Occurrence of clinical genotype *Vibrio vulnificus* in clam samples in Mangalore, Southwest coast of India

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Abstract *Vibrio vulnificus* is an opportunistic human pathogen causing gastroenteritis, wound infection and primary septicemia. *V. vulnificus* population has been divided into subpopulations based on their phenotype and genotype characteristics. In this study, 38.5% (10/26) of clam (*Meretrix meretrix*) samples obtained from Mangalore markets were seen to harbor *V. vulnificus*. Biochemical characterization of *V. vulnificus* isolates showed the strains to belong to Biotype 1 phenotype. Genotyping of strains using the 16S rRNA and virulence correlated gene (*vcg*) typing methods identified the isolates to be of 16S rRNA typeB and *vcgC* type respectively. Analysis of representative 16S rRNA and *vcg* gene sequences further substantiated that the *V. vulnificus* associated with clams in the present study to be of clinical origin, implicated as virulent type responsible for causing infection in humans.

Keywords *Vibrio vulnificus* · Clinical genotype · Biotype 1 · *vcgC* gene · 16S rRNA typeB · Clams

Introduction

Vibrio vulnificus is a Gram negative halophilic bacterium, ubiquitous to warm coastal waters throughout the world. *V. vulnificus* illness in humans is associated with gastroenteritis due to the consumption of raw or undercooked seafood particularly oysters or severe wound infections

resulting from exposing wounds to contaminated estuary or seawater (Gulig et al. 2005). The factors predisposing individuals to severe infection include underlying chronic diseases or immunocompromised health status (Oliver 2015).

Several determinants have been identified in *V. vulnificus* which contributes to its virulence (Al-Assafi et al. 2014). However, to date no single virulence factor has been described that can distinguish pathogenic from non-pathogenic strains. Reports also suggest that the putative virulence determinants although seen expressed by almost all clinical and environmental strains of *V. vulnificus*, not all have the potential to cause disease (Stelma et al. 1992).

Attempts at developing methods to detect and differentiate pathogenic and non-pathogenic *V. vulnificus*, have shown this bacterium to be heterogenic in nature and could be divided into sub-populations based on their phenotypic and genotypic characteristics. *V. vulnificus* strains are biochemically distinguished as belonging to any of the three distinct biotypes, all of which are considered virulent (Bisharat et al. 2005). Biotype 1 strains have been associated with human diseases while biotype 2 has been associated with disease in eels and fish. Biotype 3, reported from Israel is a hybrid of biotypes 1 and 2 and is associated with human wound infections (Chase and Harwood 2011).

Genotyping based on molecular techniques, such as Pulsed-field gel electrophoresis, ribotyping, RAPD-PCR, extragenomic palindromic DNA PCR (Arias et al. 1998; Chatzidakis-Livanis et al. 2006); multilocus sequence typing (Bier et al. 2013; Bisharat et al. 2005; Cohen et al. 2007; Reynaud et al. 2013) and comparative genome analysis (Morrison et al. 2012) have shown that *V. vulnificus* populations could be divided into two genotypes, the C- or the E-type. The C-type has been correlated to clinical origin responsible for human infections, while the

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E-type to those isolated from environmental origin and considered non-pathogenic.

Recent independent genotyping studies based on DNA sequence polymorphism at individual genetic loci such as the 16S rRNA (Nilsson et al. 2003) and virulence-correlated gene (*vcg*) (Rosche et al. 2005) have also demonstrated that *V. vulnificus* populations could be significantly grouped as either the clinical or environmental genotype. An analysis of the 16S rRNA gene sequences of *V. vulnificus* isolated from the environmental and clinical origin is seen to differ by a total of 17 out of 1556 bases, with most of the polymorphism being centered around the first 500 base pairs of the forward strand (Van de Peer et al. 1996). A restriction fragment analysis of the polymorphic region has further shown to generate two profiles 16S rRNA typeA or 16S rRNA typeB which significantly correlated respectively to its environmental or clinical origin (Aznar et al. 1994; Kim and Jeong 2001; Nilsson et al. 2003).

Similarly, by a simple PCR method it has been established that DNA polymorphism at the virulence correlated gene (*vcg*) locus could differentiate *V. vulnificus* into two main genotypes *vcgC* and *vcgE* with the two alleles predominantly being associated with clinical or environmental source of isolation (Rosche et al. 2005, 2010).

Molluscan seafood such as oysters and clams are filter feeders and known to concentrate large number of *Vibrio* spp. Studies so far, demonstrating the presence of clinical type *V. vulnificus* in seafood samples has mostly been isolated from oysters (Guerrero et al. 2015; Han et al. 2009; Reynaud et al. 2013; Warner and Oliver 2008b) and not clams. Therefore the objective of this study was to look for the presence of *V. vulnificus* of C-genotype in clams (*Meretrix meretrix*) harvested along this coast. We report here the occurrence of *V. vulnificus* in clams that show genetic similarity to *V. vulnificus* isolated from clinical origin.

Materials and methods

Sample collection, isolation and biochemical identification of *V. vulnificus*

A total of 26 clam (*M. meretrix*) samples which included 18 market and 8 samples collected from estuary in Mangalore were screened for the presence of *V. vulnificus*. The samples were procured on different dates for a period of three months. The samples collected were transported to the laboratory and processed within 2 h of collection. For isolation of the bacteria 25 g of the clam meat was carefully shucked aseptically into a sterile blender jar and

homogenized with 225 ml of alkaline peptone water containing polymyxin B (APWP). The homogenate was transferred to a sterile flask and incubated at 40 °C for 18 h. Following incubation a loopful of APWP enriched culture was streaked onto mCPC agar plates and incubated at 37 °C for 24 h. The plates were examined for the presence of colonies suggestive of *V. vulnificus* and a minimum of fifteen colonies picked and subjected to a battery of biochemical tests for identification of *V. vulnificus* (Food and Drug Administration 2000). *V. vulnificus* MTCC1145 was used as the reference strain in all the biochemical reactions.

DNA extraction and molecular confirmation

The isolates confirmed biochemically as *V. vulnificus* were subjected to molecular confirmation by PCR. Each *V. vulnificus* isolate was grown in 5 ml of Luria-Bertani broth (Hi-Media, Mumbai) at 37 °C overnight with shaking. Genomic DNA was extracted and purified by cetyl-trimethyl ammonium bromide-proteinase K method (Ausubel et al. 1995). An initial PCR for the molecular identity of *V. vulnificus* was performed targeting the species-specific *vvhA* gene (Lee et al. 1998). The PCR primers targeting the 704 bp fragment of the *vvhA* gene 5'-GACTATCGCAT-CAACAACCG-3' (forward) and 5'-AGGTAGCGAG-TATTACTGCC-3' (reverse) was targeted. The PCR was performed in a 30 µl mixture consisting of 3 µl of 10× buffer (HiMedia Mumbai, India), 200 µM each of the four deoxynucleotide triphosphates, 10 pmol of each primer, and 1.0 U of *Taq* DNA polymerase (HiMedia, Mumbai, India). Two micro liter of the purified DNA was used as template for PCR. The PCR assay was performed in a programmable thermocycler (MJ Research Inc., USA) having an initial delay at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. PCR products were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide (0.5 µg/mL) and photographed using a gel documentation system (BioRad, USA). *V. vulnificus*, MTCC1145 was included as reference strain in all PCR assays.

Biotyping of *V. vulnificus* isolates

The *V. vulnificus* isolates were phenotypically confirmed as belonging to biotype 1 based on a positive reaction for indole production, acid production from mannitol, lactose and salicin, decarboxylation of lysine and ornithine and growth at 42 °C (Alsina and Blanch 1994; Biosca et al. 1996).

Genotyping of *V. vulnificus* isolates

16S rRNA typing

Studying the genotypes of the isolates was based on differences in 16S rRNA and the virulence correlated gene (*vcg*) nucleotide sequences. Typing based on 16S rRNA sequences was carried out as described by Nilsson et al. (2003). A 492 bp region of the 16S rRNA gene of *V. vulnificus* was amplified using primers UFUL (5'-GCCTAACACATGCAAGTCGA-3') and URUL (5'-CGTATTACCGCGGCTGCTGG-3'). The restriction endonuclease digestion of amplified product was performed in a 20-μl reaction volume consisting of 10 μl of amplicon, 2 μl of 10× restriction buffer, 17 μl of nuclease free water and 1 μl of digestion enzyme *Hae*III (Thermoscientific, India). Digestion was carried at 37 °C for 3 h, after which DNA fragments were separated by electrophoresis on an 2% agarose gel. Representative 16S rRNA gene product was purified and sequenced (Bioserve, Hyderabad, India).

vcg Gene typing

PCR typing was carried out targeting a 277 bp fragment of the virulence correlated gene using the two primer pairs P1 (5'-AGCTGCCGATAGCGATCT-3'), and P3 (5'-CGCTTAGGATGATCGGTG-3') for *vcgC* type strains and P2 (5'-CTCAATTGACAATGATCT-3') and P3 for *vcgE* strains as described by Rosche et al. (2005). PCR was carried out in a 30 μl reaction mixture as described above with an annealing temperature of 55 °C for 1 min. The generated PCR products were purified using High Pure Purification Kit (Roche Diagnostics, Germany) and sent for sequencing (Bioserve, Hyderabad, India).

Nucleotide analysis

The generated 16S rRNA and *vcg* partial gene sequences were subjected to sequence similarity search using NCBI BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast>). Multiple sequence alignment was performed using Muscle3.8 program (<http://www.ebi.ac.uk/Tools/msa/muscle/>). Restriction Map program (http://www.bioinformatics.org/sms2/rest_map.html) was used in finding the *Hae*III restriction sites in 16S rRNA sequence. The partial sequences generated of the *vcg* gene have been deposited in GenBank with accession numbers KT936537-KT936544 and KC87864-KC875865.

Results and discussion

In this study, out of the 73 *V. vulnificus* strains isolated from clam (*M. meretrix*) samples, 57 isolates showed biochemical reactions typical of the biotype 1 phenotype.

Earlier studies have shown that the biotype 1 strains could be further sub-divided into two genotypes, clinical (C) and environmental (E) based on their genetic divergence (Warner and Oliver 2008a). It was of interest to see whether the biotype 1 phenotypes in this study belonged to the C- or E-genotype and therefore 26 biotype 1 isolates, each representing a clam sample was subjected further to 16S rRNA A/B and *vcgC/E* genotyping.

In the 16S rRNA A/B typing method, a *Hae*III restriction digest of a 492 bp fragment of the 16S rRNA is expected to give fragments of length 168, 204 and 120 bp for those of typeA, while typeB isolates should give fragments of 147, 21, 204 and 120 bp (Nilsson et al. 2003). All the 26 isolates biotype 1 *V. vulnificus* tested in this study were seen to PCR amplify a 492 bp fragment of 16S rRNA. However, on restriction digestion with *Hae*III 16 of the 26 isolates generated amplicons typical of typeA, while the remaining 10 gave amplicons of sizes 204, 147 and 120 bp (Fig. 1) typical of typeB. The presence of a 21 bp fragment not observed in the gel, probably due to poor resolution was confirmed by sequencing and analyzing the partial gene sequence of 16S rRNA (Fig. 3).

Vibrio vulnificus strains confirmed as typeB were further subjected to PCR amplification of a 277 bp of the *vcg* gene. Amplification could be achieved only with the primer set P1 and P3 specific for the *vcgC* allele (Fig. 2). Primers P2 and P3 failed to amplify the gene. A BLAST analysis of the

Fig. 1 *V. vulnificus* strain typing using *Hae*III enzyme. Lane M marker (50 bp DNA ladder); Lanes 1–10 *V. vulnificus* strains (Vv1–Vv10) from clam samples; Lane 11 reference strain MTCC 1145

Fig. 2 PCR amplification of the *vcg* gene in *V. vulnificus*. Lane M marker (100 bp ladder); Lane 2 reference strain MTCC 1145, Lane 2 Negative control; Lanes 3–12 *V. vulnificus* strains from clam samples

Fig. 3 Multiple alignment of partial 16S rRNA gene sequence of *V. vulnificus*. Vv2, Vv4, Vv6 represent *V. vulnificus* samples from this study. X76333 and X76334 are GenBank downloaded sequences representing 16S rRNA typeA and typeB respectively. *HaeIII*

partial *vcgC* gene sequences, showed closest identity to *V. vulnificus* *vcgC* sequences available in GenBank. Further, a multiple alignment of the *vcgC* sequences to *vcgC* and *vcgE* sequences downloaded from database clearly clustered the sequences in this study with *vcgC* type and was seen to be distinct from *vcgE* sequences (Fig. 4).

Studies suggest the 16S rRNA and *vcg* typing could be used as an indicator to study the environmental distribution of potentially virulent strain of *V. vulnificus*. It has been demonstrated that clinical strains are *vcg* typeC, 16S rRNA typeB and biotype 1 while the environmental type are *vcg* typeE, 16S rRNA typeA and biotype 2 (Bier et al. 2013). In this study 10 clam samples were seen to harbor *V. vulnificus* that phenotypically were of biotype 1. A PCR screening and subsequent sequencing for the distribution of polymorphic 16S rRNA and *vcg* sequences showed the strains under study to be of 16S rRNA typeB and *vcg* typeC genotypes. The markers considered in this study clearly indicate that clams harvested in Mangalore coast harbor the clinical type of *V. vulnificus* strains which may have the ability to infect humans (Figs. 3, 4).

Several factors are associated with the virulence of this pathogen which include hemolysin/cytolysin (*VvhA*), metalloprotease (*VvpE*), siderophore-encoding gene (*viuB*) and repeat-in toxin (RTX) and strains possessing these genes are assumed to be virulent (FAO/WHO 2005). The C-genotype *V. vulnificus* strains in this study were all seen

restriction site is indicated by vertical line. The shaded regions show the variations in nucleotide sequences. Arrows indicate primer binding sites

to possess the *Vvh*, *VvpE*, *ViuhB* and *rtx* genes (data not shown), which further substantiates the strains isolated from clams to be probably of the virulent type.

Conclusion

Clams species (*M. meretrix*) occur naturally in estuaries of Mangalore coast and is used as a food source. In this study 38.5% (10/26) of the clam samples tested were seen to harbor clinical type *V. vulnificus* isolates. *V. vulnificus* is an opportunistic pathogen and illness is associated to ingestion of raw or undercooked seafood or to the exposure of wounds to seafood/water contaminated by this pathogen. In southern coastal India, clams are commonly cooked and eaten and therefore pose no risk when consumed. However, ingestion of undercooked clams or exposure of wounds by shellfish handlers to pathogenic strains of *V. vulnificus* is a potential risk to human health, as it could lead to gastroenteritis or primary septicaemia. Thus the detection of clinical genotypes in clams harvested in this area assumes significance as C-genotypes are correlated to higher virulence and disease (Warner and Oliver 2008b). This study supports the presence of clinical genotype *V. vulnificus* in relatively small number of clam samples. Since no non-clinical strains were included in the study, our future work aims to do an epidemiological survey of molluscan seafood

Fig. 4 Multiple sequence alignment for partial *vcg* gene of *V. vulnificus*. AY626579–AY626583 corresponds to *vcgE* sequences from GenBank. CMCP6 and YJ016 are clinical isolates in.

including water from where it is harvested to establish the proportion of clinical and environmental genotypes present along this coast and study their clonal relatedness if any.

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MTCC1145 is the reference strain used. Strains marked asterisks are *V. vulnificus* strains from this study

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