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Establishment of challenges study system for vibriosis of Japanese tiger prawn (*Marsupenaeus japonicus*) with a pathogenic *Vibrio penaeicida* strain

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Introduction

Vibriosis is the most common bacterial disease in aquaculture farms of prawns in worldwide [1]. In Japan, vibriosis was prevalent from the late 1980s to the early 1990s when cultivation of Japanese tiger prawn (also termed Kuruma prawn) [*Marsupenaeus japonicus*] became intensive [2]. Despite of the efforts to improve culture and disease control system after that, vibriosis has been still reported at present time mainly in south western region in Japan where cultivation of Japanese tiger prawn is prosperous. However, effective ways to protect vibriosis has been limited, since development of traditional vaccines is difficult in crustacean species due to the lacking of adaptive immunity [3].

Here, we reported establishment of challenge study system for the vibriosis of Japanese tiger prawn with a high virulent *Vibrio penaeicida* strain to develop a model system for bacterial disease in crustacean species.

Materials and methods

Experimental animals
Japanese tiger prawns kindly provided by Higashimaru Co., Ltd. (Kagoshima, Japan) and Fisheries Research Center in Ehime Prefectural Research Institute of Agricultures, Forestry and Fisheries (Uwajima, Japan) were maintained in 1 or 2 t tanks with bottom sand and flow through system of sand-filtrated natural sea water. 1-4 g prawns were supplied for this study.

Bacteria and identification
Challenge strain IAYKG13-1, which was derived from lymphoid organ of Japanese tiger prawn, isolated in a farm in Kagoshima prefecture on May 2013, gram negative coccobacillus, aggregated with antiserum against *V. penaeicida* and formed green colony in TCBS ager, was provided in this study. Challenge strain was cultured with marine broth (BD) at 28ºC with vigorous agitation, suspended in marine broth containing 10 % glycerol, then bacterial suspensions were stored in liquid nitrogen until use.

Bacteria, plated on marine broth agar and incubated at 28ºC for more than 1 day, was supplied for polymerase chain reaction (PCR) specific for *Vibrio penaeicida* and 16S rRNA gene sequence.

Vibrio penaeicida specific PCR was performed by colony direct method. Primers VpF (5'- GTGTGAAGT TAATAGCTTCATAT-3') and VR (5'-CGCATCTGAG TGTCAGTATCT-3') for 16S rRNA gene amplify 310 bp product [4]. KOD FX (TOYOBO) was used to prepare reaction mixture. PCR reaction was performed with 20 µL volume in each tube. PCR products were applied for agarose gel electrophoresis and detected by UV transilluminator after immersion in ethidium bromide solution. 16S rRNA gene sequence in challenge strain was read by Sanger method with capillary electrophoresis after amplification of PCR product with a set of universal primers (27F and 1492R) and DNA templates extracted from challenge strain. Identity of approximately 500 bp sequence was searched in NCBI blast.

Challenge study
To prepare challenge inoculums, bacterial stock was aseptically injected into marine broth, then cultured for 2 days at 28ºC with vigorous agitation, then prepared seral dilutions with marine broth. Number of bacteria was back calculated from challenge inoculum plated on marine broth ager. For intramuscular injection, prawns were immersed on sea water gradually cooled down to

10-15°C for anesthesia, then each 0.1 mL of challenge inoculum was intramuscularly injected into third abdominal section of prawns. For natural rout infection, 40 mL of diluted challenge inoculum was added in 5 L sea water, then prawns were immersed at 1 hour. After infection, prawns were cultured on 45 L tanks with bottom filtration system. 5 w/w% of dried pellets per total body weight of prawns were fed once a day. Water temperature was 27-28°C during culture after infection of challenge strain.

Results and discussion

Identification of challenge strain

To identify species, challenge strain was genetically analyzed by *V. penaeicida* specific PCR and 16S rRNA gene sequence analysis. As the results, a single 310 bp band was detected in challenge strain by *V. penaeicida* specific PCR. 16S rDNA sequence of challenge strain showed 99.9% identity with *V. penaeicida* (NR_113790 and NR_042121) by blast search (Table 1). Other hits in blast search were equal or less than 98.2% identity. These genetical analysis data indicated challenge strain is typed as *V. penaeicida*.

Table 1. Top 5 hit sequences by blast search for 16S rRNA gene sequence of challenge strain

Organism	Accession No.	% Identity
Vibrio penaeicida	NR_113790	99.9
Vibrio penaeicida	NR_042121	99.9
Vibrio europaeus	AY792622	98.2
Vibrio nigripulchritudo	NR_117897	98.2
Vibrio tapetis subsp. britannicus	NR_132307	98.2

Challenge study by intramuscular injection and immersion

To confirm pathogenicity of challenge strain, challenge inoculum without dilution was intramuscularly injected. 91.6% prawns injected with challenge strain were died within 48 h. In contrast, all prawns were survived in mock control injected with marine broth. Brown or black tiny spots were often observed in gills and lymphoid organs in dead prawns, indicating typical vibriosis of Japanese tiger prawn.

To confirm effective infectious route and dose, we performed two-injection methods with several dilutions. Results of bacterial counts were 8.3×10^5 cells/dose, 8.3×10^3 cells/dose and 8.3×10^2 cells/dose in intramuscular injection groups and 4.2×10^4 cells/mL, 4.2×10^3 cells/mL, 4.2×10^2 cells/mL in immersion groups. As the results, all prawns turned to be dead within 1-3 days in dose dependent manner in intramuscular injection and within 7 days in immersion groups (Table 2). In immersion group prawns, melanin deposition was evident on body surfaces besides black tiny spots in gills and lymphoid organs. These data indicated challenge strain showed high mortality for Japanese tiger prawns not only through direct injection into muscles in abdominal section, but also through natural route infection by immersion.

Table 2. Number of survived prawns in challenge study

Infectious route	Dose[1)]	Day 0	1	2	3	4	5	6	7
Intramuscular	8.3×10^5	3	0						
	8.3×10^3	3	3	1	0				
	8.3×10^2	3	3	3	0				
Immersion	4.2×10^4	3	3	3	3	3	3	1	0
	4.2×10^3	3	3	3	3	3	3	3	0
	4.2×10^2	3	3	3	2	2	2	2	0

[1)] Unit was cells/dose (0.1mL/prawn) in intramuscular injection and cells/mL in immersion

Conclusions

Infectious disease protection in prawn cultivation has been mainly achieved by the improvement of culture and disease control system. However, it is difficult to completely eliminate bacterial pathogens including *Vibrio* spp. from production farms, since most of them forms normal bacterial flora in the ponds where prawns are cultured. Furthermore, the usage of antimicrobial agents became restricted recently in order to decrease negative impacts on natural environment. Therefore, development of the agents such can activate innate immune system of prawns themselves would be important for the protection of bacterial diseases in prawn cultivation hereafter. Recently, we isolated novel polysaccharides from some insect species, which can stimulate innate immune system of mouse macrophage RAW264 cells [5,6]. Challenge study system established in this study will be served as a model system to investigate efficacy of the immunostimulative agents including insect derived polysaccharides for bacterial disease of crustacean species.

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