Jurnal Teknologi

Full Paper

DETECTION OF VIRULENCE GENES IN *Vibrio alginolyticus* ISOLATED FROM Green Mussel, *Perna viridis*.

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Article history
Received
8 July 2015
Received in revised form
18 September 2015
Accepted
15 October 2015

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Abstract

Vibrio sp. is known as pathogenic microorganism affecting aquatic species. They are commonly isolated from wild fish, mollusc and bivalves. *V. alginolyticus* carries several virulence genes such as *ompK, toxR* and *collagenase* that can cause severe infection to the fishes, crustaceans and bivalve mollusks. Therefore, this study was carried out to detect the presence of virulence genes from *V. alginolyticus* isolated from mantle, adductor muscle, gill, hemolymph, gastrointestinal tract of infected green mussels; including from the water and rope of the cultivation areas. Further identification of the isolates was carried out using conventional method and 16S rDNA sequence analysis. The detection of virulence genes, namely *ompK, toxR* and *collagenase* were carried out using gene-targeted PCR method. A total of 21 isolates were confirmed as *V. alginolyticus*. Results showed that 67% of the isolates carry *ompK, toxR* and *collagenase* genes, while 19% carry both *ompK* and *toxR* genes. Only 5% from the total isolates carried the *ompK* and *collagenase* genes while 10% of the isolates carried *ompK* gene only. Overall, the results indicated that most of *V. alginolyticus* isolates are pathogenic bacteria as they carried the virulence factor.

Keywords: *Vibrio alginolyticus*, virulence genes, *Perna viridis*

1.0 INTRODUCTION

Vibrio alginolyticus is one of the commonest infectious pathogen affecting marine vertebrates and invertebrates which includes fish, shrimp and mollusk. Studies have shown that strains of *V. alginolyticus* are potential reservoir of many virulence genes in the aquatic environment that may contribute to the development of wound infections, enteric diseases and sepsis in humans by exposure to seawater. Previous study indicated that there are several major virulence genes contributing to the virulence of the pathogens such as *Outer membrane protein (OMP), thermolabile hemolysin (TLH), collagenase, toxR, toxRS* and also cholera toxin [6]. For instance, *OMP* is believed to play important roles in infection and pathogenicity to the host and the expression of this gene is controlled by *toxR* gene [1]. *Collagenase* has been widely utilized as biomarker in molecular identification of *V. alginoyticus* as well as capable of

degrading conjunctive tissue, basal epithelial membrane that leads to extra intestinal pathology and dissemination to blood stream [7, 10, 15].

As bivalve is a filter feeder animal, they are capable of retaining pollutants and pathogenic bacteria such as *Vibrio* sp. The consumption of raw mussels or after an insufficient process of cooking may be at risk for humans and sometimes, there are also reported cases that it causes skin infections. Although the mechanism by which the organism infects humans has yet to be comprehensively determined, *collagenase, ompK,* and *toxR* has been recognized as primary virulence factors in *V. alginolyticus*. In the present study, we isolated *V. alginolyticus* from clinically diseased mussels that were confirmed based on conventional and 16S rDNA sequence analyses. Further, the isolates' ability to infect host was determined by detection of several virulence genes [6].

2.0 EXPERIMENTAL

2.1 Bacterial Isolation from Perna Viridis's Organs, Water and Debris

P. viridis were collected from Setiu wetland areas. Sterile swabs were used to isolate the bacteria from mantle, adductor muscle, foot, gills, gastrointestinal (GIT) and debris before cultivation on Marine Nutrient Agar (MNA) (Merck KGaA, Germany). About 100 µl was taken from each of water samples from the wetland areas before spreading onto MNA. Colonies obtained were grown onto Thiosulphate-citrate-bile salt-sucrose (TCBS) (Becton, Dickinson and Company France). Presumably, 2 to 3 mm in diameter of large uniform yellow colonies were selected as *Vibrio alginolyticus* colonies *were* transferred to TSA (Tryptic Soy Agar) supplemented with 2% of NaCl. All the isolates were incubated at 28 ℃ for 24 hours. After that, the morphology of isolates was studied through Gram staining and subjected to the VITEK system for identification process [16].

2.2 Bacterial Strain

V. alginolyticus reference strain from the American Type Culture Collection (ATCC 17749) that produce *toxR, collagenase* and *ompK* genes was used as positive control. TSA supplemented with 2 % of NaCl was used to grow this reference strain and incubated at 28 ℃ for 24 hours [16].

2.3 DNA Extraction

Isolates identified as *V. alginolyticus* in VITEK System Biomérieux SA, USA (biochemical identification) underwent second confirmation through molecular work.

To prepare the DNA template, a 10 µl aliquot of bacterial culture was centrifuged at 6000 rpm for 10 to 15 minutes after overnight incubation in Tryptic Soy Broth (TSB). The pellet was heated in the microwave for three times. DNA template was stored at -20 ℃ for prolong storage [17].

2.4 Polymerase Chain Reaction (PCR) of 16S rRNA gene

Amplification of 16S rRNA gene was carried out by PCR and performed in a final volume of 50 µl using universal primer; 5'- AGAGTTTGATCCTGGCTCAG - 3' (8F) and 5'- GGTTACCTTGTTACGACTT - 3' (1492R) [2]. The PCR was carried out with 1 µl of DNA template, 25 µl of MyTaq™ Mix (Bioline,USA), 1µl of each forward and reverse primers and sterile distilled water. The cyclic conditions for PCR were explained in Table 1. The PCR products were then directly sequenced by FirstBase Sdn. Bhd., Malaysia. Sequences obtained were analyzed using the EzTaxon server [9, 18].

2.5 Polymerase Chain Reaction (PCR) of Virulence Associated Genes

The PCR were performed in a final volume of 25 µL for *collagenase* and 50 µl for outer membrane protein k (*ompK*) and *toxR* genes using thermal cycler (Eppendorf, Germany) as described in Table 1. The sequence of virulence genes were shown in Table 2. For amplication of collagenase gene, the reaction mixtures consisted of 2.5 µl of 10X PCR reaction buffer, 1 µl of each of the four dNTPs, 1.25 u of Taq™ polymerase, 2 µl of DNA template, sterile distilled water and 10 µM of each primer as shown in Table 1. While for other genes, the PCR were carried out using 1 µl of DNA template, 25 µl of MyTaq™ Mix (Bioline, USA), 1µl of each forward and reverse primers and sterile distilled water [22]. PCR products were separated in 1.5% agarose gel at 110 V for 35 minutes. Gel extraction of *ompK* and *toxR* PCR product were purified and done by 1st Base service.

Table 1 Cycling condition for PCR of 16S rRNA and virulence genes of *V. alginolyticus*

Genes	Initial Denaturation	Denaturation	Annealing	Extension	Cycles
16S rRNA	94 ℃ for 3 min	94 ℃ for 1 min	55 ℃ for 1 min	72 ℃ for 1 min	30
Collagenase	95 ℃ for 5 min	94 ℃ for 30 sec	57 ℃ for 30 sec	72 ℃ for 50 sec	34
ToxR	94 ℃ for 4 min	94 ℃ for 1 min	54 ℃ for 1 min	72 ℃ for 1 min	35
OmpK	94 ℃ for 4 min	94 ℃ for 1 min	58.1 ℃ for 1 min	72 ℃ for 1 min	35

Table 2 Primers used for amplication of virulence genes of *V. alginolyticus.*

Target genes	PCR primer sequences (5'-3')	Product sizes	References
Collagenase	VA-f:CGAGTACAGTCACTTGAAAGCC	737 bp	Di pinto *et al.*,
	VA-r: CACAACAGAACTCGCGTTACC		2004,
ToxR	f:GATTAGGAAGCAACGAAAG	658	Xie *et al.*, 2005
	r:GCAATCACTTCCACTGGTAAC		
Ompk	f:GGCGGTCGCTCTGGTATT	319	Cai, *et al.*, 2009
	r:TTGCCATCGTAAGTGCTGTA		

Tox R codes for Toxin R, Omp K codes for outer membrane protein K.

3.0 RESULTS AND DISCUSSION

Twenty one isolates of *V. alginolyticus* were obtained after morphological, VITEK system identification with the range identification between 86 % to 99 % of *V. alginolyticus* before reconfirmation through sequence analysis. After 1.5% agarose gel electrophoresis, result showed that PCR products of *ompK* genes turned out to be well conserved in the strain of *V.alginolyticus* as they could be detected from all samples. Detection of virulence gene using specific primer; *collagenase* showed positive results by amplification of single clear band in lane 3 and 4 (Figure 1).

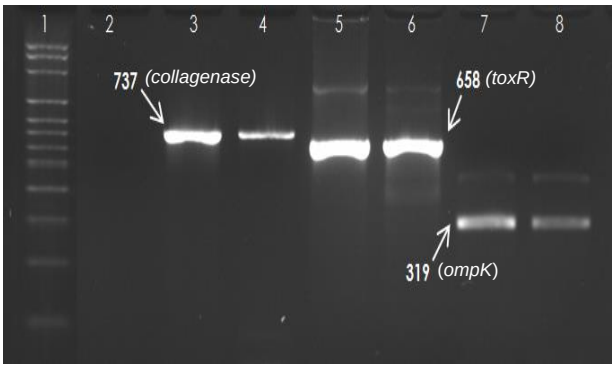

Figure 1 1.5 % agarose electrophoretic profiles. Lane 1: 100 bp DNA hyperladder; Lane 2: Negative control; Lane 3: positive control; Lane 4: Positive sample; Lane 5: Positive control; Lane 6: Positive sample; Lane 7: Positive control; Lane 8: Positive sample

Meanwhile, for detection of *toxR* and *ompK* genes, DNA sequencing analysis confirm the results with high identification (99 % and 98 %). Eventhough there were faint and multiple bands in lane 5, 6, 7 and 8, the bands could not be identified through BLAST analysis.

Qian *et al.* [19] reported that OmpK was highly conserved in *V. alginolyticus*. These results are in agreement with this study based on the detection of *ompK* gene. For 16S rRNA gene, fragment size of 1300 base pairs was produced with 98% identification of *V. alginolyticus.* Thus, this results are within the pairwise similarities range of 97 % and higher as stated by Stackebrandt *et al.* [20, 21].

It had been observed in Table 3 that from all *V. alginolyticus* isolates, 14 strains (67%) possessed all three genes; *collagenase, ompK* and *toxR*; *and* 4 strains (19%) carried two genes; *toxR* and *ompK*. Apart from that, 2 isolates (10%) possessed only *ompK* gene and only 1 isolates (5%) carried both of *ompK* and *collagenase* gene.

Among the virulence genes, all isolates acquire *ompK* followed by *toxR* and *collagenase* genes as confirmed by PCR and subsequence analysis.

Table 3 Percentage of virulence genes presence in *V. alginolyticus*

Virulence Genes	Percentage (%)
toxR* and *ompK	19
ompK	10
collagenase* and *ompK	5
collagenase*, *toxR* and *ompK	67

Furthermore, this result showed that *ompK* gene could be used as gene marker for *Vibrio alginolyticus* compared to *collagenase* [7,12]. Besides, in previous study by Zulkifli *et al*. [24] *toxR* function as regulator for expression of the virulence factor genes in other species of *Vibrio* such as *V. parahaemolyticus.* In this study, 18 strains out of 21 isolates gave the amplification product for the *toxR* gene which suggested that the toxin is able to regulate the expression of *ompK* [5]. In spite of that, secretion of toxins into extracellular milieu is one of the bacterial manipulation mechanism towards the host organism [11]. Thus, role of *ompK* gene in adaptation to the changes of external environment can be related with *toxR* regulation.

Another common virulence gene of *V. alginolyticus* commonly found in green mussel is *collagenase* [10]. Moreover, Miyoshi *et al.* [15] stated *collagenase* plays a role in the wound infection as the enzyme is capable to fasten the bacterial dissemination through the protein components of the

extracellular matrix and cause hemorrhagic tissue damage through destruction of the basement membrane by digestion of type IV collagen. Further effect of *collagenase* towards the tissue can be determined by the bacterial attachment.

V. alginolyticus had been reported to infect other marine organisms such as, oyster, sea horse scallop, white shrimp, fish, silver sea bream, sea mullet, stone crab and prawn [13, 23, 8, 22]. In general, *toxR* and *ompK* was found to cause bacillary necrosis and mortality in larval and juvenile mollusks [3]. In addition, omp was isolated from diseased shrimp, *Penaeus monodon* which indicated that this virulent factor could produce harmful effects to aquatic organism [14].

Based on molecular studies of several proteins in *V. alginolyticus*, the data showed that one strain of several virulence factor could be acquired at a time [4]. Regardless either *ompK*, *toxR* and *collagenase* virulent genes could cause severe clinical illness in the marine organisms. Hence, these particular genes are also useful for disease detection in the aquatic animals. Consumption of mussel that contained this pathogenic bacteria can lead to the risk of human health. Thus, the way of handling the raw seafood and preparation is very vital to avoid from getting any infection from this bacteria. it is recommended that continuous monitoring of potentially pathogenic *V. alginolyticus* to be done intensively in order to reduce the health risk that arised from shellfish consumption.

4.0 CONCLUSION

In conclusion, on the basis of molecular data presented in this study, *V. alginolyticus* is a pathogenic bacteria as they carry several virulence factor that responsible in causing harmful effects to *P. viridis* tissues.

Acknowledgement

This work was supported by the grants from NRGS (VOT 53131) and Ministry of Education (MoE). The authors wish to acknowledge full gratitude to University Malaysia Terengganu (UMT) and Institute of Marine Biotechnology (IMB) for providing facilities to do the lab work.

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