Journal of Nutrition & Food Sciences
ISSN: 2155-9600
Gargouti et al., J Nutr Food Sci 2015, 5:5
DOI: 10.4172/2155-9600.1000405
Research Article Open Access

Detection of *tdh* and *trh* Toxic Genes in *Vibrio Alginolyticus* Strain from Mantis Shrimp (*Oratosquilla Oratoria*)

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Abstract

Vibrio alginolyticus is a halophilic Gram-negative marine bacterium. The current study investigated the presence of the *tdh* and *trh* virulence factors in isolates of *Vibrio alginolyticus.* Five samples of Mantis Shrimp (*Oratosquilla oratoria*) were collected from local markets around Selangor, Malaysia. Samples were then examined using the MPN and multiplex PCR methods. By targeting the species-specific gene *toxR,* 2 (40%) were found to be positive. The two samples were positive for *Vibrio alginolyticus*, and one of these samples contained *tdh/trh* toxin. The *Vibrio alginolyticus* isolated, possessed and expressed the *trh*, *tdh* and *toxR* toxic genes. *Vibrio* strain identification was determined by *16S rRNA* sequence; the results showed 99% homology to *Vibrio alginolyticus*. The sequencing of the isolated toxin genes indicated a homology of 97-98% to the *Vibrio parahaemolyticus* toxin genes. To our knowledge, this is the first report of *Vibrio alginolyticus* possessing three toxin genes of *Vibrio parahaemolyticus*.

Keywords: *Vibrio alginolyticus;* Multiplex PCR; Toxin genes

Introduction

Vibrio alginolyticus is a Gram-negative halophilic bacterium, widely spread geographically in marine and estuarine waters [1]. *Vibrio alginolyticus* is recognized as a causative agent of gastroenteritis, wound infection and septicemia inhuman, and skin ulcer in marine animals [2].

Vibrio species express a variety of hemolysis toxins. Some of these toxins are very similar, however, not necessarily identical [3]. Many studies have reported *Vibrio alginolyticus* strains carrying virulence genes derived from other *Vibrio* species. In the United States (USA), during a 2004 investigation of a *Vibrio parahaemolyticus* outbreak, researchers isolated *Vibrioalginolyticus* that possessed and expressed a *trh* gene with 98% homology to the *trh2* gene of *Vibrio parahaemolyticus* [4]. In Morocco, researchers isolated *Vibrio alginolyticus* carrying a *trh* gene [5]. *Vibrio alginolyticus* can be a reservoir of many virulence genes of other *Vibrio species* in the marine environment [6]. The *tdh* and *trh* are considered the two major pathogenic virulence factors of *Vibrio parahaemolyticus* [7]. In order to differentiate between pathogenic and non-pathogenic strains, *Vibrio parahaemolyticus* were examined for the presence of *tdh* and/or *trh* genes [7]. In this study, the virulence genes of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* in shrimps were examined, targeting the specific *toxR* genes considered to be the global regulator of *Vibrio* species.

Materials and Methods

Sampling

Five samples of shrimp were purchased locally from hypermarket located in Selangor, Malaysia. All samples were transferred to sterile plastic bags for transportation and were processed and tested on the same day.

Most probable number method (MPN)

A quantity of 10 g of shrimp was mixed with 90 mL of Alkaline Peptone Water (APW) in a sterilized stomacher bag, and homogenized for one minute using a stomacher (Inter-science, France), as described in the U.S. Food and Drug Administration, Bacterial Analytical Manual (BAM) [8]. The sample was determined by the three-tube MPN method with slight modifications as described previously [9]. Briefly, a 10-fold serial dilution was applied by adding 1 ml of the homogenate to 9 ml of APW. A 1 ml of dilution was transferred into sterile centrifuge tubes; each tube contained 9 ml of APW with final dilution samples of 1:10 to 1:10000 in triplicates. The tubes were incubated at 37°C for 18-24 h, followed by plating of each dilution on the CHROMTM *Vibrio* agar. The total number of *Vibrio* was determined by PCR method.

DNA preparation

The DNA extraction was carried out using the boiling cell method [10]. Briefly, one milliliter of inoculated APW was incubated at 37°C for 18-24 h, and centrifuged at 10,000 RPM for 3 minutes. The supernatant was discarded, and 200 µl of sterile distilled water was added to the pellet and mixed gently using a vortex mixer. The resulting mixture was then boiled in a dry bath for 15 minutes followed by a centrifuge run at 10,000 RPM for one minute. The supernatant was used for PCR.

Multiplex PCR assay

The sets of primers (Sigma, USA) used are shown in Table 1; the primer sets have been reported to be effective in detecting *Vibrio parahaemolyticus* toxin genes [11,12]. *Vibrio parahaemolyticus* (ATCC17802) was used as a reference strain in the current study [13]. A total PCR mixture reaction of 25 µL was used following the protocol.

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Received August 07, 2015; **Accepted** August 18, 2015; **Published** August 21, 2015

Citation: Gargouti AS, Ab-Rashid MNK, Ghazali MF, Mitsuaki N, Haresh KK, et al. (2015) Detection of *tdh* and *trh* Toxic Genes in *Vibrio Alginolyticus* Strain from Mantis Shrimp (*Oratosquilla Oratoria*). J Nutr Food Sci 5: 405. doi:10.4172/2155-9600.1000405

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Gene	Primers	Band	Reference
toxR	F 5'-GTCTTCTGACGCAATCGTTG-3'	368 bp	-9
	R 5'-ATACGAGTGGTTGCTCATG-3'		
trh	F 5'-TTGGCTTCGATATTTTCAGTATCT-3'	484 bp	-3
	R 5'-CATAACAAACATATGCCCATTTCCG-3'		
tdh	F 5'-CCACTACCACTCTCATATGC-3'	251 bp	-16
	R 5'-GGTACTAAATGGCTGACATC-3'		
16S rRNA	1492 R 5'-TACGGYTACCTTGTTACGACTT- 3'	1485 bp	(13, 15)
	27 F 5'-AGAGTTTGATCMTGGCTCAG-3'		

Table 1: Oligonucleotide primers used in PCR reaction and 16S rRNA sequencing.

The reaction mixture was applied with the following cycles: pre-denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 sec, then annealing at 60°C for 45 sec, and extension at 68°C for 1 min. The final extension was at 72°C for 3 min. The PCR products were separated by agarose gel electrophoresis stained with ethidium bromide, and were visualized and photographed with a UV trans illuminator (SYNGENE, USA). A 100 bp DNA ladder was used as a marker (PROMEGA, USA).

Identification of Vibrio alginolyticus

The amplified PCR products were sequenced by using full-length 16S ribosomal RNA sequences in order to identify the isolated *Vibrio* strain. The primers used are as shown in Table 1. Sequencing was carried out by First Base Asia Laboratory, Selangor, Malaysia.

Results

Isolation and enumeration of total Vibrio

A total of 2 (40%) out of 3 samples were found to be positive. The thermostable Direct Hemolysin toxin (TDH) is encoded by the *tdh* gene whereas the TDH-Related Hemolysin toxin (TRH) is encoded by the *trh* gene. One sample of the *Vibrio alginolyticus* carried the *tdh*/*trh* toxin, and the two samples carried toxR toxin gene. The colour of the colonies on CHROM TM *Vibrio* agar was colour less to creamy and light mauve, indicating the existence of a mixed culture. In the first plating, more than 90% of the colonies were colour less to creamy colour, indicating the presence of *Vibrio alginolyticus*. However, a few colonies were of light mauve colour, indicating the presence of *Vibrio parahaemolyticus*. Purification was carried out by sub culturing twice. Furthermore, the enrichment was tested on an alternative agar to confirm the results, through plating on TCBS agar at 37C for 18 h. The colonies were largely yellow in colour, indicating the presence of *Vibrio alginolyticus*. The two samples were isolated and saved in glycerol under -20°C, for further experiment.

Multiplex PCR

The agarose gel electrophoresis of PCR products determined the presence of *trh*, *tdh* and *toxR* genes at bands 484 bp, 251 bp and 368 bp respectively (Figure 1). The results clearly indicate the presence of virulence toxins (*trh* and *tdh*) and a regulator toxin (*toxR*). The PCR method was repeated five times to optimize the reaction conditions. According to the culture morphology and PCR results, it was assumed that *Vibrio alginolyticus* possessed the toxin genes of *Vibrio parahaemolyticus*. In order to identify the *Vibrio* species, we tested a DNA template for both samples by using 16S rRNA 1.5Kb full length sequencing. The sequencing reaction was carried out by the First Base Laboratory, Malaysia. Universal Primers (First Base Laboratories Sdn Bhd) were used as shown in Table 1. At the same time, the PCR products were tested with DNA sequencing to identify the toxin genes. The results revealed a 98% homology to the *tdh* gene, a 99% homology to the *toxR* gene, and a 98% homology to the *trh* gene of *Vibrio parahaemolyticus*. The *trh* toxin gene which was isolated showed 98% homology to *Vibrio* strain Vp 93A-5807 (accession number DQ359748). The *Vibrio alginolyticus* reported possessed homologues of the virulence gene *trh1* [5].

Identification of *Vibrio alginolyticus*

According to NCBI blast sequence analysis, the *Vibrio* strain was confirmed and identified by 16S rRNA sequences to be 99% homologically identical to the *Vibrio alginolyticus* strain ATCC17749 16S ribosomal RNA gene, partial sequence as shown in Figure 2.

Discussion

This study was carried out with the main goal of quantifying pathogenic and non-pathogenic *Vibrio* toxin factors in fresh shrimps in Selangor, Malaysia. Three toxin genes were determined, which were isolated from live shrimp samples (directly from the tank). When plated on CHROMTM *Vibrio* agar and incubated overnight at 37°C, it was found that 90% of colonies were white in colour, related to the presence of *Vibrio alginolyticus,* and a few colonies were light mauve in colour which indicated the presence of *Vibrio parahaemolyticus*. The results of multiplex PCR confirmed the presence of toxin genes. *Vibrio alginolyticus* and *Vibrio parahaemolyticus* are considered as causative organisms of gastrointestinal disease and are both responsible for substantial financial damage in the aquaculture industry [14,2]. *Vibrio*

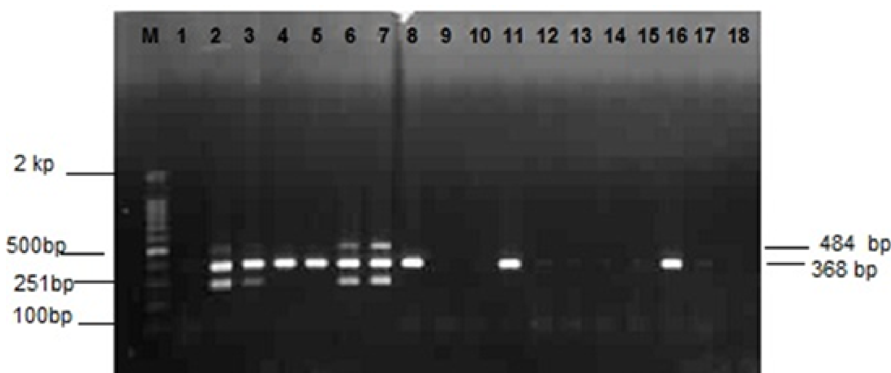

Figure 1: Agarose gel electrophoresis of PCR products, lane M=DNA ladder, lane 1=negative control, lane 2=positive control (Vp. ATCC17802), lane 9, 10, 12, 13, 14, 15, 17, 18=negative results, lane 3, 4, 5, 6, 7, 8, 11, 16=*toxR* positive at band 368bp, lane 6, 7=*tdh* & *trh* positive at bands 251bp and 484bp, respectively.

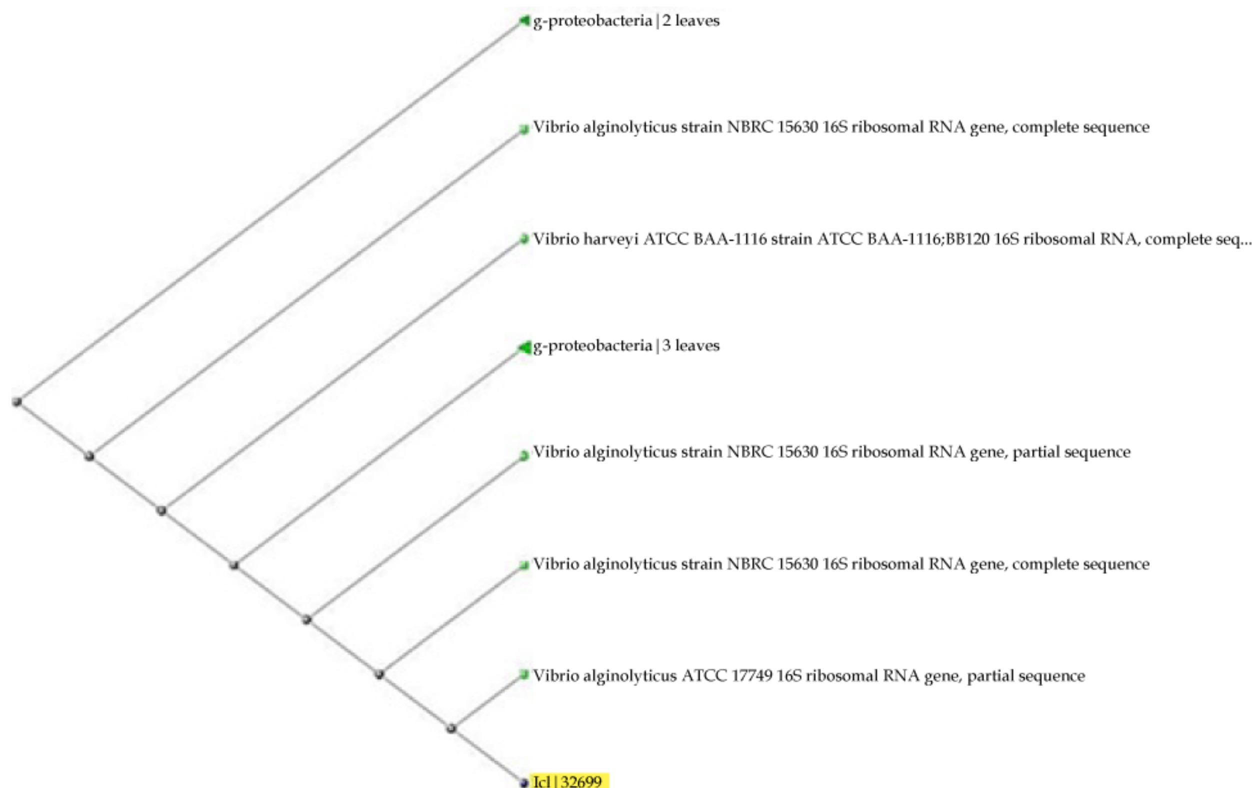

Figure 2: Phylogenetic tree-neighbor joining of 16S rRNA sequencing result, ID number (lcl32699) was generated automatically by the NCBI blast tree method.

alginolyticus was found to have a high similarity to *Vibrio* species [2]. There are many reports that *Vibrio* species possess and express *Vibrio parahaemolyticus* toxin genes, such as *V. hollisae* which has been reported to be capable of producing TDH toxin [9]. In addition, *Vibrio alginolyticus* might act as a reservoir of virulence factors from other *Vibrio* species [15]. In relation to this hypothesis, researchers involved in the investigations of an outbreak in the USA in 2004 isolated *Vibrio alginolyticus* expression *trh 2* toxin gene [5]. Under starvation, *Vibrio alginolyticus* and *Vibrio parahaemolyticus* were reported to exhibit morphological changes and changes in expression of their toxins [16]. Reports of isolating *Vibrio alginolyticus* carrying *trh* toxic gene have been increasing in frequency. The first few reports that identified the *trh* gene in *Vibrio alginolyticus* occurred in Alaska [5] and Tunisia [17]. In addition, a strain of *Vibrio alginolyticus* carrying the *trh* gene has also been reported in Morocco [18] is this all upper case or it starts with an upper case-please standardize!. In this study, *Vibrio alginolyticus* possessed and expressed the main three pathogenic and non-pathogenic genes (*trh*, *tdh* and *toxR*) of *Vibrio parahaemolyticus*, isolated from their shrimp sample and this supports our hypothesis mentioned above. However, the detection of these virulence genes, or one of them, in a mixed culture does not always imply that pathogenic *Vibrio parahaemolyticus* is present [5]. In conclusion, increasing number of reports from various locations around the world on *Vibrio alginolyticus* expression and possession of virulence genes suggest that this organism might be a reservoir for these genes. To our knowledge, this is the first report indicating that *Vibrio alginolyticus* possessed and expressed *tdh* and *trh* toxin genes of *Vibrio parahaemolyticus* in an environmental sample. This phenomenon needs further investigation by researchers [19]. Nucleotide sequence accession numbers. The gene toxin sequences have been deposited in GenBank under accession numbers as following:

- *toxR* gene sequence accession number **KP146109**
- *trh* gene sequence accession number **KP146110**
- *tdh* gene sequence accession number **KP146112**
- *toxR* gene sequence accession number **KP146111**

The *toxR* gene, with accession number KP146111, isolated from other samples of *Vibrio alginolyticus,* was positive to *toxR* gene toxin and negative to *tdh* and *trh* gene toxins [20,21].

Acknowledgments

This study was funded by the Government of Libya, and laboratory research was funded by the E-Science fund from the Ministry of Science, Technology and Innovation, Malaysia (E-Science 5450683) and the RP 026/2012F grant (under sub-program food security and safety), Asia Africa Development, University of Malaya, Kuala Lumpur and, in part, by the Kakenhi Grant-in-Aid for Scientific Research (KAKENHI 24249038), Japan Society for the Promotion of Sciences and Grant-in-aid of the Ministry of Health, Labour and Welfare, Japan.

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