Isolation and identification of *Vibrio spp.* with potential ability to produce polysaccharide monooxygenase from diseased *Penaeus monodon*

Ho Ta Giap[1*], Ngo Nguyen Vu[1], Vu Van Van[1]

[1]Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam
[*]Corresponding author: htgiap@ntt.edu.vn

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ABSTRACT

Polysaccharide monooxygenases (PMOs) and Glycoside Hydrolases (GH) play a crucial role in breaking down chitin and cellulose. Research on PMOs has mainly focused on their role in the production of bioethanol from biomass. Still, the potential of PMOs as virulence factors in microorganisms is not been fully studied. Some pathogenic bacteria secrete PMOs, which have been identified as virulence factors. The ability of *Vibrio* species to metabolize chitin is well-known, but the question of which Vibrio species contains the PMO gene remains unanswered. Materials and methods: Black tiger shrimp *Penaeus monodon* was collected from a culture pond in Long An, Vietnam. Thiosulfate-citrate-bile salts-sucrose and CHROMagar Vibrio agar were used to isolate, Vibrio bacteria from the shrimp samples. Mixed bases primers were designed to detect PMOs gene from Vibrio strains. Results: Total of 35 different strains of bacteria were isolated from the guts and pancreas of the shrimp sample. Based on biochemical tests, there were a total of 20 different Vibrio strains identified among 35 isolated strains. The PCR results showed that six isolates could have the PMOs gene. The partial 16S rDNA fragments of six isolate Vibrio strains were sequenced. Comparing 16S rDNA sequences with sequences available in NCBI databases revealed that all the sequences are *Vibrio spp.* 16S rDNA sequences.

1. Introduction

Chitin, the second most abundant biopolymer after cellulose, is derived from the exoskeletons of crustaceans and cell walls of fungi and insects (Elieh-Ali-Komi & Hamblin, 2016). In parallel, pathogenic bacteria have developed a variety of pathways for the capable conversion of cellulose and chitin into energy. Cellulose-degrading bacteria are prevalent on land and the ground, while chitin-degrading bacteria can be found in both land and water environments (Brzezinska, Jankiewicz, Burkowska, & Walczak, 2014). Polysaccharide monooxygenases (PMOs) and Glycoside Hydrolases (GH) are primarily responsible for the breakdown of chitin and cellulose (Ngo & Vu, 2021). The utilization of microbial enzymes has been widely published, and the commercial use of enzymes also has been recently adopted (Elieh-Ali-Komi & Hamblin, 2016). Accordingly, GH has been utilized in various commercial sectors, including agriculture, brewing, laundry, paper, and textile industries (Ejaz, Sohail, & Ghanemi, 2021). Whereas PMOs were only introduced as supporting enzymes for the GH (Vaaje-Kolstad et al., 2010). According to the classification of the CAZy database (http://www.cazy.org/), PMOs are classified as Auxiliary Activities (AA) enzymes. From 2010 to 2019, a total of seven PMOs families were

published, including AA9, AA10, AA11, AA13, AA14, AA15, and AA16 (Couturier et al., 2018; Filiatrault-Chastel et al., 2019; Harris et al., 2010; Hemsworth, Henrissat, Davies, & Walton, 2014; Sabbadin et al., 2018; Vu, Beeson, Span, Farquhar, & Marletta, 2014; Vaaje-Kolstad et al., 2010). Trends in the production of bioethanol from biomass are the main focus of PMO research. Meanwhile, the research direction of PMOs as the virulence factor of microorganisms is still a new but promising subject. In fact, some pathogenic bacteria secrete AA10 PMOs identified as a virulence factors. An example is the GlcNAc-binding protein A (GbpA) of *Vibrio cholerae*, which was reported as a crucial factor in letting the bacteria form adhesion to both intestinal human and the chitinous surface (Sabbadin et al., 2018). In 2014, GbpA was described as a chitin-active PMO and a virulence/infection factor of *V. cholerae* (Loose, Forsberg, Fraaije, Eijsink, & Vaaje-Kolstad, 2014). Using GbpA as mediates adhesion to the exoskeletons of zooplankton and invertebrates, *V. cholerae* is able to survive in oligotrophic environments (Sabbadin et al., 2018). Similarly, to infect human *V. cholerae* also uses GbpA to interact with mucus in the human intestine, an organ with mucin glycoprotein as the main component. GbpA of *V. cholerae* binds to GlcNAc residues of mucin and contributes to intestinal colonization and virulence in a mouse model (Sabbadin et al., 2018). Therefore, GbpA is also known as a mucin-binding protein. When GbpA was removed, *V. cholerae* was unable to cause disease in a mouse model, thereby confirming that *V. cholerae* needs to have GbpA in order to cause infection (Sabbadin et al., 2018). Many studies showed evidence of other *Vibrio* species causing disease on the surface of chitinaceous animals, for example *Vibrio vulnificus* colonizes on the surface of shellfish (Froelich & Noble, 2014). *Vibrio parahaemolyticus* used chitinase to colonize particulate chitin of copepods (Fennell, Blackwell, Thomson, & Dorman, 2021). *Vibrio* species are proved to metabolize chitin which is a favorite substrate for this group of bacteria. On the other hand, "which *Vibrio* species has the gene encoding PMOs?" is an unanswered question.

Using bioinformatics tools to search for PMOs from *V. cholerae* showed that PMOs were also present in *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, and *V. harveyi* species (https://www.ebi.ac.uk/Tools/hmmer/search/phmmer). These *Vibrio* species are prevalent in shrimp grow-out ponds. Diseases caused by *Vibrio* bacteria are major problems for businesses and households farming brackish water shrimp in Vietnam, such as Acute Hepatopancreatic Necrosis Disease (AHPND) (Tran et al., 2020). PirABvp toxin, homologous to Photorhabdus insecticidal toxin (Pir), has been shown to be the main toxin for AHPND in shrimp (Choi, Stevens, Smith, Taylor, & Kuhn, 2017). However, the infection pathway and the main factors that help *Vibrio* infect the host have not been published. This study aims to determine the presence of genes encoding PMOs in several isolated *Vibrio* species from cultured shrimp, thus strengthening the hypothesis of PMOs role as a virulence factor in this group of bacteria.

2. Materials and methods

2.1. Primer design for Vibrio PMOs gene detection

Because of the highly divergent sequence composition of PMOs and other families of the PMOs found in bacteria, we employed a supervised homology detection protocol to find PMOs similarity based on iterative reciprocal similarity searches using jackhammer and hmmsearch (HMMER 3.3). The GbpA protein sequence (A9Y370) was used as input data. Gene sequences encoding PMOs homologous GbpA were used to design primers for PCR reaction to detect PMO from *Vibrio*.

2.2. Isolation and identification of Vibrio

Fresh shrimp sample (*Penaeus monodon*) was collected from a culture pond in Long An,

Vietnam. The sample was stored at 4°C until transported to the Microbiology laboratory, NTT Hi-tech Institute for bacterial isolation process.

Each shrimp sample was dissected with an aseptic scalpel blade. The guts and hepatopancreas of each shrimp were separated and homogenized in NaCl 0.85% following the ratio of 0.5 g/ml to form a homogenate sample. The homogenate solution was diluted 10 - 10^6 times with NaCl 0.85% solution. 100µL of each homogenate at the concentrations 10^{-4}, 10^{-5}, and 10^{-6} was spread on thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Oxoid, france) and CHROMagar Vibrio (CV) agar (CHROMagar, France) and i ncubated at 35°C for 24h. The water sample on the same culture pond was also spread on both CHROMagar and TCBS agar, 100µl sample for each agar plate. After incubation period, grown bacterial colonies were identified and classified by their morphology and color, then sub-cultured until pure-strain was archived.

2.3. Detection of PMOs genes

Each *Vibrio* colony grown on an agar plate was picked and mixed with 1mL of sterile deionized distilled water in 2mL eppendorf tube. Subsequently, the mixture was boiled at 100°C for 10min and centrifuged at 16,000 xg for 10min. Following, 1µL of supernatant was added to the PCR reaction. The sequences encoding AA10 PMOs in Vibrio species were searched and downloaded from Uniprot. Then, all available sequences were aligned using MUSCLE embedded in Geneious Prime v2022.2. The alignment result was used to identify a suitable region for designing primer pairs manually. The length of the selected regions ranged from 18bp to 20bp, which has a maximum number of three different nucleotides in the sequences. PCR products of the designed primers were supposed to be 150 - 200bp in length. Each primer pair was checked for GC content, melting temperature, molecular weight, extinction coefficient, µg/OD, nmol/OD, hairpin, self-dimer, and hetero-dimer using the Oligo Analyzer tool of Integrated DNA Technologies (https://sg.idtdna.com/calc/analyzer). Consequently, two primer pairs, including primerF1-380 3'-GAC TGG AAA TAY TAC ATY AC-5', primerF2-193 3'-GAA CCG CAA AGT GTA GAA GG-5', primerR1-600 3'-ACG TCG ATA ACA TTR TAR AA-5', and primerR2-560 3'-GCA GTA TCA CCC ACA TCC CA-5' were selected and used for further experiments in this study. 16S rDNA fragments were amplified with primers 27F:5'-AGA GTT TGA TCC TGG CTC AG-and 1492R: 5'-GGT TAC CTT GTT ACG ACT T-3' (Kawai et al., 2002). A procedure to detect 16S rDNA was performed with each sample to test the efficiency of DNA extraction as an initial positive control. The conditions of PCR for PMO gene amplification were set at one cycle of 95°C for the 30s, followed by 35 cycles of amplification consisting of denaturation at 95°C for 15s, annealing at 55°C for 30s, and extension at 68°C for 2min, and then followed by one cycle of 68°C for 5min. After that, the PCR amplified products were stained with GelRed, checked in 1% agarose gels by electrophoresis, and then photographed under a UV transilluminator.

3. Results

3.1. PMO-encoding gene sequences and primers for PCR

Jackhmmer results showed that PMOs also present in *Vibrio mimicus, Vibrio metoecus, Vibrio tapetis, Vibrio tubiashii, Vibrio europaeus, Vibrio alfacsensis, Vibrio bivalvicida, Vibrio sinaloensis, Vibrio brasiliensis, Vibrio alginolyticus, Vibrio diabolicus, Vibrio neocaledonicus, Vibrio chagasii, Vibrio splendidus, Vibrio tasmaniensis, Vibrio crassostreae, Vibrio gigantis, Vibrio coralliirubri, Vibrio lentus, Vibrio atlanticus, Vibrio scophthalmi, Vibrio ichthyoenteri, Vibrio mediterranei, Vibrio shilonii, Vibrio thalassae, Vibrio aestuarianus, Vibrio vulnificus, Vibrio parahaemolyticus, and Vibrio barjaei*. 31 gene sequences encoding PMO (29 sequences above plus Vibrio spp. and Vibrio cholera) were aligned (Figure 1). Genes encoding PMOs of

Vibrio exhibit sequence diversity. Specifically, the identical sites are 424/1,497 (28.4%); the pairwise identity is 67.8%. Therefore, we designed mixed bases primers for the PCR reaction to detect PMOs in *Vibrio* isolated from shrimp.

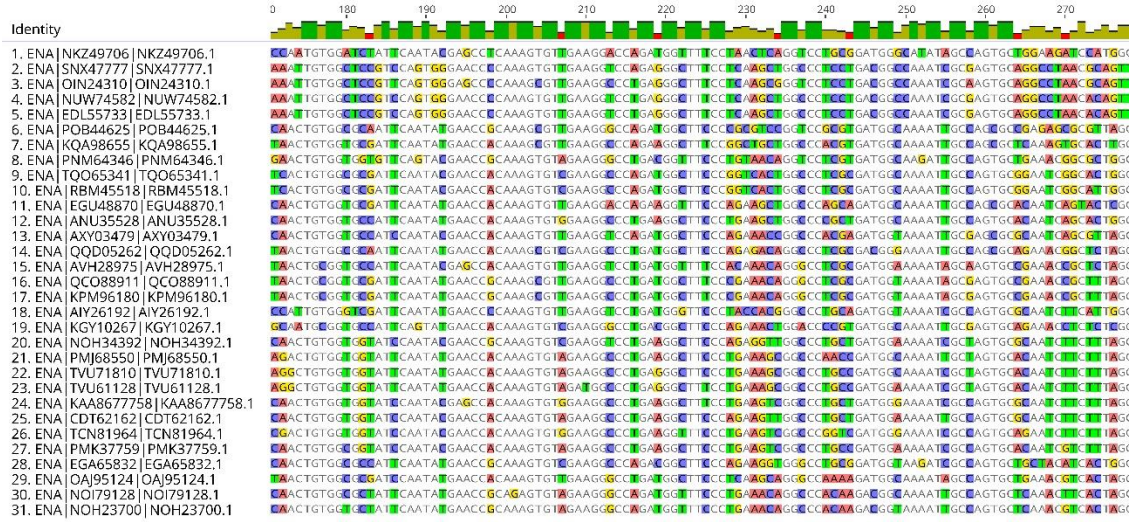

Figure 1. The divergent of PMOs gene sequences

3.2. Isolation and identification of Vibrio from shrimp

Vibrio strains can be identified and classified using their colony morphology and color when cultured on differentiate mediums such as TCBS and CHROMagar (Pfeffer & Oliver, 2003). On TCBS agar plate, colonies with flat yellow morphology, about 2 - 3mm in diameter, are suspected to be *V. cholerae*, *V. fluvialis*, *V. furnissii*; flat green colonies, 2 - 3mm in diameter, suspected to be *V. vulnificus*, *V. parahaemolyticus,* and *V. mimicus*. On CHROMagar agar plate, mauve colonies are suspected to be *V. parahaemolyticus*, turquoise are suspected to be *V. vulnificus* or *V. cholerae*, and white/transparent colonies are suspected to be *V. alginolyticus* (Nigro & Steward, 2015).

In summary, there was total 35 different strains of bacteria isolated from the guts and pancreases of the shrimp sample. Based on the Oxidase test, Motility test and Gram staining procedure (data not shown), there were total of 20 different *Vibrio* strains identified among 35 bacterial strains and were used for the next step (Table 1).

Table 1

List of isolated Vibrio strains in this study

Strain code name	***Isolation source***	***Colony color on CHROMagar***	***Colony color on TCBS agar***
GG1	Gut	Green	Yellow
GM2	Pancrease	Mauve	Yellow
GM3	Pancrease	Mauve	Yellow
GM4	Gut	Mauve	Yellow
GW1	Gut	White	Yellow
GW2	Gut	White	Yellow green
GW3	Gut	White	Green
WG1	Culture water	Green	Green
WG2	Culture water	Green	Yellow

Strain code name	***Isolation source***	***Colony color on CHROMagar***	***Colony color on TCBS agar***
WG2	Culture water	Green	Yellow
WM1	Pancrease	Mauve	Yellow
WM2	Pancrease	Mauve	Yellow
WM3	Pancrease	Mauve	Yellow
WM4	Pancrease	Mauve	Yellow
WW1	Culture water	White	Yellow
WW2	Culture water	White	Yellow
WW3	Culture water	White	Green
WW4	Culture water	White	Yellow
WW5	Culture water	White	Yellow
WW6	Culture water	White	Yellow

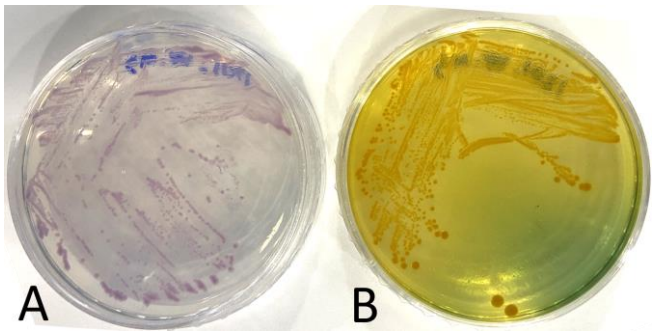

Figure 2. Colonies of bacterial strain WM3 with mauve color on CHROMagar (A) and yellow color on TCBS agar (B)

3.3. Identification Vibrio carry PMOs gene

Out of the 20 Vibrio colony samples showed positive with 16s rDNA gene, a single PCR product of the expected size (1.5kb) was detected (Figure 3) in all of the 20 collected *Vibrio* samples. Thus, the genomic DNA isolation of all samples qualified to be able to investigate the presence of PMO by PCR.

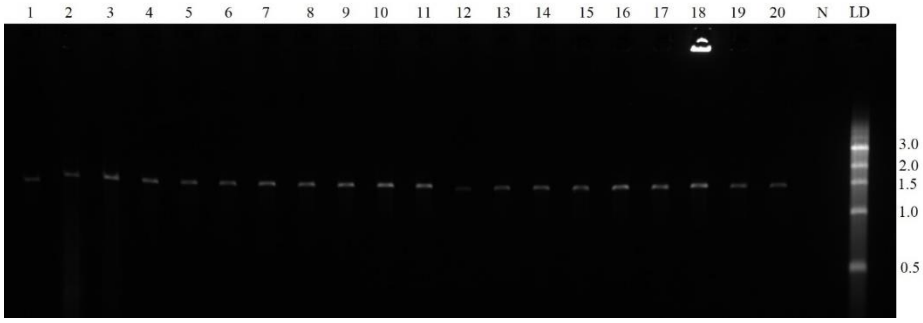

Figure 3. PCR amplification of 16S rDNA

The primerF1-380 and primerR1-600 primers were designed to bind to PMOs gene of *Vibrio* and the PMO-amplified product has a length of about 220bp. The results showed only 06 (30%) *Vibrio* samples suggesting PMOs gene (Figure 4), include sample numbers 12, 13, 15, 16, 17, and 18. We also designed the second PMOs detection primers (primerF2-193 and primerR2-560). These primers were also designed to bind to PMOs gene but did not overlap with the primerF1-380 and primerR1-600 primer targets.

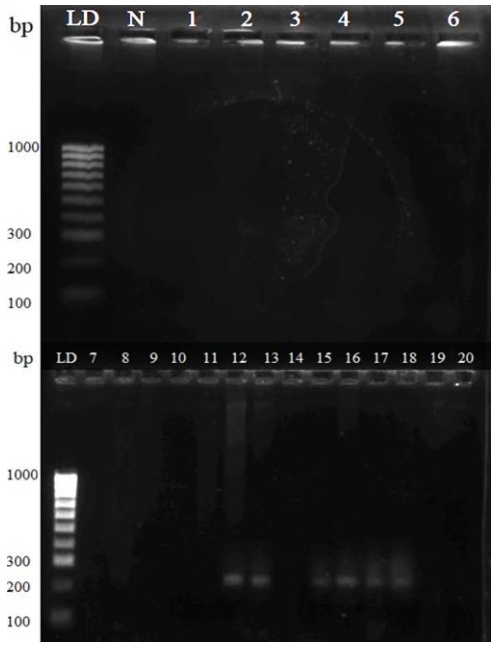

Figure 4. Detection of PMOs with primerF1-380 and primerR1-600 primers

In the PCR reaction using primerF2-193 and primerR2-560 primers, there were 07 (35%) samples suggesting PMOs gene (Figure 5), include sample numbers 12, 13, 15, 16, 17, 18, and 19. Samples number 17 and 19 showed the fuzzy band and smear product.

Based on results from Figures 4 and 5, there were total of 6 *Vibrio* strains suggesting PMOs gene when detecting with both primers pairs. The strain code names of these strains are GG1 (13), GM4 (12), GW1 (16), WW2 (15), WW3 (17), and WW6 (18). The partial 16S rDNA fragments of 06 isolated *Vibrio* strain were sequenced. A comparison of 16S rDNA sequences with sequences available in NCBI databases revealed that all the sequences are V*ibrio spp.* 16S rDNA sequences (see supplementary file).

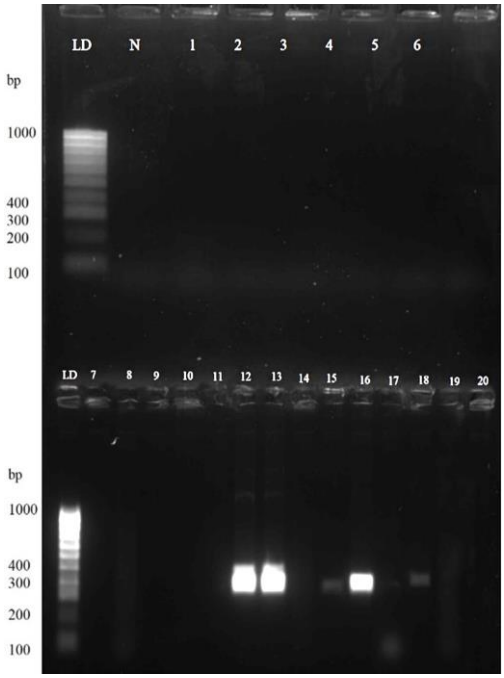

Figure 5. Detection of PMO with primerF2-193 and primerR2-560 primers

4. Discussion and conclusion

4.1. Discussion

Our results suggest several requirements for obtaining PMOs gene sequence. The first requirement is enough data to present the most amplified PMOs gene segments. The regularity of primer amplification, species abundance, and sequencing depth all play a role in determining the sufficiency of data. Primers detected multiple sequences are usually not specific. Therefore, PMOs gene from *Vibrio* spp. was detected by amplifying two regions. Each Vibrio strain is identified as carrying the PMOs gene when PCR products are positive on both designed sequence regions. By this method, in this study, we detected 06 strains of Vibrio carrying the PMOs gene.

Second, bacterial 16s rDNA detected by PCR is an essential step. In this study, we suggested a simple DNA extraction protocol. For the research presence of a gene in bacteria, the price of the chemicals, the duration of the procedure, and the complexity of the protocol need to be taken into consideration. Therefore, a simple protocol is necessary. Accordingly, we suggested qualitative bacterial 16s rDNA as an initial positive control. Full-length 16s rDNA was amplified by PCR using 27F and 1492R primers. Our results showed all Vibrio colony samples positive with the 16s rDNA gene.

Finally, the most important requirement for studying PMOs from *Vibrio* spp. is obtaining *Vibrio* bacteria with potential virulence affinity. As suggested above, *Vibrio* spp. with PMOs can be found abundant in diseased shrimp ponds, especially AHPND disease, with a high appearance of many different species of *Vibrio* in culture water. Detection and isolation of *Vibrio* spp. from such source are quick and simple with the help of modern isolation agar, such as TCBS and CHROMagar. The average isolation procedure for each batch of shrimp samples is 02 - 03 days, yielding 10 - 20 different strains of Vibrio bacteria each.

In a future study, Vibrio strains carrying the PMOs gene have to be identified as species. The *recA, rpoA, gapA*, and 16s rDNA are the genes needed to identify the *Vibrio*. Based on bioinformatics tools, we found 30 different PMOs gene. As mentioned, these gene sequences encode GbpA and only GbpA of *V. cholerae* has been proven to be PMO. Using the BLAST tool, WW6 strain as *V. rotiferianus*, WW2 and WW3 as *V. alginolyticus*, GW1 as *V. mediterranei*, GM4 as *V. owensii*, *V. jasicida*, or *V. harveyi*, and GG1 as *V. alginolyticus* or *V. diabolicus* were identified. In a further study, we will amplify the full-length PMOs gene from these 06 Vibrio strains. After that, the PMOs gene will be cloned and expressed.

4.2. Conclusion

Most of the studies related to shrimp diseases caused by *Vibrio* have focused on virulence factors. However, to understand the pathway of infection and prevent Vibrio from causing disease in shrimp, it is necessary to pay attention to different means Vibrio can utilize to attack host. PMOs (know as GbpA) of *V. cholerae*, which were reported as a critical component to help the bacteria create adhesion to both intestinal human tissue and the chitinous surface. This study suggests that, not only *V. cholera* but other *Vibrio* species may have used PMOs as a mean to colonize on the host tissue. The synergies of different *Vibrio* species attack on the same host could promote the breakdown of the host protection, promoting the chance of pathogenic *Vibrio* species to attach to host cells. This study has created a protocol for detection of *Vibrio* carrying PMOs gene, and detected six strains with potential virulence PMOs using the protocol. These Vibrio samples will be used for further research to understand more about the way Vibrio bacteria use PMOs and ultimately find a solution to inhibit virulence PMOs, thereby preventing *Vibrio* from attaching to the host.

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