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Phylogenetic Diversity of the Causative Agents of Vibriosis Associated with Groupers Fish from Karimunjawa Islands, Indonesia

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Abstract: A molecular-based study was conducted to estimate the richness of the causative agents of vibriosis associated with groupers from Karimunjawa islands, North Java Sea, Indonesia. Moribound grouper fish were collected from the cage cultures and a total of 32 isolates were isolated from external wound and kidney of groupers. Based on the repetitive sequence-based PCR (rep-PCR) and Koch postulate test, eight isolates were chosen for further sequencings. On the basis of the sequence analysis, the data showed that the causative agents are closely related with *Vibrio natriegen*, *V. oliviceaus*, *V. fortis*, *V. alginolitycus*, *V. harveyi*, *V. parahemolitycus*, *V. damsela* and *V. carchariae*, respectively. Present study highlighted the effectiveness of rep-PCR in rapid grouping and estimating the richness of the causative agents of vibriosis associated with the groupers.

Key words: Rep-PCR, *Vibrio*, causative agents

INTRODUCTION

Groupers are high priced and popular for seafood fishes among the major maricultured fish species in Indonesia and Southeast Asia. However, with the rapid development of grouper aquaculture in the recent years, the marine environment is becoming more and more stressful. The industry faces serious threat owing to a variety of diseases problem, especially caused by vibrosis.

Vibriosis is one of the most serious bacterial diseases affecting in the fish culture (Liao *et al*., 1996) and a major disease problem in mariculture causing high mortality and severe economic loss in many production countries (Goarant *et al*., 1999). Vibriosis has also been recorded in cultured of groupers (Lee, 1995). This disease can cause more than 70% mortality in maricultured grouper in the disease season.

Recently, various molecular methods have been developed for the detection of this pathogen (Urakawa *et al*., 1999a, b) and some studies have also been carried out to determine the causative agent of vibriosis in the grouper from geographically different regions (Lee, 1995; Yii *et al*., 1999), which confirmed the persistent occurrence of vibriosis among groupers.

Molecular based-approaches have been successfully applied, such as RFLP (Restriction Fragment Length Polymorphism) for rapid grouping of vibrios (Urakawa *et al*., 1999a, b; Radjasa *et al*., 2001). Recently, repetitive sequence-based PCR (rep-PCR) has also been used to group a number of bacterial isolates that produced complex fingerprint profiles from gram negative bacteria. However, this

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technique has not been employed to estimate the diversity of the causative agent of vibriosis among groupers. Recently, Radjasa *et al*. (2007b) successfully applied repetitive-PCR for grouping marine psychrotrophic bacteria collected from deep-sea waters of Makasar strait, Indonesia as well as the bacterial symbionts of sponge *Haliclona* sp. (Radjasa *et al*., 2007c).

To best our knowledge, there has no report been documented so far on describing diversity of vibrios, the causative agent of vibriosis associated with groupers from Indonesian marine culture using molecular tools. The research regarding the molecular diversity of vibrios as the causative agents is important for creating health management of groupers culture. In this study, we reported the phylogenetic diversity of vibrios bacteria as a causative agent vibriosis isolated from groupers from Karimunjawa Islands, Indonesia assessed by 16S rDNA approach.

MATERIALS AND METHODS

Sampling of Grouper Fish

The moribund groupers were collected from cage cultures at Karimunjawa Islands, Java Sea, Indonesia (Fig. 1) and identified as tiger grouper (*Epinephelus fuscoguttatus*); humpbeck grouper (*Cromileptus altivelis*) and coral cod (*Plectropomus maculates*). After collection, groupers were put into the containers and immediately brought to the Marine Centre Laboratory of Fisheries and Marine, Science Faculty, Diponegoro University in Jepara, North Java for bacterial isolation.

Bacterial Isolation

Bacteria were isolated directly from the external lesion and kidney of groupers by streak plate on TCBS agar (composition (g L^{-1}): Peptone from casein 5.0; peptone from meat 5.0; yeast extract 5.0; sodium citrate 10.0; sodium thiosulfate 10.0; ox bile 5.0; sodium cholate 3.0; sucrose 20.0; sodium chloride 10.0; iron (III) citrate 1.0; thymol blue 0.04; bromothymol blue 0.04 and agar-agar 14.0).

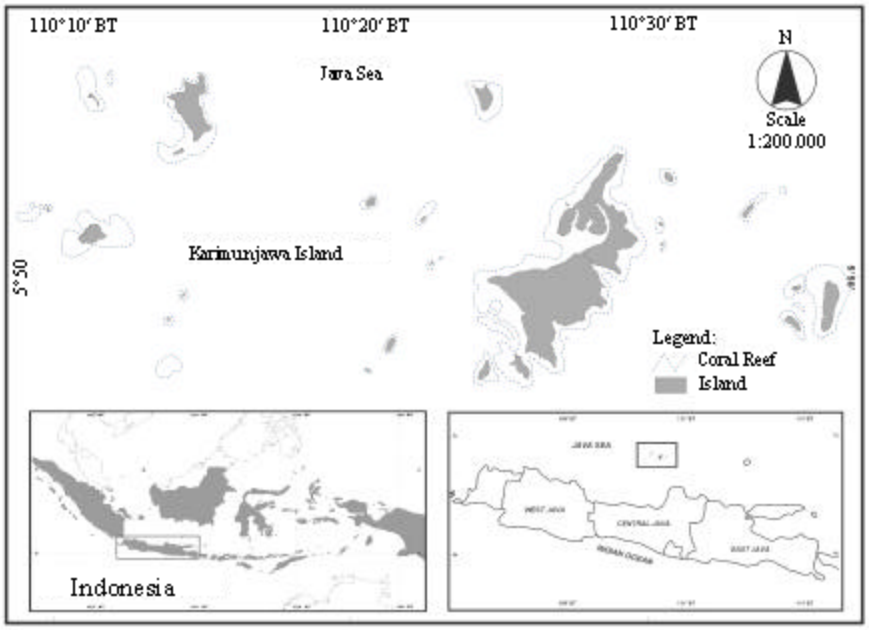

Fig. 1: Sampling site for the collection of grouper fish from Karimunjawa Islands, Java Sea, Indonesia

Bacterial isolation was also conducted from the inner part of kidney and part of tissue lesions, which were scraped off with a sterile knife. The resultant tissues were serially diluted, spread on TCBS agar medium and were incubated at room temperature for 24-48 h. On the basis of morphological features, colonies were randomly picked and purified by making streak plates (Madigan *et al*., 2000).

Repetitive-PCR

The procedure was carried out according to a method previously described by Radjasa *et al*. (2007b). In the rep-PCR, BOX A1R (5'-CTACggCAAggCgACgCTgACg-3') (Versalovic *et al*., 1994) was used. The REP 1R-I and REP 2-I primers contain the nucleotide inosine (I) at ambiguous positions in the REP consensus. PCR reaction contained of 1 μL DNA template (diluted 100x), 1 μL primer, 7.5 μL Megamix Royal dan sterile water up to total volume of 15 μL.

Amplifications were performed with a thermal cycler model Gene Amp PCR System 9700 with the following temperature conditions: initial denaturation at 95°C for 5 min; 30 cycles of denaturation (92°C for 1 min), annealing (50°C for 1, 5 min), extension (68°C for 8 min) and final extension at 68°C for 10 min. Five microliter aliquot PCR products were run using electrophoresis on 1% ethibium bromade gel by using 1X TBE buffer.

Grouping of Isolates

Grouping was carried out according to a method of Radjasa *et al*. (2007c) by making matrixes from the positions of bands on the gel which were then analyzed by using Free Tree program by using UPGMA method for constructing the tree. Resampling was performed by bootstrapping with 1000 replications.

PCR Amplification and Sequencing of 16s rRNA Gene Fragments

PCR amplification was carried out according to method of Radjasa *et al*. (2007a). Two primers, GM3F (5'-AGAGTTTGATCMTGGC-3') and GM4R (5'-TACCTTGTTACGACTT-3') were used to amplify nearly complete 16S rRNA gene (Muyzer *et al*., 1995). Genomic DNA of causative agent of vibriosis-strains for PCR analysis were obtained from cell materials taken from an agar plate, suspended in sterile water (Sigma, Germany) and subjected to five cycles of freeze (-80°C) and thaw (95°C). PCR amplification of partial 16S rRNA gene of bacteria, purification of PCR products and subsequent sequencing analysis were performed according to the method of Radjasa *et al*. (2007b). The determined DNA sequences of strains were then compared for homology to the BLAST database.

Koch Postulate Test

Between two-three isolates representing each branch in the dendogram constructed from the result of rep-PCR analysis were used in the test against healthy groupers (45 days old with an average of 7 cm in length).

Test was performed in 10 L aquariums, in which each aquarium was filled with 9 L filtered seawater and 5 groupers, respectively and was acclimated for 7 days before the injection. Aliquots of 0.2 mL culture of each causative agent in the logarithmic growth phase (ca. 10^9 cells mL^{-1}), was injected in intraperitonial of tiger grouper (*Epinephelus fuscoguttatus)*. Control was carried out on tiger grouper with an injection of 0.2 mL saline phosphate buffer. Mortality of groupers was observed in time series of 6, 9, 12, 15, 18, 21, 24, 30, 39 and 54 h incubation on each aquarium.

RESULTS

Characteristics of the Bacterial Isolates

The clinical features of moribund groupers affected by vibriosis from Karimunjawa Islands, Indonesia were dark skin, pale gill, haemorhagic area near mouth and the base of the fins, haemorhage

Table 1: Characteristics of isolates associated with groupers from Karimunjawa Islands, Indonesia

Groups	Isolate sources	Colony color on TCBS	Colony form	Isolat code
C. altivelis	Red mouth (septicemia)	White with yellow outer ring	Round	JT-04
C. altivelis	Red mouth	Yellow	Round	JT-03
C. altivelis	Red mouth	Green	Round	JT-26
C. altivelis	Kidney	Yellow with black outer ring	Round	JT-20
C. altivelis	Kidney	Black	Round	JT-29
C. altivelis	Kidney	Yellow	Round	JT-10
C. altivelis	Kidney	Green	Round	JT-07
C. altivelis	Kidney	Green	Round	JT-24
C. altivelis	Kidney	Yellow	Round	JT-09
C. altivelis	External lesion	Black	Round	JT-30
C. altivelis	External lesion	Yellow	Round	JT-11
C. altivelis	Kidney	Green	Round	JT-25
C. altivelis	Kidney	Dark yellow	Round	JT-02
C. altivelis	External lesion	White	Round	JT-21
C. altivelis	External lesion	Yellow	Round	JT-01
C. altivelis	External lesion	Black	Round	JT-31
C. altivelis	External lesion	Black	Round	JT-32
C. altivelis	External lesion	White	Round	JT-22
C. altivelis	External lesion	Yellow	Round	JT-12
C. altivelis	External lesion	Yellow	Round	JT-13
E. fuscoguttatus	External lesion	Yellow	Round	JT-14
E. fuscoguttatus	External lesion	Green	Round	JT-27
E. fuscoguttatus	External lesion	White	Round	JT-08
E. fuscoguttatus	Kidney	Yellow	Round	JT-15
E. fuscoguttatus	Kidney	White	Round	JT-23
E. fuscoguttatus	Kidney	Green	Round	JT-28
P. maculates	External lession	Yellow	Round	JT-16
P. maculates	Kidney	Yellow	Round	JT-18
P. maculates	External lesion	Black	Round	JT-05
P. maculates	External lesion	Yellow	Round	JT-17
P. maculates	Kidney	Yellow	Round	JT-19
P. maculates	Kidney	Black	Round	JT-06

in the kidney, liver and intestinal wall, ascites in the body cavity, yellow wish-bloody fluid; empty stomach, skin lesion and ulcer in the surface skin.

Bacterial isolation resulted in a total of 32 vibrio isolates (JT01-JT32) obtained from both of skin lesion and ulcer of surface skin (external lesion) as well as from kidney of moribund groupers (Table 1).

Repetitive-PCR Analysis

Based on the repetitive-PCR result and constructed dendogram of the causative agents of the groupers, eight groups were formed. Eight different isolates representing different groups (Fig. 2) as well as the result of confirmation as the causative agent using Koch Postulate, were further selected for DNA sequencing

Sequencing of Representative Causative Agents

Based on molecular characterization it is shown that all isolates are the members of the genus *Vibrio* as presented in the Table 2.

Phylogenetic Analysis

A comparison of the 16S rRNA gene sequence of representative strains of JT 02; JT 07: JT 10; JT 13; JT 20; JT 24; JT 27 and JT 31 with sequences from GenBank demonstrated that these isolates are affiliated with vibrionales within the family Vibrionacea (Fig. 3) with a homology in the range of 97-100%, respectively.

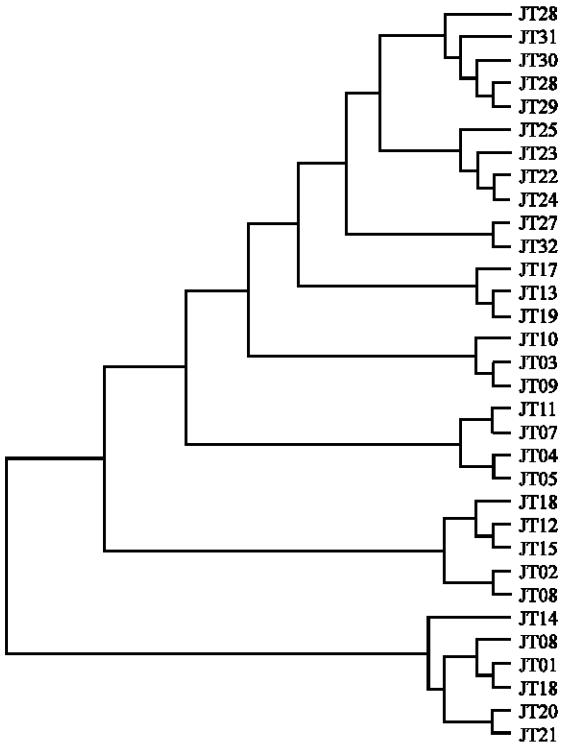

Fig. 2: Dendrogram of bacterial isolates associated with groupers (JT.02, JT 07; JT 10, JT 13, JT 20, JT 27 and JT 31) were selected for further DNA sequencings

Table 2: Molecular characterization of 8 representatives of the causative agents

Isolates	Closest relative	Homology (%)	Acc. No.
JT 02	*Vibrio natriegens*	99	AJ 874352
JT 07	*Vibrio olivaceus*	98	AY827859
JT 10	*Vibrio damsela*	99	AY827492
JT 13	*Vibrio fortis*	97	AJ514914
JT 20	*Vibrio alginolitycus*	99	AY332566
JT 24	*Vibrio harveyi*	100	DQ146936
JT 27	*Vibrio parahaemolitycus*	98	EF 467290
JT 31	*Vibrio carchariae*	99	AF134581

Table 3: Pathogenicity test of the causative agents of vibriosis in groupers

	Mortality of grouper at the different time of incubation (h)										
Isolates	6	9	12	15	18	21	24	30	39	54	Percentage
JT-2	3	2	0	0	0	0	0	0	0	0	100
JT-7	4	1	0	0	0	0	0	0	0	0	100
JT-10	0	0	1	3	0	0	1	0	0	0	100
JT-13	0	1	1	1	0	0	0	0	0	0	60
JT-20	0	3	2	0	0	0	0	0	0	0	100
JT-24	0	3	2	0	0	0	0	0	0	0	100
JT-27	0	1	2	0	0	0	0	0	0	0	60
JT-31	0	0	2	0	2	0	0	0	0		80

No mortality was observed in the control

Koch Postulate Test

Based on the result of the test, it is indicated that eight isolates were confirmed as the main causative agents of vibrisosis of groupers from Karimunjawa Islands, Indonesia (Table 3).

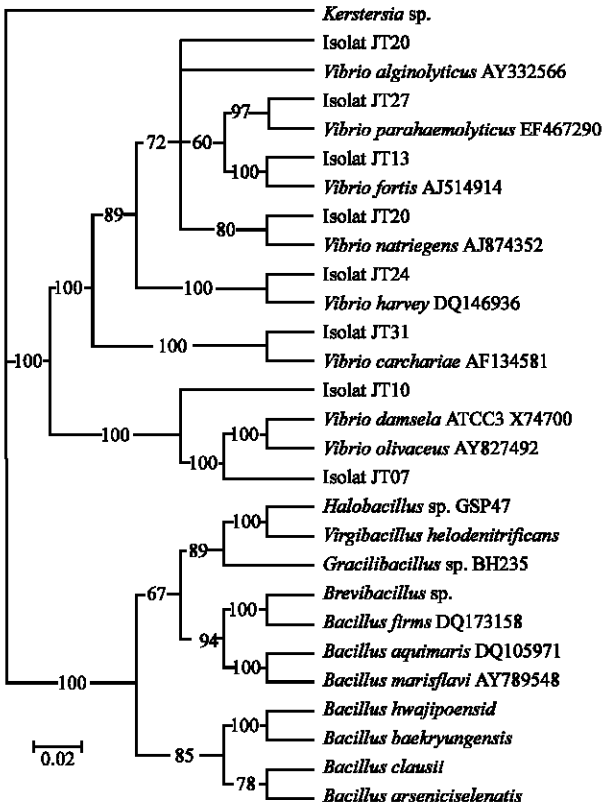

Fig. 3: Phylogenetic tree based on comparative of partial 16S rRNA gene sequence analysis of *Vibrio* species showing the phylogenetic affiliation of strain JT 02 ; JT 07; JT 10; JT 13; JT 20; JT 24; JT 27 and JT 31. Selected sequences from the alpha subclass of *Kertersia* were used to root the tree. The bar indicates 2% sequence divergence

The causative agents of vibriosis from groupers positively confirmed based on the test, in which eight isolates representing different branches were found to cause mortality on groupers in the range of 60-100% (Table 2).

DISCUSSION

We investigated marine bacteria isolated from the groupers of Karimunjawa Islands, Indonesia. The attention was focused on the occurrence of the causative agents followed by PCR-based approach, i.e., rep-PCR for estimating the richness of the causative agents of vibriosis associated with the groupers.

Understanding the causative agents of vibriosis has important implications for analysis of microbial taxonomic studies and identification, in particular for strain differentiation among the causative agents of vibriosis are essential for epidemiology and pathogenicty.

In the field of marine diseases, it is very important to identify bacterial species accurately. In addition, Somarny *et al*. (2002) mentioned that phenotypic identification of *Vibrio* sp. relies on time-consuming techniques such as studies on morphology, nutrient requirement, antibiotic resistance, enzyme comparison that have limited discriminatory powers.

Generally, the term vibriosis at grouper are described as *Vibrio alginolyticus*, *V. parahaemolyticus*, *Vibrio* sp. (Lee, 1995) and *V. charchariae* (Yii *et al*., 1999). Other members of the genus *Vibrio* are *V. anguillarum*, *V. ordalii*, *V. salmonicida*, *V. vulnificus* and *V. harveyi* that were also isolated from disease of fish.

Vibrio species are natural habitants of seawater and brackish water and widely distributed throughout the world. However, some species have exhibited clinical significance for aquatic animals and are recognized as potential pathogens. The large number of vibrios fish pathogens causing epizootic outbreaks in aquaculture has made it necessary to develop efficient, fast and sensitive methods for their detection. Both detection and identification of vibrios have been traditionally depended on their growth on the Thio-Sulphate Citrate Bile Salt Sucrose selective medium and subsequent characterization by biochemical tests (Diggles *et al.*, 2000).

Vibriosis was characterized by dark skin, pale gill, exopthalmia, haemorhagic area near mouth and the base of the fins, corneal opacity and ulcers on the skin surface. Internally moribund fish also showed anemia, haemorhage in the abdominal fat, kidney and liver full of yellow-wish bloody fluid (Yii *et al*., 1999). The results of the present study were similar to clinical sign findings reported as shown in the Table 1. However, the corneal cavacity and exopthalmia in the moribund groupers was not detected in this study.

The results of this study revealed that the application of rep-PCR has been a reliable tool for strain rapid grouping and differentiation among the causative agents of vibriosis in groupers from Karimunjawa Islands (Fig. 2, 3). This molecular approach may be used for the analysis of other *Vibrio* species related to mariculture diseases.

Molecular identification of the causative agents of vibriosis (Table 2, Fig. 3) shows that the identified strains were nicely in accordance with the dendrogram constructed from the rep-PCR analysis. The identified strains include the species of *V. natriegens*, *V. olivaceus*, *V. damsela*, *V. fortis*, *V. alginolitycus*, *V. harveyi*, *V. parahaemolyticus* and *V. carchariae* (Table 2). The diverse species reported in this study has further confirmed the role of *Vibrio* as the dominant genus responsible for much of the observed mortality among maricultures (Jayaprakash *et al*., 2005).

In conclusion, the application of the rep-PCR method is useful for rapid grouping and estimation of the phylogenetic diversity of the causative agents of vibriosis from groupers with high discriminating power and offers an alternative technique for grouping of numerous of marine bacterial isolates.

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REFERENCES

Diggles, B.K., G.A. Moss, J. Carson and C.D. Anderson, 2000. Luminous vibriosis in rock lobster *Jasus verreauxi* (Decapoda: Palinuridae) phyllosoma larvae associated with infection by *Vibrio harveyi*. Dis. Aquat. Organ., 43: 127-137.

Goarant, C., F. Merien, F. Berthe, I. Mermoud and P. Perolat, 1999. Arbitrarily primed PCR to type *Vibrio* sp. pathogenic for shrimp. Applied Environ. Microbiol., 65: 1145-1151.

Jayaprakash, N.S., S.S. Pai, A. Anas, R.Preetha, R. Philip and I.S.B. Singh, 2005. A marine bacterium, Micrococcus MCCB 104, antagonistic to vibrios in prawn larval rearing systems. Dis. Aquat. Org., 68: 39-45.

Lee, K.K., 1995. Pathogenesis studies on *Vibrio alginolyticus* in the grouper, *Epinephelus malabaricus* Bloch et Schneider. Microb. Pathogen., 19: 39-45.

Liao, I.C., K.K. Lee and Y.H. Chien, 1996. Practical Approaches to Marine Fish Health Problems in Taiwan. In: Aquaculture Health Management Strategies for Marine Fish in Asia and the United States. Main, K.L. and C. Rosenfeld (Eds.). The Oceanic Institute, Honolulu, USA., pp: 57-67.

Madigan, M.T., J.M. Martinko, J. Parker and T.D. Brock, 2000. Biology of Microorganisms. Prentice-Hall, Inc., New Jersey, USA.

Muyzer, G., A. Teske, C.O. Wirsen and H.W. Jannasch, 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol., 164: 165-172.

Radjasa, O.K., H. Urakawa, K. Kita-Tsukamoto and K. Ohwada, 2001. Characterization of psychrotrophic bacteria in the surface and deep-sea waters from northwestern Pacific Ocean based on 16S ribosomal DNA approach Mar. Biotechnol., 3: 454-462.

Radjasa, O.K., T. Martens, H.P. Grossart, T. Brinkoff, A. Sabdono and M. Simon, 2007a. Antagonistic activity of a marine bacterium *Pseudoalteromonas luteoviolacea* TAB4.2 associated with coral *Acropora* sp. J. Biol. Sci., 7: 239-246.

Radjasa, O.K., D. Nasima, A. Sabdono, K. Kita-Tsukamoto and K. Ohwada, 2007b. Characterization of psychrotrophic bacteria from sea waters of makasar strait, Indonesia. J. Boil. Sci., 7: 658-662.

Radjasa, O.K., A. Sabdono, Junaidi and E. Zocchi, 2007c. Richness of secondary metabolite-producing marine bacteria associated with sponge *Haliclona* sp. Int. J. Pharmacol., 3: 275-279.

Somarny, W.M.Z., N.S. Mariana, V. Neela, R. Rozita and A.R. Raha, 2002. Differentiation of pathogenic *Vibrio* species by RAPD. J. Medical Sci., 2: 165-169.

Urakawa, H., K. Kita-Tsukamoto and K. Ohwada, 1999a. 16S rDNA restriction fragment length polymorphism analysis of psychrotrophic vibrios from Japanese coastal water. Can. J. Microbiol., 45: 1001-1007.

Urakawa, H., K. Kita-Tsukamoto and K. Ohwada, 1999b. Restriction fragment length polymorphism analysis of psychrophilic and psychrotrophic *Vibrio* and *Photobacterium* from the North-Western Pacific Ocean and Otsuchi bay, Japan. Can. J. Microbiol., 45: 67-76.

Versalovic, J., M. Schneider, F.J. de Bruijn and J.R. Lupski, 1994. Genomic fingerprinting of bacteria using repetitive sequence based-Polymerase Chain Reaction. Meth. Cell. Mol. Biol., 5: 25-40.

Yii, K.C., T.I. Yang and K.K. Lee, 1999. Isolation and characterization of *Vibrio carchariae*, a causative agent of gastroenteritis in the groupers, *Epinephelus coicoides*. Curr. Microbiol., 35: 109-115.