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DETECTION OF *VIBRIO PENAEICIDA* FROM APPARENTLY HEALTHY KURUMA PRAWNS BY RT-PCR

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Abstract

An RT-PCR technique, which was developed previously based on a species specific sequence of 16S RRNA of *Vibrio penaeicida,* revealed a higher carrier rate than that by a conventional culture method in apparently healthy kuruma prawns *(Penaeus japonicus).*

Introduction

Vibrio penaeicida (Ishimaru *et al.* 1996), the causative bacterium of vibriosis in cultured kuruma prawn *Penaeus japonicus* in Japan, is ubiquitous in apparently healthy kuruma prawns and in their culture environment (de la Peña *et al.* 1992). The disease possibly occurs when kuruma prawns are stressed and thus diagnosis at carrier stages or earlier stages of infection is most important for prophylaxis of the disease. Our previous study (de la Peña *et al.,* 1995) by a culture method indicates that V. *penaeicida* multiplies in the stomach, hepatopancreas, and lymphoid organs of experimentally infected kuruma prawns. However, detection of *V. penaeicida* is. often difficult due to the lack of a selective culture technique which discriminates *V. penaeicida* from other bacteria residing normally in such organs. We developed a reverse transcription polymerase chain reaction (RT-PCR) based on a species-specific sequence in the 16S rRNA of V. *penaeicida* as a specific and sensitive detection method for V. *penaeicida* (Genmoto *et al.,* 1996). This study demonstrates that the RT-PCR is applicable for detection of the pathogen from apparently healthy kuruma prawns.

Materials and Methods

Extraction methods of bacterial nucleic acids: V. penaeicida strain KH-1 was used to examine the efficiency of extraction methods of the bacterial nucleic acids. The bacteria were grown at 25°C in tryptic soy broth (TSB; Nissui) and the culture (OD 600=0.7) was diluted with TSB to prepare bacterial cells at concentrations of 10^3, 10^2, 10^1, 10^0 and 10^{-1} CFU (colony forming unit) per µ1. A 200 ul of each bacterial suspension was centrifuged at 15,000xg and the resultant pellets were lysed with 200µl of proteinase K (1 mg/ml) and 1% sodium dodecyl sulphate (SDS), 5% Tween 20, or 4.2M guanidine isothiocyanate (GITC). After incubation at 37°C for 15 min or at 60°C for 1 h for GITC, bacterial nucleic acids were extracted by TE buffer (100 mM Tris-HCI, 1 mM EDTA, pH8.0) saturated phenol and chloroform-isoamylalcohol (24:1) mixture. The nucleic acids extracted by SDS or GITC were further precipitated with sodium acetate (pH5.2) and ethanol, and resuspended in DEPC (diethyl pyrocarbonate)-treated water. In another experiment, the extraction of nucleic acids was carried out in the presence of the viscera consisting of stomach, hepatopancreas and intestine, which were freshly prepared from kuruma prawns. A 100 ul of the bacterial suspensions was mixed with a 1 mi of visceral homogenate (100 mg/ml) and then centrifuged at 15,000xg. The total nucleic acids were extracted from the resultant sediment by the above method using Tween 20 or GITC.

RT-PCR amplification of 16S rRNA*:* A primer pair, R-GEN (5'GATTACCAGGG-TATCTAATC-3') as an antisense primer

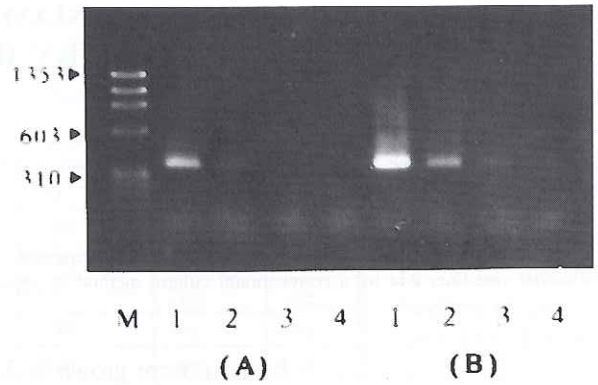

Figure 1. Effects of prawn visceral homogenate on the detection of *V. penaeicida* by RT-PCR. The nucleic acids of *V. penaeicida cells* (KH-1) were extracted in the presence (A) or absence (B) of visceral homogenate by the GITC method. M: length marker (0 X 174/HaeIII digest), 1: 200 CFU, 2: 20 CFU, 3: 2 CFU, 4: 0 CFU/ µ

and P-PJF (5'GTGTGAAGTTAATAGCTTCATATC-3' as a sense primer both developed in the previous study (Genmoto *et al* 1996), was used here for the amplification of a 349 bp rNA fragment from *V. penaeicida.* The cDNAs to the I6S rNA were synthesised in a reaction mixture containing 10 mM Tris-HCl (pH8.3), 50 mM KCI, 5m MgCl, 0.2 mM each dTP, 10 pmol antisense primer, 1U µl RNase inhibitor (Toyobo), and 2.5U µ1 reverse transcriptase (M-MLV, USB) at 42°C for 30 min. PCR amplification of the CDNA was carried out in a 100µ1 reaction mixture containing the extracted bacterial nucleic acids, 10 mM TrisHCI (pH 8.3), 50 mM KCI, 2.5m $MgCl_2$ 0.2 mM each dNTP, 10 pmol each primer and 2.5 U Ampli *Taq* DNA Polymerase (Perkin-Elmer) with an automatic thermal cycler (Astec PC-700). The thermal profile involved 35 cycles of denaturation at 95°C for 60 s, annealing at 62°C for 60 s and extension at 72°C for 60 s. The results of amplification were analysed by 1.5% agarose gel electrophoresis.

Detection of V. penaeicida from kuruma prawn:

A total of 100 cultured kuruma prawns weighing 10-20 g each was used to detect V. *penaeicida.* The prawns were randomly sampled from a private farm in Hiroshima Prefecture in June (N=30), September (N=60) and October (N=10), 1996. Ten prawns per day were sampled and immediately examined as follows. The viscera

Table 1. Detection of *V. penaeicida* from healthy kuruma prawns by culture and RT-PCR methods

Sampling date (1996)	No. of prawns examined	No. of *V. penaeicida* - positive prawns	
		Culture method	RT-PCR method
Jun. 4,5,6	30	0	4
Sep. 17,18,19	30	1	3
Sep. 24,25,26	30	1	1
Oct. 14	10	2	2
Total	100	4	10

containing stomach, hepatopancreas and intestine was homogenised with 10 mM PBS (pH7.2) at a ratio of 0.1g/ml. A 100 µl of the diluted homogenate was inoculated on ZoBell's 2216e agar and incubated at 25°C for 2 d, and then *V. penaeicida-like* colonies were tested by a slide agglutination with an anti-V. *penaeicida* (KH-1) rabbit scrum. The total nucleic acids were extracted from the homogenate by the GITC method and 16S rRNA-targeted RT-PCR was performed according to the method described above.

Results and Discussion

A single RT-PCR product of the predicted size (ca. 350 bp) was amplified in the nucleic acids extracted from bacterial suspension containing 2 to 5 cells per ul, irrespective of the extraction methods. However, the SDS extraction method gave variable results when the test was repeated and therefore the other two extraction methods, Tween 20 and GITC, were used in the following experiments. In addition, as confirmed previously (Genmoto *et al.,* 1996), the higher sensitivity of RT-PCR targeted both rDNA and rRNA compared with PCR targeted rDNA in the detection of *V. penaeicida* was also confirmed in the present study, since PCR needed the nucleic acids extracted from more than 10^2 cells per µl to yield a visible fragment on agarose gel electrophoresis. In the presence of prawn's visceral homogenate, the detection limit of *V. penaeicida* by RT-PCR increased to 10^2 CFU/µl from 10^0 CFU/µl in the absence of the homogenate (Fig. 1).

There were no apparent outbreaks of *V. penaeicida* infection in the farm during the sampling periods. The results of detection of *V. penaeicida* by both culture and RT-PCR methods are shown in Table 1. Out of 100 prawns examined, ten prawns were positive in RT-PCR and *V. penaeicida* was isolated from only four of ten RT-PCR positive prawns. As expected, the prawn viscera contained bacteria at 10^5 to 10^6 CFU per g, from which it was highly difficult to find V. *penaeicida* colony for identification by the antiserum. These results indicate that the present RT-PCR procedure is a useful tool to detect V. *penaeicida* from asymptomatic carrier kuruma prawns.

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