JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2009. Vol. **3**(1), p. 75-82

Use of Random Amplified Polymorphic DNA and Restriction Fragment Length Polymorphism for Typing of *Vibrio alginolyticus* Isolated from Black Tiger Shrimp *Penaeus monodon*

N.P. Marhual and B.K. Das

Central Institute of Freshwater Aquaculture, PO. Kausalyaganga,
Bhubaneswar - 751 002, India.

(Received: 12 November 2008; accepted: 21 February 2009)

From 20 isolates representating one strain of *Vibrio alginolyticus* isolated from *Penaeus monodon* were selected for the genotyping properties using RAPD-PCR. The strains were isolated from different organs as haepatopancreas, external surface, pleopod and telson from prawn and confirmed on Thiosulphate-Citrate Bilesalt-Sucrose agar with supplemented with 2% NaCl. For the RAPD study, 10 decamer random primers e.g. OPA, OPB, OPC, OPG, OPH were used for screening. Out of 100 primer tested only 34 primers shows amplification of the genomic genomic DNA of *V. alginolyticus* with more than 4 bands. Maximum no of bands were produced by OPA 20 i,e 15. DNA finger printing pattern of *V.alginolyticus* showed 3-15 polymorphic bands in 5 series of selected primers. Their molecular weight ranging from 0.212-5.238 kbp. RFLP analysis has been widely developed as an effective and sensitive method for strain identification. Out of 34 primers of RAPD-PCR only 9 primers of OPA (2, 4, 6, 18 and 20) and OPG series (3, 4, 11) shows RFLP product. PCR-RFLP finger printing pattern of *V. alginolyticus* showed 1-6 polymorphic bands in 2 series (OPA, OPG) of selected primers and their molecular weight ranging from 0.510-3.867 kbp. RAPD and RFLP technique appeared to be a reliable and fast technique for genomic study of *Vibrio alginolyticus* on the basis of their form of isolation and therefore, represents a powerful tool for epidemiological studies of these prawn pathogens.

Key words: DNA fingerprinting, RAPD-PCR, RFLP, *Vibrio alginolyticus*.

The members of the family Vibrionaceae have been frequently isolated and detected from seawater, estuarine, and freshwater environments[1]. They include several species pathogenic for humans and marine animals[2]. Bacteria of the genus *Vibrio* belong to the family Vibrionaceae are ubiquitous in marine and estuarine aquatic ecosystems in which shrimp occur naturally and are farmed. Several *Vibrio* spp. form part of the natural biota of fish and shellfish[3]. Some of the *Vibrio* constitutes one of the most important shrimp pathogen, often causing heavy mortalities in aquaculture facilities worldwide[4]. *Vibrio parahaemolyticus* and *Vibrio alginolyticus* are two important pathogenic species[5]. Vibriosis, diseases caused by *Vibrio* species, are known to be major disease problems in farmed marine animals, including penaeid

* To whom all correspondence should be addressed.

shrimp, in which they have been recognized as potential pathogens since the beginning of shrimp farming activities[6,7,8]. *Vibrio alginolyticus,* a halophilic Gram-negative bacterium, which cause serious episode to marine animals, such as marine fish, shrimp and shellfish[9,10], and bring a large damage in economy. Thus, it is essential to explore an effective protective pathway against the infection of this microorganism.

To describe the epidemiology and pathobiology of different *Vibrio* specis and strains accurately typing methods have been used like phenotypic characteristics, biochemical test and serogrouping. All these method have its own limitation and time consuming with poor sensitivity. On the other hand technique used to analyze the bacterial DNA such as Ribotyping, Randomly Amplified Polymorphic DNA (RAPD) finger printing, Polymerase Chain reaction (PCR), Restriction Fragment length Polymorphism (RFLP), Pulsed field Gel Electrophoresis (PFGE) , Plasmid profiling are gaining important these days as these method are fast, sensitive and accurate results can be obtained[11,12].

The PCR technique is extremely powerful that generate microgram quantities of DNA copies of the desired DNA, present even as a single copy in the initial preparation. It is rapid reliable and sensitive tool for the molecular based diagnosis and detection. Randomly amplified polymorphic DNA (RAPD) analysis of *Vibrio* species is based on the ability of a single primer of arbitrary nucleotide sequence to generate polymorphic amplification products for any genome in question. RAPD is a rapid method to characterize genetic differences and has been used to fingerprint variety of bacterial species[13,1,14]. The procedure is unique in that no prior sequence information of the genomic DNA sample is needed. This method was first utilized by William et al.,[15] to produce unique genetic fingerprints. The PCR product can be digested with a restriction enzyme to confirm the presence of restriction endonuclease recognition sequence at unique location, and then the resultant fragment size pattern can be indicative of a particular species. Restriction fragment length polymorphism (RFLP) analysis has been gradually increasing because of their relative simplicity and rapidity[2].

MATERIAL AND METHODS

Sample collection

Diseased shrimp, *Penaus monodon* showing whitish musculature and inactivity were collected from farms in CIFA, Bhubaneswar. The different organs as hepatopancreas, external surface, pleopods and telson were collected and macerated in phosphate buffered saline (PBS). Haemolymph from diseased or moribund shrimp was drawn aseptically.

Isolation and Identification of Bacteria

All the samples were diluted serially and 0.1 ml aliquots were spread onto NA The plates were incubated at 37°C for 24 hr. Dominant colonies observed on the NA plates were further screened on Thiosulfate citrate bile salts sucrose (TCBS) agar (Himedia). The biochemical test was done according to Mac Fadden,[16], West and Colwell,[17].

Bacterial morphology and Biochemical characteristics

Gram stained smears from pure culture of bacterial isolates were examined by light microscopy to determine cell size and morphology. Test cultures were grown on Tryptic Soya Broth (TSB) supplemented with 2% NaCl for 24 hr at 37°C and then inoculated into test media for biochemical tests. The biochemical test was done according to Mac Fadden, 1976 and West and Colwell, 1984.

Genus identification was made on the basis of gram reaction colony and cell morphologies, motility. Fermentation of glucose, growth on TCBS agar and sensitivity to the vibriostatic compound 2, 4 diamino 6, 7 diisopnopy pteridine (0/129). The biochemical tests were conducted to determine the physiological & metabolic activities such as carbohydrate metabolism and production of nitrogenous compounds. The physiological characters include catalase production, methyl red and Voges-Proskaeur, tolerance to NaCl. Observation on carbohydrate metabolism reaction included production of acid and gas in glucose, glycerol, production of acid in arabinose, xylose, mannose, lactose, maltose, mannitol, salicin and production of β-galactosidase. Metabolisms of nitrogenous compounds were determined by

performing production of urease, lysine decarboxylase, ornithine decarboxylase, arginine dehydrolase, hydrogen sulphide production and indole production (Table 1).

DNA Isolation from *Vibrio alginolyticus*

For isolation of genomic DNA by Williams *et al*.[15] with slight modification was followed. *V. alginolyticus* was grown at 37°C for 24 hours in 20 ml of tryptone soya broth added 1.5% NaCl (Hi Media, India). The culture was transferred to an eppendorf tube and centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant fluid was discarded and the pellet was washed in 0.5 µl of TE buffer, Centrifuged again and the pellet resuspended in 567 µl ml of TE, 30 µl of 10% SDS and 3 µl of Proteinase K (20 mg/ml). The samples were incubated at 37°C for 1-2 hours. To it 100 µl of 5 M NaCl was added. The samples were mixed by inversion. Then 90 µl of CTAB solution was added. The samples were incubated at 65^0 C for 15 minutes. Equal volume (790µl) of phenol: chloroform: isoamyl alcohol (25:24:1) was added to it. The sample was centrifuged at 13,000 rpm for 20 mints. The top aqueous phase layer was recovered without disturbing the interface and organic phase (bottom layer proteins and other debris) in a fresh tube with the help of a micropipette. Then exactly 0.6 V of Isopropanol was added and the solution was gently mixed to precipitate DNA. Then it was centrifuged at 13,000 rpm for 15 minutes. The supernatant was decanted slowly and the pellet was air –dried. Then 25-30µl TE buffer was added and kept for over night. Then stored at 4°C for further analysis. The absorbance of the DNA solution was read at 260 and 280 nm in UV-spectrophotometer (Biorad, SmartSpec 3000) to determine the concentration and purity. The genomic DNA samples was also examined on an 0.8% (w/v) agarose gel to check that, the genomic DNA had not become excessively fragmented and stored at -20°C for further use.

RAPD Assay

Prior to amplification DNA sample were diluted to a concentration of 25ng/µl. DNA sample was mixed with 2.5µl 10X (Banglore genei Pvt.Ltd), 1µl of a deoxynucloside triphosphate (dNTP) mixture (Banglore genei Pvt.Ltd), 5pmol primer (Operon Technologies, INC., Alameda, USA). 1U Taq DNA polymerase (Banglore genei Pvt.Ltd). The final volume was 25 µl was adjusted with sterilized double distilled water. The tube were then placed in the thermal cycler with programme being set as follows: one cycle of initial denaturation step at 94°C for 4 min followed by 45 cycles of 45 second of 94°C(denaturing temp), 36°C(annealing temp) for 45 second and 72°C(extension temp) for 1.30 minute. The cycling was concluded by an additional final extension at 72°C for 7 minute and the reaction products were stored at 4°C until further analysis.

RAPD Primers

100 randomly designed 10-mer oligonuclotide primers (operon Technologies, INC., Alameda, USA) were used for generating RAPD fingerprints. These primers had a G+C content of 60 to 70% and that they have no self-complementary ends. Out of 100 primers tested for RAPD fingerprinting, only 34 primers were selected for better amplification (Table 2).

Agarose gel Electrophoresis of RAPD products

4 µl of the PCR product was added 6 µl of 1X TBE buffer and 2 µl of gel loading dye. Molecular weight marker 1kb DNA ladder was used as a size standard. This mixture was electrophoreses on 1.2% agarose at 100V for 1h[18]. The gel were stained with ethidium bromide and photographed on gel documentation (Sambrook *et al*., 1989).

Analysis of RAPD finger print patterns

The molecular weight of the PCR product was determined by using software, UVI soft. Data analysis was performed using one-way ANOVA for the determination of molecular weight of amplified bands by comparing it with known molecular weight DNA bands. The comparison of the band was done by using the equation S_{AB}- = $2N_{AB}$ / (N_A+N_B)

where, S_{AB} =Shared DNA bands between two species A and B/genetic similarity between A and B.

N_{AB} =Number of DNA bands shared in common between species A and B.

N_A and N_B=Total number of bands possessed by the species A and B, respectively

PCR- RFLP Assay

The PCR products were further digested by restriction endonuclease. The restriction endonuclease that was used for the present study was EcoRI (20 U/µl, Bangalore Genei Pvt, Ltd). 5 µl of PCR amplified product was mixed with

2 µl of 10 X RE buffer, 1 µl of restriction enzyme. The final volume makes up to 20 µl by adding sterilized distilled water. The mixture was mixed gently and incubated at 37°C for 4-6 h in a water bath. After that the reaction was stopped by adding 0.5µl of 0.5M EDTA (pH 8.0) to the mixture. Reaction products were stored at 4°C for further analysis.

Agarose gel electrophoresis

4 µl of restriction enzyme digested product was mixed with 6 µl of 1X TBE buffer and 2 µl of gel loading dye and the preparation was electrophoresed. This mixture was electrophoreses on 1.2% agarose at 100V for 1h. Molecular weight marker of 1kb DNA ladder was used as a size standard. The gel was stained with Ethidium bromide and photographed on gel documentation (Sambrook *et al.*, 1989).

Analysis of RFLP fingerprinting Pattern

The molecular weight of the digested PCR product was determined by using software UVI soft by comparing it with known molecular weight DNA bands. Data analysis was performed using one-way ANOVA for the determination of molecular weight. The comparison of the band was done by using the equation

$$S_{AB}- = 2N_{AB} / (N_A+N_B)$$

where, S_{AB} =Shared DNA bands between two species A and B/genetic similarity between A and B.

N_{AB} =Number of DNA bands shared in common between species A and B.

N_A and N_B=Total number of bands possessed by the species A and B, respectively

RESULTS AND DISCUSSION

The shrimp aquaculture industry suffers great economic losses due to disease outbreaks. Mass mortalities are encountered in culture and hatchery operations, which are associated with *Vibrios*. *Vibrio alginolyticus* is an important and dominant pathogenic *Vibrio* present in the shrimp aquaculture system. The infections caused by this microorganism greatly hampered the commercial culture practices. The present study is aimed at characterization of *V. alginolyticus* with the biochemical and molecular level so that further identification of this microorganism from diseased shrimp become easier and subsequent control measure can be adopted to prevent these diseases.

Biochemical characteristics of isolates

Bacteriological isolation on the TCBS agar plates reveled that there are both yellow colony and green colony. *V. alginolyticus* produces a yellow colony on TCBS agar. *V. alginolyticus* showed positive reaction for oxidation fermentation, oxidase test, catalase test, motility test, Simmon's citrate test, Lysine decarboxylase test, Ornithine decarbozylase test, liquification of gelatin, Hydrolisis of Starch ,Fermentation of maltose, mannitol etc. This organism is negative to urease test, production of, H_2S, Arginine decarboxylase test, fermentation of Arbinose, Salicin. (Table 1)

Table 1. Biochemical tests of *Vibrio alginolyticus*

S. No.	Biochemical Test Parameter	*Vibrio alginolyticus*
1.	Oxidation fermentation test	+
2.	Oxidase	+
3.	Catalase	+
4.	Motility	+
5.	Urease	-
6.	TCBS growth	Y
7.	Indole	+
8.	Methyl red	-
9.	V-P test	+
10.	Citrate	+
11.	Hydrolysis of starch	+
12.	Gelatin	+
13.	Casein	+
14.	Gas production	-
15.	H_2S production	-
16.	Arginine dehydrolase	-
17.	Lysine decarbonxylase	+
18.	Ornithine decarboxylase	+
19.	Acid from	
20.	Sorbitol	+
	Sucrose	+
	Manitol	+
	Trehalose	+
	Salicin	-
	Arabinose	-
	Glycerol	-
	Maltose	+

Note: Y: Yellow; D: Variable; +: Positive; - : Negative

Quantification of DNA

The quantitative estimation of the *V. alginolyticus* DNA was based on the spectrophotometric determination. The DNA samples of *V. alginolyticus* corresponded to 450-1500 mg/ml.

RAPD-PCR band Analysis

In the present study RAPD assay was chosen to type *V. alginolyticus.* The genomic diversity of V. *alginolyticus* was determined by using random amplified polymorphic DNA[5]. 5 operon primer series as OPA, OPB, OPC, OPG and OPH were originally assessed for RAPD-PCR and 34 primers were used on their ability to produce consistent and distinguishable fragment pattern. The result of RAPD analysis are shown in (Table 2). Maximum no of bands were produced by OPA 20 i.e. 15. In OPA series of primer OPA-2 and OPA -20, OPA-4 and OPA -7 amplified bands of *V. alginolyticus* are quite similar, but bands amplified by primers OPA-14 and OPA-18 were quite different (Fig. 1). In OPC series bands amplified using OPC -7 and OPC-10 were quite similar but bands amplified using OPC-1, OPC-2,

Table 2. Summary of the number and molecular weight range of amplified products of *Vibrio alginolyticus*

Name of primers	Sequence (5'-3') of primers	Number of amplified bands	Molecular weight range (kbp) of amplified DNA fragments
OPA 2	TGCCGAGCTG	9	0.463-3.452
OPA4	AATCGGGCTG	5	0.356-3.753
OPA6	GGTCCCTGAC	12	0.414-3.091
OPA7	GAAACGGGTG	4	0.281-3.591
OPA14	TCTGTGCTGG	12	0.528-4.094
OPA18	AGGTGACCGT	7	0.406-5.254
OPA20	GTTGCGATCC	15	0.469-3.988
OPB5	TGCGCCCTTC	8	0.223-0.848
OPB9	TGGGGGACTC	6	0.466-1.391
OPB12	CCTTGACGCA	6	0.557-3.385
OPB15	GGAGGGTGTT	5	0.400-2.037
OPB20	GGACCCTTAC	6	0.212-5.214
OPC1	TTCGAGCCAG	5	0.566-2.856
OPC2	GTGAGGCGTC	5	0.431-2.383
OPC4	CCGCATCTAC	5	2.40-2.788
OPC5	GATGACCGCC	7	0.874-3.184
OPC7	GTCCCGACGA	4	1.087-2.721
OPC8	TGGACCGGTG	5	0.823-1.849
OPC9	CTCACCGTCC	5	0.765-2.788
OPC10	TGTCTGGGTG	4	0.439-2.754
OPC11	AAAGCTGCGG	4	1.023-3.681
OPC13	AAGCCTCGTC	3	1.023-3.681
OPC15	GACGGATCAG	5	0.813-2.689
OPG2	GGCACTGAGG	3	2.845-5.238
OPG3	GAGCCCTCCA	6	.865-5.050
OPG4	AGCGTGTCTG	3	1.833-5.050
OPG11	TGCCCGTCGT	6	1.071-4.751
OPG12	CAGCTCACGA	6	1.613-5.112
OPH2	TCGGACGTGA	4	0.600-2.259
OPH5	AGTCGTCCCC	3	0.607-1.123
OPH12	ACGCGCATGT	7	0.369-1.287
OPH13	GACGCCACAC	3	0.226-1.150
OPH16	TCTCCAGCTGG	2	0.579-1.378
OPH18	GAATCGGCCA	6	0.322-1.738

OPC-4, OPC-5, OPC-8, OPC-9, OPC-10, OPC-11, OPC-13 and OPC-15 were different from each other (Fig. 2). The molecular range of the amplified product of *V. alginolyticus* against OPA, OPB, OPC, OPG, and OPH ranged 0.990-4.497 Kbp, 0.212-5.214 Kbp, 0.394-3.435 Kbp, 0.469-5.238 kbp and 0.226-2.259 kbp. Over all the amplified bands in all primers varied between 0.212-5.238 kbp.

There was close similarity in the amplified fragment of *V. alginolyticus,* when analyzing with several random primers. 1.2 kb fragment was common when amplified using primer as OPA-7, OPB-5, OPB-9, OPB-15, OPB-20 OPC-2, OPC-4, OPC-5, OPC-8,OPC-11,OPC-15, OPH-5, OPH-13, OPH-16, OPH- 18. 1.4 kbp fragment was common when amplified using primer as OPA-2, OPA-20, OPB-12, OPC-2, OPC-7, OPC-9, OPC-10, OPC-13 and OPH-2. 1.8 kbp fragment was common in OPA-6, OPA-14, OPC-1 etc. The technique describe here can be used for initial identification of species or strain of *V.alginolyticus*.After an initial isolation and characterization by plating or presumably contaminated samples from fish, reliable results confirming the presence or absence of the pathogenic organism can be confirmed by RAPD-PCR[19].Genotyping system include PFGE and RAPD analysis may prove to be useful tools for epidemiological studies of *V. alginolyticus.* As expected, the arbitrary primers used in this study for *V. alginolyticus,* can be used to study and characterize the different strains of *V. alginolyticus.*

RFLP band analysis

RFLP analysis has been widely developed as an effective and sensitive method for strain identification[1]. In the present study RAPD-PCR product were subjected to digestion with EcoRI restriction enzyme. Each individual products of RAPD-PCR were tested against EcoRI. Among those products only OPA and OPG series of PCR products shows bands with restriction enzymes for the two bacteria. Out of 34 primers of RAPD-PCR only 9 primers of OPA, and OPG shows RFLP product. These are included in OPA series (2, 4, 6, 7, 18 and 20) and OPG series (3, 4, and 11). PCR-RFLP finger printing pattern of *V. alginolyticus* showed 1-6 polymorphic bands in 2 series (OPA, OPG) of selected primers (Figure). Primer OPA-2 and OPA- 4 are quite similar, but bands produced

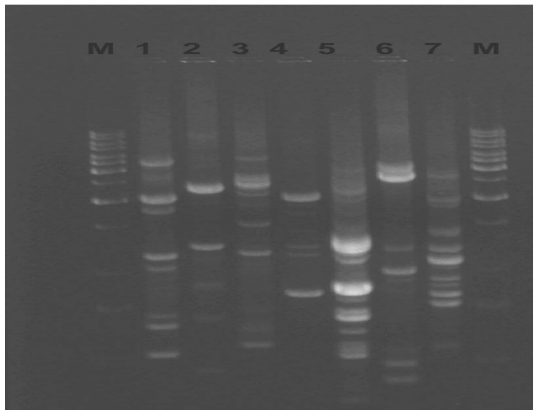

Fig. 1. RAPD band patterns of *V. alginolyticus* using OPA- 2, 4, 7, 14, 18, 20 random primers (L-R), M- 1kb DNA ladder

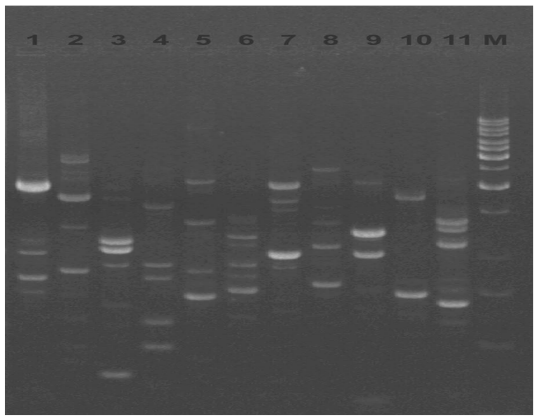

Fig. 2. RAPD band patterns of V. alginolyticus using OPC- 1,2,4,5,7,8,9,10,11,13,15 random primers.12- M- 1kb DNA ladder

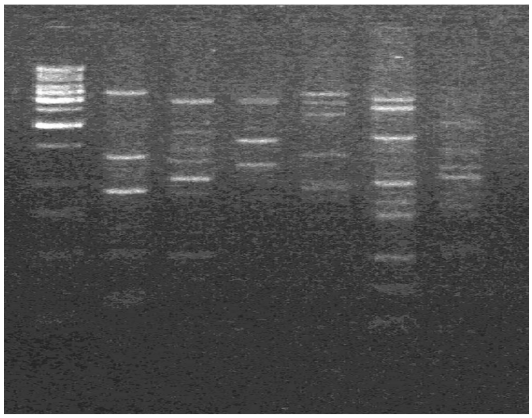

Fig. 3. RFLP pattern of *Vibrio alginolyticus* using OPA seris-2, 4, 6, 18 and 20 (L), M- 1kb DNA ladder

by digestion of OPA-6, OPA-14, OPA-18 and OPA-20 are quite different. Their molecular weight ranging from 0.510-3.867 kbp. RFLP product was absent in OPA -7 primer

CONCLUSION

In the present study Genotyping systems including RAPD and RFLP analysis may prove to be useful tools for epidemiological studies of *V. alginolyticus* RAPD–PCR is easier to perform and more sensitive, inexpensive than other molecular based techniques. (Wang *et al.* 1993). The common typing bands as revealed by various primers will be useful for preliminary identification purpose for unknown stains of *V. alginolyticus*

ACKNOWLEDGMENTS

The authors are thankful acknowledged the assistance given by the Indian Council of Agricultural Research in the form of Lal-Bahadur Shastri young scientist award and Director, Central institute of Freshwater Aquaculture, for providing the necessary facilities to carry out this work.

REFERENCES

1. Mahmud, Z. H., Kassu, A., Mohammad, A., Yamato, M., Bhuiyan, N.A., Balakrish Nair, G. and Ota, F. Isolation and molecular characterization of toxigenic *Vibrio parahaemolyticus* from the Kii Channel, Japan. *Microbiol Res*., 2006; **161**: 25-37
2. Urakawa ,H.,, Kita-Tsukamoto, K., Ohwada ,K. 16S rDNA genotyping using PCR/RFLP (restriction fragment length polymorphism) analysis among the family Vibrionaceae. *FEMS Microbiol Lett*., 1997; **152**: 125-132.
3. Otta, S.K., Karunasagar, I., Karunasagar, I.,. Bacterial flora associated with shrimp culture ponds growing Penaeus mondon in India. *J Aquacul. Tropics.,* 1999; **14**: 309-318.
4. Austin, B and Austin, D.A. *Vibrionaceae* representative. In Bacterial Fish Pathogens 2nd edition, Chichester, Engaland Ellis Horwood Ltd. 1993; 165-307.
5. Sudheesh, P. S., Jie, K., Xu, H. S. Random amplified polymorphic DNA –PCR typing of *Vibrio parahaemolyticus* and *V. alginolyticus* isolated from cultured shrimps., *Aquacultur.,* 2002; **207**:11-17.
6. Lightner, D.V., Lewis, D.H. A septicemic bacterial disease syndrome of penaeid shrimp. *Marine. Fishery Rev.,* 1975; **37**: 25-28.
7. Bondad-Reantaso, M.G., Subasinghe, R.P., Arthur, J.R., Ogawa, K., Chinabut, S., Adlard, R., Tan, Z. Shariff, M. Disease and health management in Asian aquaculture. *Vet Parasitol*., 2005; **132**: 249-272.
8. Goarant, C., Reynaud, Y., Ansquer, D., Decker, S.D., Merien, F. Sequence polymorphism-based identification and quantification of *Vibrio nigripulchritudo* at the species and subspecies level targeting an emerging pathogen for cultured shrimp in New Caledonia. *J .Microbiol Methood*., 2007*;* **70**: 30-38.
9. Zorrilla, I., Arijo, S., Chabrillon, M., Diaz, P. Vibrio species isolated from diseased farmed sole (Solea senegalensis, Kaup), and evaluation of the potential virulence role of their extracellular products. *J. Fish Dis.,* 2003; **26**: 103-108.
10. Du , M., Chen, J., Zhang, X., Li ,A., Li ,Y., Characterization and resuscitation of viable but nonculturable *Vibrio alginolyticus* VIB283. *Archial Microbiol* 2007; **88**: 283–288
11. DePaola, A., Ulaszek, J., Kaysner,C. A., Tenge, B. J., Nordstrom, J. L, Wells, J., Puhr, N. and Gende, S. M.,. Molecular, Serological, and Virulence Characteristics of *Vibrio parahaemolyticus* Isolated from Environmental, Food, and Clinical Sources in North America and Asia. *App. Enviro.n Microbiol*., 2003; **69**: 3999-4005.
12. Kimura, B., Sekine, Y., Takahashi, H., Tanaka, Y., Obata, H. , Kai, A. , Satoshi , M., Fujii, T.,. Multiple-locus variable-number of tandem-repeats analysis distinguishes *Vibrio parahaemolyticus* pandemic O3:K6 strains. *J Microbiolo. Meth.* 2008; **72**: 313–320.
13. Ripabelli, G., Sammarco, M.L., McLauchlin, J. and Fanelli, I., Molecular Characteriozation and Antimicrobial Resistance of *Vibrio vulnificus* and *Vibrio alginolyticus* isolated from Mussels .*Sys App. Microbiol*., 2003; **26**:119-126.
14. Bhowmick, P.P., Khushiramani, R., Raghunath, P., and Karunasagar, I. Molecular typing of *Vibrio parahaemolyticus* isolated from seafood harvested along the south-west coast of India . *Lett App Microbiol* 2007; **46**: 198-204.
15. Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, JA, Tingey, S.V. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res*., 1990; **18**: 6531-6535.

16. Mac Fadden, J.F. Biochemical tests for the identification of Medical Bacteria. Willims and Wilkens Co. Baltimore 1976; 310. ISBN-0683053183.
17. West, P.A.,Colwell, R.R. Identification of *Vibrionaceae* : an overview. *In*: Colwell, R.R. Ed., Vibrios in the environment. New York, USA, John Wiley, 1984; 205-363. ISBN-0471873438
18. Sambrook, Fristisch, Maniatis,. Molecular cloning- A laboratory manual. 2nd Edition, Cold Spring Harborv Laboratory Press 1989.
19. Samal, S. K., Samantaray, B.R., Das, B.K., Gnetic analysis of *Aeromonas hydrophila* MTCC 646 by Random Amplfied.