クルマエビ類で見つかった病原微生物Vibrio nigripulchritudoの性状解析およびゲノム解析

[課程博士・論文博士共通]

博士学位論文内容要旨
Abstract

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論文題目 Title	Identification and genomic characterization of a bacterial pathogen *Vibrio nigripulchritudo* isolated from Penaeid shrimps		

In shrimp farming industry, the occurrence of bacterial and viral diseases has a severe environmental and economic significance. Substantial risks are found in amplification of the existing pathogenic strains and spreading of the virulence to commensal bacteria with the intensification of culture systems. Therefore, to control and mitigate the impact of the shrimp diseases, viral and bacterial agents should be mainly concerned. Vibriosis is one of the major bacterial diseases occurred in Penaeid shrimp creating spectacular economic losses in the industry. Vibriosis is caused by several *Vibrio* sp. which are categorized as opportunistic pathogens in shrimp farming environments.

Vibrio nigripulchritudo belonged to family Vibrionaceae is a halophilic gram negative, oxidative positive and O/129 sensitive bacteria. The first isolation of *V. nigripulchritudo* was performed in New Caledonia in 1995 from diseased white leg shrimp *Penaeus stylirostris* affected by winter vibriosis syndrome followed by the second isolation in 1997 causing summer syndrome in *P. stylirostris*. First reported event of mass mortality of Kuruma shrimp *Penaeus japonicus* due to *V. nigripulchritudo* infection in Japan was occurred in 2005. Summer Syndrome caused by *V. nigripulchritudo* is occurred with high temperature ranging from 20 -30 °C implicating that the severity of the disease is enhanced when the water temperature is high. Hence, this disease must be seriously addressed in present along with the global warming.

We isolated the *Vibrio nigripulchritudo* from a mass mortality event occurred in a closed marine aquarium in the Tokyo University of Marine Science and Technology, Tokyo, Japan in 2018. For the conventional therapy of the vibriosis disease and for proper epidemiological characterization, rapid, sensitive, and specific identification and characterization of the causative organism is required. The use of proper culture medium for presumptive identification, biochemical identification, serological identification using polyclonal antibodies and identification using molecular methods can be applicable for the identification of *V. nigripulchritudo.*

For the identification of *Vibrio* sp. having similar phenotypical and biochemical properties, use of polyclonal antibodies prepared against sodium azide- killed cells can be applied as a rapid and specific method due to their high specificity. Also, 16S rRNA gene sequence is widely used as a common housekeeping genetic marker to study the bacterial phylogeny and taxonomy. Therefore, in the presents study, molecular diagnostic methods including PCR amplification with 16S rRNA and hemolysin primers along with the Sanger sequencing and the use of polyclonal antibodies for agglutination test were used for the identification of the causative agent of the bacteria. Simultaneously, standard challenge tests were conducted to determine the pathogenicity of the isolated bacterial strains for kuruma shrimp *P. japonicus* through injection and infection challenge models.

The results of the agglutination test showed that all the isolated bacterial strains have shown the positive result with the antiserum produced by using FKC- VN fed kuruma shrimp *P. japonicus* forming coagulants. Therefore, these results indicate that the isolated bacterial strains are *V. nigripulchritudo* based on the antiserum positivity. After the obtained sequences were applied for homology search using the nucleotide Basic Local Alignment Search Tool, the results showed that all the isolated strains of the bacteria are having the highest homology with the available reference genomes of *V. nigripulchritudo* in the GenBank database based on both 16S rRNA and hemolysin primers. The isolated strain TUMSAT_H_18 was identified as *V. nigripulchritudo* which shows

99.87% of percentage identity with *Vibrio nigripulchritudo* strain F77028 in 16S ribosomal RNA gene (Gene bank accession number: JF281755.1). The pairwise alignment view shows that the isolated TUMSAT_H_18 strain was aligned with F77028 16S ribosomal RNA gene with a coverage of 99.9% with no mismatches. Also, all the other isolated bacterial strains (TUMSAT_R_9, TUMSAT_R_20, and TUMSAT_R_56) from the diseased white leg shrimps were identified and confirmed as *V. nigripulchritudo* based on PCR diagnosis using 16S rRNA and hemolysin primers. Concurrently, the results of the challenge tests showed that the TUMSAT_H_18 was pathogenically virulent for Kuruma shrimp *P. japonicus* in both immersion and injection infection.

With the development of technological advances over the last two decades, a remarkable rising of DNA sequencing technology has contributed to the new generation of sequencing methods which were targeted to complement and replace the Sanger sequencing eventually. The sequencing techniques are used to compare the gene content, genomic organization, and gene expression within species of multiple strains. Therefore, the comparative study of closely related genomes improves our understanding about the evolutionary processes involved in the emergence of new infectious diseases.

There are very few scientific publications in the literature on genomic characterization and identification of *V. nigripulchritudo.* Therefore, it is a critical need to study the whole genome sequence of the isolated strains of *V. nigripulchritudo* to fill this information gap while providing a proper knowledge on the virulence genes responsible for their pathogenicity. We performed the whole genomes sequencing to identify the genomic characterization of the isolated bacterial strain of *V. nigripulchritudo* (TUMSAT_H-18, TUMSAT_R_9, TUMSAT_R_20, and TUMSAT_R_56) using the Illumina Miseq sequencing for short read assembly and nanopore sequencing for long read assembly. After making the hybrid genomic assembly from the short reads and long reads, the genome of the strains was annotated using RAST and Prokka to gain insight into the genomic features and pathogenicity of isolated *V. nigripulchritudo.* The virulence factors were identified using VFDB.

The sequencing results showed that the total genome length is 6749370 bp, 6338956 bp, 6402855 bp, and 6346147 bp in TUMSAT_H-18, TUMSAT_R_9, TUMSAT_R_20, and TUMSAT_R_56 respectively while the percentage GC content is in the range of 45.60 – 45.86%. After annotating of the genome sequences, it has been found that many of the stains possess T6SS secretory system, Phosphonate ABC transporter, Multidrug Resistance Efflux Pumps etc., which may be responsible for its virulence.

Compared to the other bacterial pathogens belonging to family Vibrionaceae, the whole genome sequence and annotation of *V. nigripulchritudo* is yet to be done to fulfill the breakthrough of *V. nigripulchritudo* infection research. This study presents the first whole genome sequencing of *V. nigripulchritudo* making hybrid assemblies by Illumina and nanopore sequencing techniques. Therefore, the study of genes and their functions, host-pathogen interactions and comparative genomics with other related species would allow a complete understanding on pathogenicity and virulence of *V. nigripulchritudo.* Concurrently, the information based on the whole genome characterization of *V. nigripulchritudo* would help to identify the preventive and controlling measures of the vibriosis disease of penaeid shrimps which would help to secure the seafood production.