *V. harveyi*, strain to strain variation in the presence of these suggests that more than one factor may be responsible for the virulence of *V. harveyi* to fish and shellfish (6). Extracellular products such as hemolysins, phospholipases and proteases play important roles in the virulence of *V. harveyi* to *P. monodon* (19). At present, the hemolysin VHH is the only well characterized virulence determinant of *V. harveyi* pathogenic to fish (30). The role of VHH in shrimp pathogenicity and its mode of action on invertebrate cells have not yet been demonstrated. VHH has been identified as a phospholipase B (26). Phospholipases are major virulence factors of many pathogenic bacteria that act on eukaryotic cells by hydrolyzing the cell wall phospholipids, thereby leading to the loss of cell integrity and cytolysis (25). The mechanism of action of VHH on crustaceans may be similar, though this remains to be experimentally proven.

In India, PCR for *vhh* gene is employed to identify *V. harveyi* strains isolated from *P. monodon* hatcheries during routine hatchery operations or during outbreaks of vibriosis. However, the results of our study indicate that nucleotide sequence variations within *vhh* may not permit detection of this gene in all isolates of *V. harveyi* by *vhh*-specific PCR. Further, the *vhh* gene is present in the majority of *V. harveyi* irrespective of whether they are isolated from healthy or infected *P. monodon* larvae. Therefore, detection of *vhh* gene alone may not help in determining the infection status of *P. monodon* larvae. Studies have shown that varying temperature and salinity also play a role in the infectivity of *V. harveyi* to *P. monodon* larvae (2). Further, scientific evidence suggests that quorum sensing regulates bioluminescence and virulence gene expression in *V. harveyi* (15). Recent studies have demonstrated that quorum sensing regulates virulence of *V. harveyi* to brine shrimp (*Artemia franciscana*) and rotifers (*Brachionus plicatilis*) (11, 27). All *V. harveyi* isolates used in our study were luminescent and harbored the *vhh* gene. However, the expression of VHH itself may be dependent on the density of *V. harveyi* or other cues in the hatchery environment. More studies are necessary to prove this hypothesis and determine the precise role of VHH in the virulence of *V. harveyi* to *P. monodon*.

Acknowledgements

The authors are grateful to the Director, NIO, Goa and the Scientist in charge, NIO (RC), Cochin for their support and advice. Financial support from supra institutional project SIP 1302 is gratefully acknowledged. S.K is grateful to Prof. (Dr). Manuel Varela, Department of Biology, Eastern New Mexico University, USA for scientific advice and guidance. This is NIO contribution no. 4508.

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Table 1 Oligo nucleotide primers used in this study

Primer	Sequence (5'-3')	Binding site	Product size (bp)	Reference
LCF	atgaataagaccattactgtact	1-23	1254	This study
LCR	gaacggatgggtcगतगctggt	1254-1231		
F1	atcatgaataaaactattactgtact	1-26	1254	Zhang et al. (2001)
R1	gaaaggatggttgacaat	1235-1254		
F2	tcagtgcctctcaagtaaga	92-111	216	Conejero and Hedreyda (2004)
R2	gcttgataaacactttgcggt	307-287		
16S-F	ccgcataatacctacgggtc	175-194*	967	Fukui and Sawabe (2007)
16S-R	acccgaagtctggcaaca	1223-1142*		

Amplicon sizes with primer combinations F1 & R2 =308 bp, LCF & R2 =308 bp, F2 & R1 =1162 bp, F2 & LCR= 1162 bp

* correspond to nucleotide positions of *E. coli* 16S rDNA

Table 2 PCR detection of *vhh* in *V. harveyi* (n=70) using different primer combinations

Sources of <i>V. harveyi</i> isolates	Number positive by PCR with forward/reverse primer								
	F1/R1	F1/R2	F1/LCR	F2/R1	F2/R2	F2/LCR	LCF/R1	LCF/R2	LCF/LCR
Seawater (n=18)	12	8	0	18	10	0	17	7	0
Infected larvae (n=30)	16	9	4	17	4	1	14	4	2
Healthy larvae (n=22)	15	7	2	20	8	3	9	5	1
Total	43	24	6	55	22	4	40	16	3

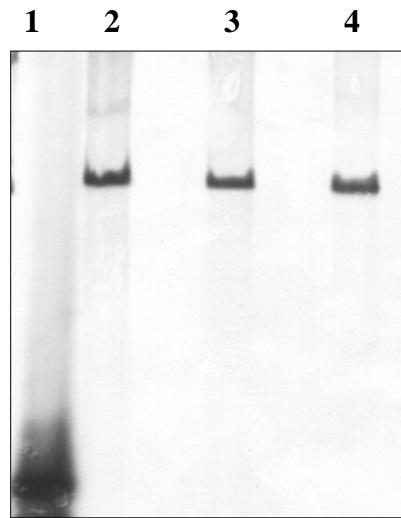


Fig. 1 Result of Southern blot hybridization of HindIII digested genomic DNA of selected *V. harveyi* strains with DIG-labeled probe. The probe was derived from a 308 bp PCR product obtained with primers F2-R2. Lane 1: unlabeled PCR product from primers F2-R2; lane 2: *V. harveyi* VH34; lane 3: *V. harveyi* VH39; lane 4: *V. harveyi* VH56.

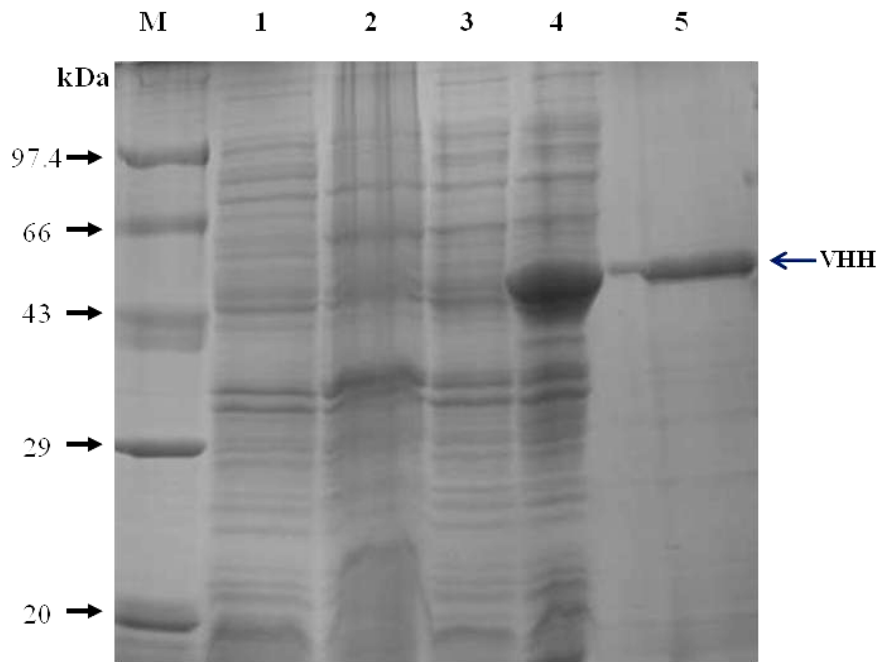


Fig. 2 SDS-PAGE analysis of recombinant VHH protein purified by Ni-NTA affinity chromatography. Lane M, Protein molecular weight marker (PMW-B, Bangalore Genei); lane 1, Non-recombinant *E. coli* SG13009 without IPTG induction; lane 2, non-recombinant *E. coli* SG13009 cells induced with IPTG; lane 3, *E. coli* SG13009/pVhh without IPTG induction; lane 4, *E. coli* SG13009/pVhh induced with IPTG; lane 5, purified recombinant VHH.