African Journal of Biotechnology Vol. 12(14), pp. 1624-1629, 3 April, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.683
ISSN 1684–5315 ©2013 Academic Journals

Full Length Research Paper

Occurrences of pathogenic *Vibrio parahaemolyticus* from Vellar estuary and shrimp ponds

K. M. Alagappan[1,2]*, B. Deivasigamani[2] and S. Balamurugan[2]

[1]Centre of advanced study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai -608502, Tamil Nadu South India.
[2]Department of Biochemistry and Biotechnology, Pondicherry University, Puducherry – 605014, India.

Accepted 16 November, 2012

***Vibrio parahaemolyticus* is the predominant seafood pathogen associated with human gastroenteritis. Samples were collected from Vellar estuary, shrimp ponds and shrimp for characterization of *V. parahaemolyticus*. A total of 26 blue green centre (BG) *Vibrio* strains were isolated and characterized through biochemical tests, *toxR* gene and 16S rRNA gene sequencing. Based on pathogenic characteristics, six strains were confirmed as pathogenic *V. parahaemolyticus*. This report implies that preventative measure must be taken before consumption of fish and shrimp.**

Key words: Estuary, shrimp pond, *Vibrio parahaemolyticus*, protease, haemolysis, *toxR* gene.

INTRODUCTION

Vibrio harveyi and *Vibrio parahaemolyticus*, are opportunistic pathogens that cause symptomatic infections when the shrimp are stressed. *V. parahaemolyticus* is the dominant species in shrimp affected by red disease and tail necrosis (Jayasree et al., 2006). Most of the pathogenic *Vibrio* sp. has the virulence characteristic like haemolytic activity and proteolytic activity. Potentially, pathogenic *V. parahaemolyticus* strains could be differentiated from the related nonpathogenic marine vibrios based on haemolytic and non haemolytic activity. Moreover, extracellular products secreted by the *V. parahaemolyticus* were found to be highly toxic to tiger prawn (Sudheesh and Xu, 2001). However, gastroenteritis caused by *V. parahaemolyticus* has been associated with foods prepared with seaweeds (Mahmud et al., 2007). In 2006, an outbreak of *V. parahaemolyticus* infections resulted in 177 cases and was linked to the consumption of contaminated raw shellfish including oysters (Balter et al., 2006).

Identification and detection of total and pathogenic *V. parahaemolyticus* by polymerase chain reaction (PCR) have been extensively studied. A toxin regulatory gene (*toxR*) sequence specific to *V. parahaemolyticus*, which is present in all the strains irrespective of their ability to produce thermostable direct hemolysin (tdh) or tdh related hemolysin (trh), has been applied for definitive identification of the bacterial isolates by several workers (Jacksic et al., 2002; Kim et al., 1999). Vellar estuary is situated in Parangipettai, Southeast coast of India. This estuary is the water hub for ~150 shrimp ponds and it is used as the nursery ground for fish fry and fingerlings. Studying of pathogenic *V. parahaemolyticus* is vital for seafood consumption, therefore, this work was scrutinized to enumerate *Vibrio* sp. from Vellar estuary, shrimp ponds, healthy shrimp tissues and infected shrimp tissues; characterization of isolated *Vibrio* strains for haemolytic activity and protease activity and identification of *V. parahaemolyticus* through specific *toxR* gene and partial sequencing of 16S rRNA gene.

MATERIALS AND METHODS

Sample collection and enumeration of *Vibrio* sp.

Samples (water, W and sediment, S) were collected from five different stations (A to E) in Vellar estuary (Figure 1). All the samples were collected during monsoon season which usually occur in the month of October to first part of December 2009. Healthy and infected shrimp (*P. monodon*), water, and sediment samples were collected

*Corresponding author. E-mail: alagumicro@yahoo.co.in. Tel: 91+0413-2654421 or +91 8608264301.

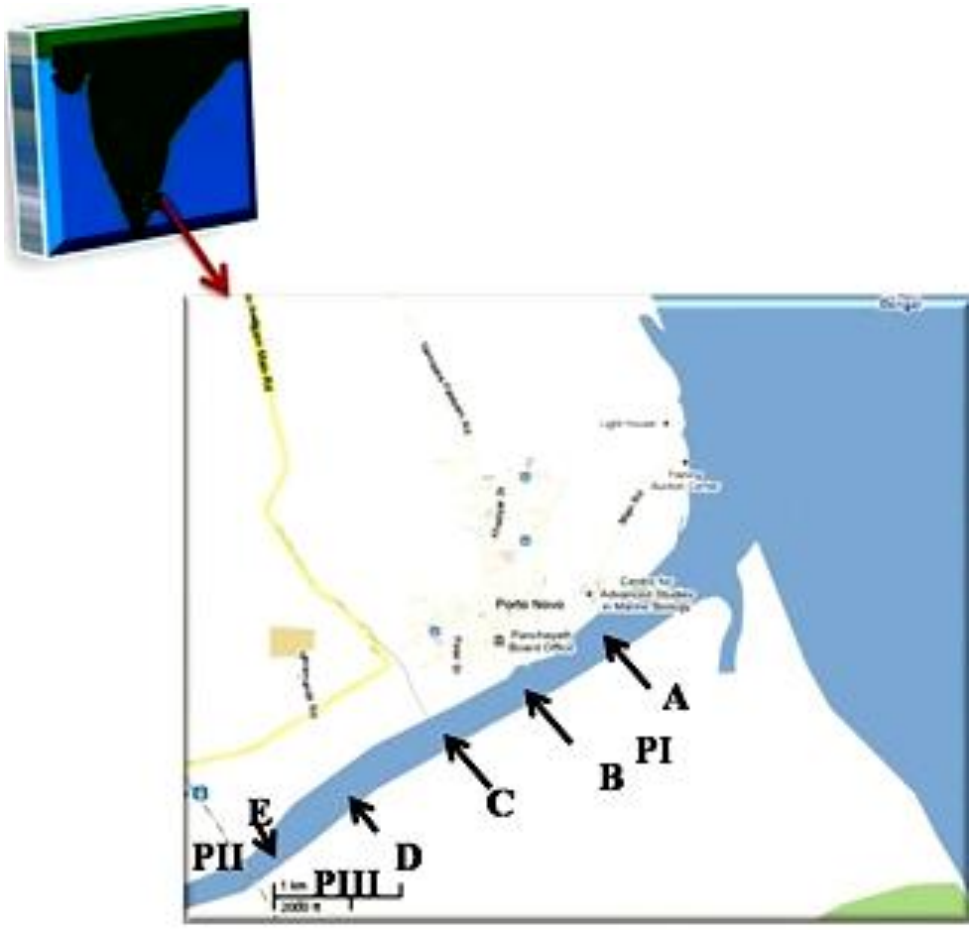

Figure 1. Sample collection stations in Vellar estuary (A to E) and shrimp ponds (PI to PIII).

separately from three shrimp culturing ponds after 70 days of culture, Parangipettai, Tamil Nadu, India and brought to the laboratory with sterile containers. However, heavy mortality in shrimp was not observed while collecting samples. Samples from water and sediment of three ponds (P) were designated as PW-I, PS-I, PW-II, PS-II, PW-III and PS-III and samples from shrimp surface (SS) and infected shrimp tissues (IST). All the samples were collected in sterile glass vials and brought to the laboratory under cold conditions for the isolation of *Vibrio* sp. and *V. parahaemolyticus*. One gram of sediment samples was added to 100 ml of sterile 50% sea water and mixed well. The filtrate was serially diluted and plated on thiosulphate citrate bile salt sucrose (TCBS) agar medium. Similarly, 1 ml of water was serially diluted and plated on TCBS agar medium. Animal tissue (1 g wet weight) were ground, homogenized on a glass homogenizer with a small amount of 1% peptone water with 3% NaCl, and covered with a fourfold amount of peptone water and simultaneously was aseptically pipette into TCBS agar. For isolation of *V. parahaemolyticus*, previously used methodology was followed (Alagappan et al., 2010)

Haemolytic activity and protease activity for BG strains

The Kanagawa phenomenon (KP) or tdh phenotype detection was carried out on Wagatsuma agar as described by Elliot et al. (1992). Protease enzyme activity for isolated BG strains was characterized on skim milk agar (Himedia, Mumbai). All the strains were streaked on the skim agar plates and incubated at 28°C for 24 h. Proteolytic bacteria exhibiting zones of clearance on skim milk agar was confirmed as positive.

Bacterial DNA isolation

The biochemically identified isolate was streaked on nutrient agar plates and the colonies were inoculated in the nutrient broth supplemented with NaCl (2% w/v), and incubated at 37°C under shaking (120 rpm) for 16 to 18 h. The broth cultures were centrifuged (10 000 rpm, 4°C, 10 min) to obtain the pellet and DNA was isolated by followed the method of Sambrook et al. (1989).

Amplification of *toxR* gene

The PCR targeting *toxR* gene of *V. parahaemolyticus* was performed for isolated BG strains based on the method of Kim et al. (1999) using a thermocycler (Thermalcycler, Genei, Bangalore, India) and PCR amplification was done by using PCR mastermix (Medox^{TM}, Chennai, India). Primers like toxR forward (5´-GTCTTCTGACGCAATCGTTG-3´) and reverse (5´-ATACGAGTGGTTGCTTGCTGTCATG-3´) were purchased from Bioserve Biotechnologies (India) Pvt Ltd, Hyderabad, India. Each cycle consisted of three step reactions that is, initial denaturation (95ºC, 1 min) followed by 35 cycles of denaturation (94°C, 30 s), annealing temperature was calculated with T_m value of primer

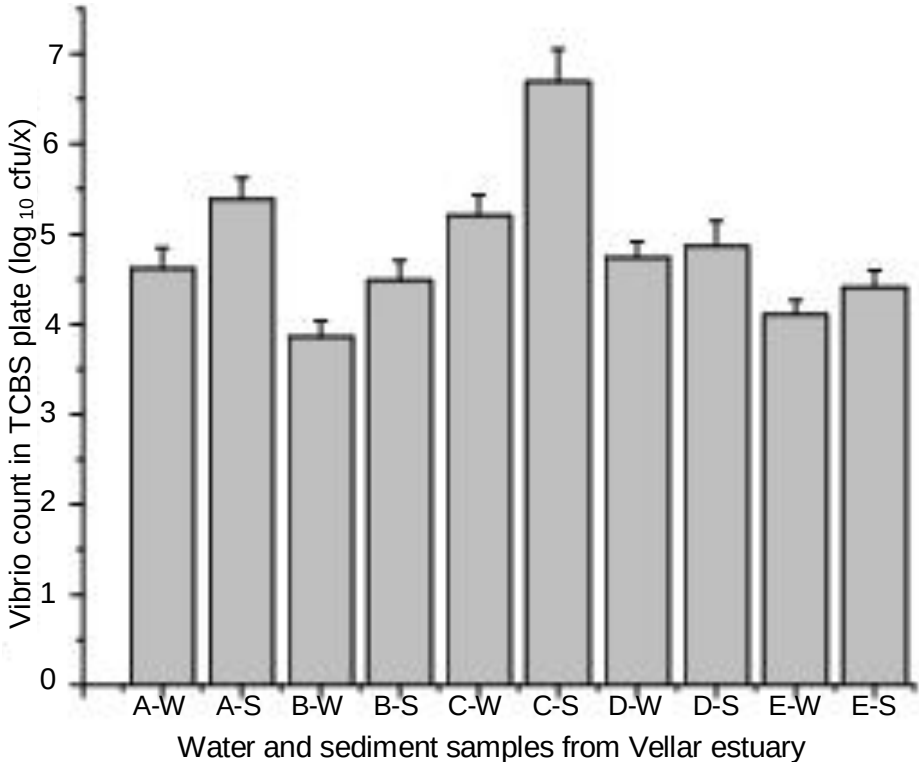

Figure 2. Total *Vibrio* spp. count in TCBS plate of water and sediment samples collected from five different stations in Vellar estuary. Each bar represents the mean value from three determinations with the standard deviation (mean ± SD). X = ml or g.

(51°C, 1 min) and extension (72°C, 1 min 30 s) followed by final extension (72°C, 10 min). The PCR products were resolved on agarose (1.5% w/v) gel electrophoresis. The gel was stained with ethidium bromide (0.5 mg/ml) and visualized under a ultraviolet (UV) transilluminator (Alpha Imager, Innotech Corporation, USA). Genei™ 100 bp DNA Ladder (Genei, Bangalore, India) was used as a molecular size marker.

16S rRNA gene amplification and phylogenetic tree construction

After *toxR* gene PCR reactions for BG strains, two toxR positive and negative strains were confirmed as *V. parahaemolyticus* or other species, respectively through 16S rRNA gene sequence (Kumaran et al., 2010). The amplified PCR products were purified using a Medox PCR product purification kit (Medox™, Chennai, India). All the PCR 16S rRNA gene products were sequenced by using ABI 3130 (four capillary) or 3730Xl (96 capillary) electrophoresis instruments (VIMTA LABS Pvt. Limited, Hyderabad, India). Four bacterial sequences BG24-1446 nt, BG6-1394 nt, BG17-1243 nt and BG12-1441 nt were performed. The data were analyzed using applied biosystem DNA editing and assembly software and sequence comparisons were obtained using the Micro Seq Software. Sequence similarity search was made for the four bacterial 16S rRNA gene sequences by applying their sequence to blast search of the NCBI, then phylogenetic tree was constructed for four strains with Gram positive bacteria *Rhodococcus* sp. SH15 (HM590053) and Gram negative bacteria *Pseudomonas* sp. KUMS3 (FJ596148) served as out group.

RESULTS AND DISCUSSION

For isolation of *Vibrio* spp. and *V. parahaemolyticus*, samples from five different stations in Vellar estuary, three shrimp ponds and healthy and infected animal were collected and characterized. During the monsoon season, samples were collected which was highly influenced by freshwater and salinity was also low compared to other seasons. This condition may induce the invasion of pathogenic forms into estuary and shrimp culturing ponds due to rainfall and inflow of fresh water with sewage contamination. Seasonal changes affect the culturing shrimp in ponds and diseases were more during monsoon and post monsoon period (Muralidhar et al., 2010).

When compared with all the estuary and shrimp pond samples, *Vibrio* sp. count was observed as slightly higher in sediments than in waters (Figures 2 and 3). High *Vibrio* count was observed in station C water and sediment samples when compared with other stations and minimum was observed in stations B and E. Station C waters are connected with sewages and highly contaminated with fecal matters. Eja et al. (2008) also reported that the estuary was constantly faecally polluted, coupled with high rates of infection of shellfish by *V. parahaemolyticus*, *V. cholerae* non-01 and *V. alginolyticus*. Moreover, plankton composition plays an important and independent

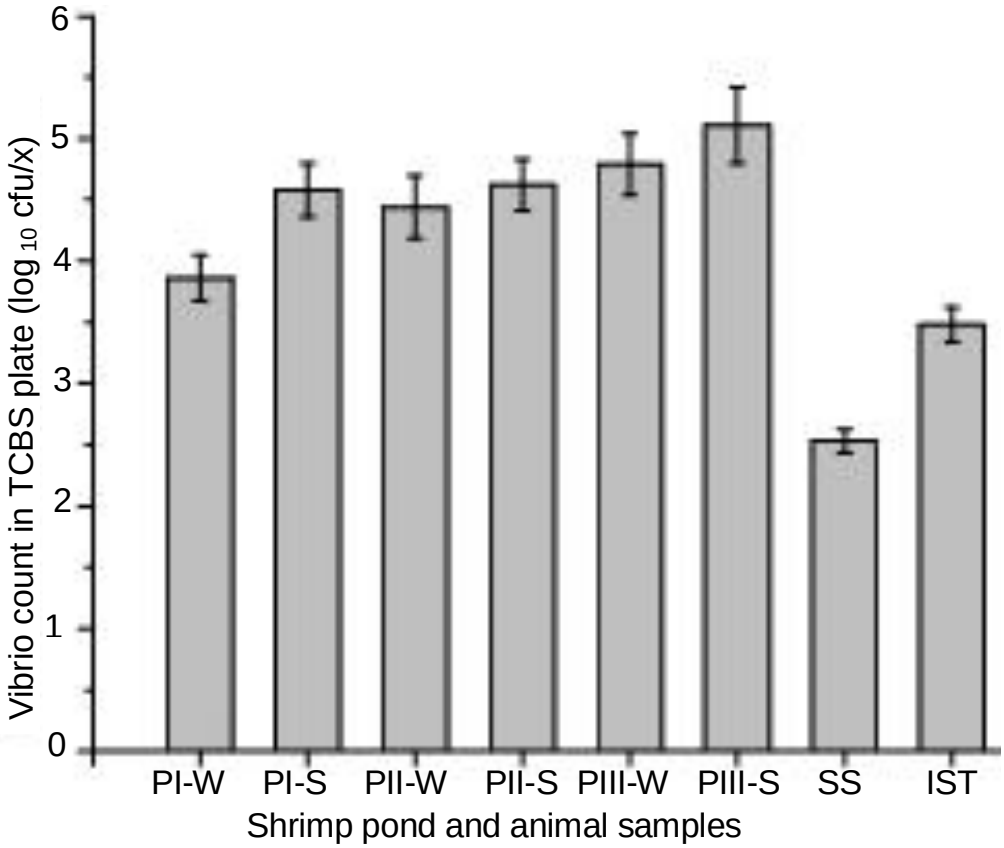

Figure 3. Total *Vibrio* count in TCBS plate of three different shrimp ponds and animal samples. Each bar represents the mean value from three determinations with the standard deviation (mean ± SD). In $\log_{10}$ cfu/x: X for ml in PI, II, III (W); for g in PI, II, III (S) and IST; for ml in one shrimp (15 g weight) surface (ss) was completely swabbed and dipped into 10 ml alkaline peptone water.

role as a driver of the total culturable *Vibrio* community in natural estuarine systems (Turner et al., 2009). Shrimp pond III sediment and water samples showed high count of *Vibrio* when compared with other ponds. A total of 26 BG strains were isolated and ~70% of the strains obeyed all the biochemical tests for *V. parahaemolyticus*.

Pathogenic characteristics like Kanagawa phenomenon, protease and toxR were used as validating factors of *V. parahaemolyticus*. Totally 17 protease and 13 β –haemolyic strains were characterized. In that four strains were isolated from infected shrimp with 2 β -haemolysis and 3 protease production. Moreover, different *Vibrio* extracellular products (ECP) have been identified and proposed as putative virulence factors that are pathogenic to shrimp (Harris and Owens, 1999). Ten (10) BG strains showed positive for both protease and β –haemolysis and considered to be pathogens. However, Sudheesh and Xu (2001) reported that extracellular products of *V. parahaemolyticus* were toxic to *Penaeus orientalis* and it was found to be a poor producer of hemolysins. Environmental strains of *V. parahaemolyticus* are typically not human pathogens; therefore, it is important to have data which states that the occurrence of virulent strains like *V. parahaemolyticus* and *V. cholerae* infection through consumption of cultured shrimp. Due to the marine environmental conditions, identification of marine vibrios is difficult. However, BG colonies also produced by *V. vulnificus* could not easily identify the causative agent whether *V. parahaemolyticus* or *V. vulnificus*. Moreover, *V. vulnificus* is also an important *Vibrio* spe-cies which can cause wound infections and septicemia with a high mortality rate and also associated with disease outbreaks, either with ingestion of contaminated seafood or infectious wounds by contaminated sea water (Nascimento et al., 2001).

Therefore, molecular techniques like PCR amplification for species specific gene have given higher importance than biochemical tests. All strains of *V. parahaemolyticus* harbor the *toxR* gene and it has been suggested that PCR amplifying the *toxR* gene could be used for detection of *V. parahaemolyticus* (Kim et al., 1999).

Some authors suggested that tdh and trh are used to characterize its identification and pathogenicity associated with human disease (DePaola et al., 2003). Moreover, virulence factor genes called tdh and trh are usually found in strains isolated from clinical specimens and less

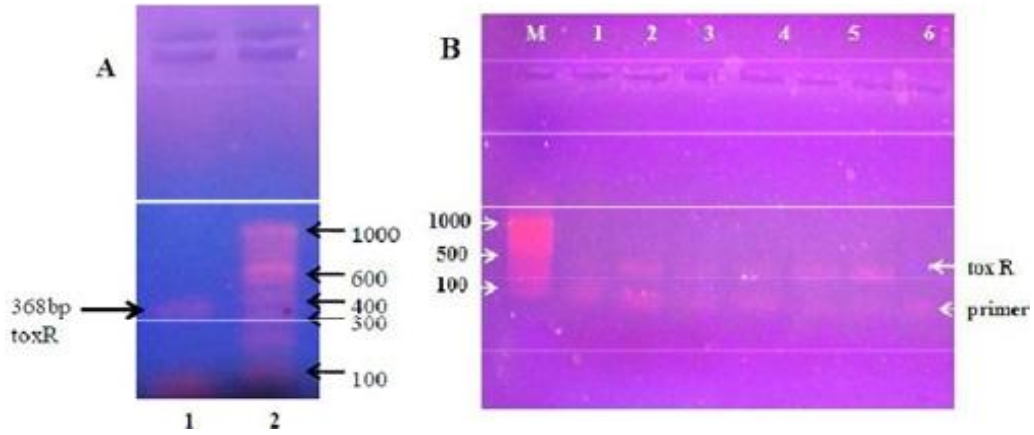

Figure 4. PCR product of amplified *toxR* gene from BG strains. **A.** Agarose gel electrophoresis of PCR amplified products for *toxR* gene (368 bp). Lane 1, *toxR* gene of BG24 strain; Lane 2, DNA ladder (1000 - 100 bp). **B.** Lane M, DNA ladder (1000 - 100 bp). Lanes 1 to 6, BG 1, 3, 6, 8, 15 and 19 strains toxR gene products.

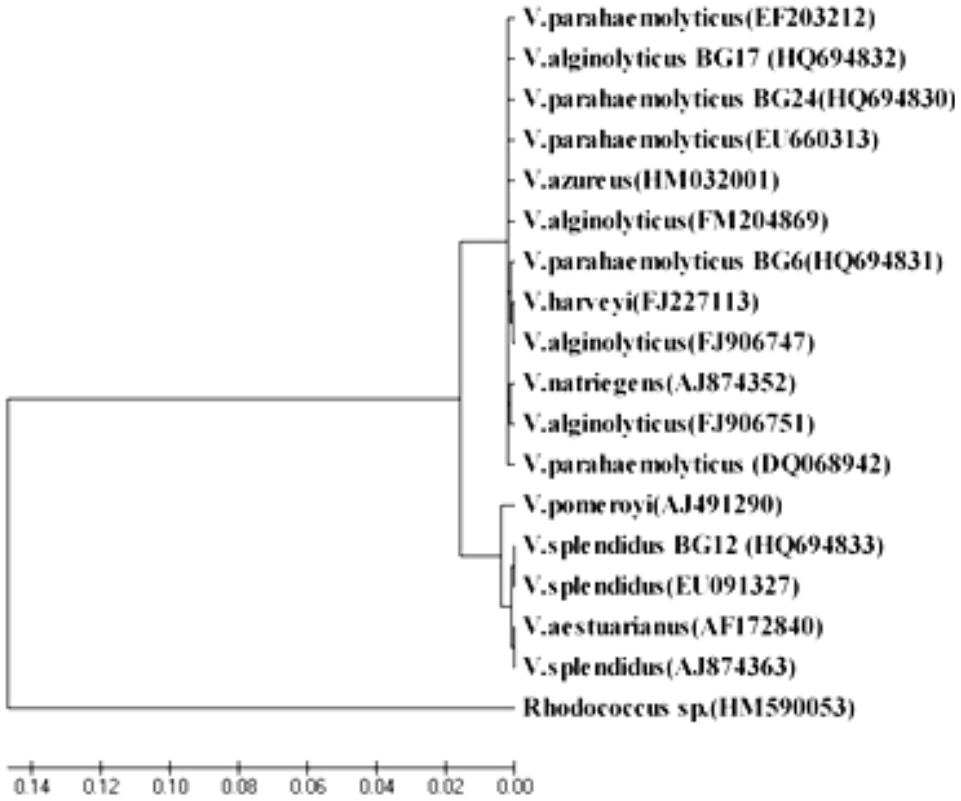

Figure 5. Phylogenetic tree constructed through Kimura 2-parameter model using the neighborhood- joining method. Bar scale 0.02. *Pseudomonas* sp. KUMS3 and *Rhodococcus* sp. SH15 were used as an out group. Queried sequence: BG24, *V. parahaemolyticus* (HQ694830); BG6, *V. parahaemolyticus* (HQ694831); BG17, *V. alginolyticus* (HQ694832); BG12, *V. splendidus* (HQ694833).

than 5% of environmental isolates producing thermostable direct hemolysin (Wong et al., 2000). However, Johnson et al. (2009) isolated *V. parahaemolyticus* containing tdh and trh from Mississippi coastal environments and they found that there was no source-specific sequestering in oysters, water or sediment.

From 26 BG strains, 11 (BG: 1, 3, 6, 8, 15, 16, 18, 19, 23, 24 and 25) were found to be positive for *toxR* gene and identified as *V. parahemolyticus* (Figures 4A and B). Similarly, Chakraborty and Surendran (2008) also confir-

med that 24% of their 72 *V. parahaemolyticus* cultures showed positive for 368-bp *toxR* gene fragment. In that of the 11 BG strains, six (BG1, BG3, BG6, BG15, BG19 and BG24) produced positive for protease activity, β –haemolysis and *toxR* gene which were confirmed as highly pathogenic to shrimp and also humans. Biochemical results for identification of *V. parahaemolyticus* from clinical samples may produce 100% good results but in marine strains different types of sugars utilization occurred. Therefore, biochemical identification of marine strains may be a suitable method but however, the results may value up to genus level and not to species level. Therefore, two positive and two negative toxR *gene* strains were sequenced for 16S rRNA gene to confirm its species level. Similarity between these strains and previously identified strains were compared with NCBI Blast and the 16S rRNA sequenced four strains matching maximum percentage were observed. After blasting, maximum percentage similarity (98%) were observed and two toxR positive strains were belongs to *V. parahaemolyticus* (BG24 and BG6) and negative strains were *V. alginolyticus* (BG17) and *V. splendidus* (BG12) (Figure 5). From the results blue green centered colonies were also belongs to other *Vibrio* species other than that of *V. vulnificus*. Moreover, identification of toxR gene in marine samples is the assured technique to confirm marine *V. parahaemolyticus*. Moreover, six BG strains (BG1, BG3, BG6, BG15, BG19 and BG24) were confirmed as pathogenic *V. parahaemolyticus* based on three virulence characteristics.

TCBS plating and biochemical tests are not correct tools to identify pathogenic marine *V. parahaemolytcius*. When compared with terrestrial bacterial strains, marine bacterial strains may differ with biochemical characteristics. Moreover, pathogenic *V. parahaemolyticus* isolated from estuary, shrimp pond and shrimp may pose human health risk when consumed. During favorable conditions, pathogenic strains may cause infection in estuarine fishes and culturing shrimp in shrimp ponds.

ACKNOWLEDGEMENT

We thank our Dean Prof. T. Balasubramanian, Annamalai University for his kind cooperation and encouragement during our research. First author want to thank University Grand Commission, India for his fellowship.

REFERENCES

Alagappan KM, Deivasigamani B, Somasundaram ST, Kumaran S (2010). Occurrence of *Vibrio parahaemolyticus* and Its Specific Phages from Shrimp Ponds in East Coast of India. Curr. Microbiol. 61: 235-240.

Balter S, Hanson H, Kornstein L, Lee L, Reddy V, Sahl S, Stavinsky F, Fage M, Johnson G, Bancroft J, Keene W, Koepsell J, Williams M, MacDonald K, Napolilli N, Hofmann J, Bopp C, Lynch M, Moore K, Painter J, Puhr N, Yu P (2006). *Vibrio parahaemolyticus* infections associated with consumption of raw shellfish - three states. Journal of the American Medical Association. 296: 2309-2310.

Chakraborty RD, Surendran PK (2008). Occurrence and distribution of virulent strains of *Vibrio parahaemolyticus* in seafoods marketed from Cochin (India). World. J. Microbiol. Biotechnol. 24: 1929-1935.

DePaola A, Ulaszek J, Kaysner CA, Tenge BJ, Nordstrom JL, Wells J, Puhr N, Gendel SM (2003). Molecular, serological, and virulence characteristics of *Vibrio parahaemolyticus* isolated from environmental, food, and clinical sources in North America and Asia. Appl. Environ. Microbiol. 69(7): 3999-4005.

Eja ME, Abriba C, Etok CA, Ikpeme EM, Arikpo GE, Enyi-Idoh KH, Ofor UA (2008). Seasonal Occurrence of Vibrios in Water and Shellfish Obtained from the Great Kwa River Estuary, Calabar, Nigeria. Bull. Environ. Contam. Toxicol. 81: 245-248.

Elliot EL, Kaysner CA, Tamplin ML (1992). Media and reagents. In: USFDA bacteriological analytical manual, (Eds.) 7th ed. AOAC International, Arlington, Va. p. 508.

Harris LJ, Owens L (1999). Production of exotoxins by two luminous *Vibrio harveyi* strains known to be primary pathogens of *Penaeus monodon* larvae. Dis. Aquat. Org. 38: 11-22.

Jacksic S, Suncica U, Petrak T, Bazulic D, Lada GK (2002). Occurrence of *Vibrio* spp. In: Sea fish, shrimps and bivalve mollusks harvested from Adriatic sea. Food Control. 13: 491-493.

Jayasree L, Janakiram P, Madhavi R (2006). Characterization of *Vibrio* spp. associated with diseased shrimp from culture ponds of Andhra Pradesh (India). J. World Aquac. Soc. 37: 523-532.

Johnson CN, Flowers AR, Young VC, Gonzalez-Escalona N, DePaola A, Noriea NF. 3rd., Grimes DJ (2009). Genetic Relatedness Among tdh+ and trh+ *Vibrio parahaemolyticus* Cultured from Gulf of Mexico Oysters (*Crassostrea virginica*) and Surrounding Water and Sediment. Microb. Ecol. 57: 437-443.

Kim YB, Okuda J, Matsumoto C, Takahashi N, Hashimoto S, Nishibuchi M (1999). Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the toxR gene. J. Clin. Microbiol. 37: 1173-1177.

Kumaran S, Deivasigamani B, Alagappan KM, Sakthivel M, Guru Prasad S (2010). Isolation and characterization of *Pseudomonas* sp. KUMS3 from Asian sea bass (*Lates calcarifer*) with fin rot. W. J. Microbiol. Biotechnol. 26: 359-363.

Mahmud ZH, Neogi SB, Kassu A, Wada T, Islam MS, Nair GB, Ota F (2007). Seaweeds as a reservoir for diverse *Vibrio parahaemolyticus* populations in Japan. Int. J. Food. Microbiol. 118: 92-96.

Muralidhar M, Kumarana M, Muniyandia B, Aberyb NW, Umeshc NR, De Silvab SS, Jumnongsongd S (2010). Perception of climate change impacts and adaptation of shrimp farming in India: Farmer focus group discussion and stakeholder workshop Report, 2nd ed. Network of Aquaculture Centers in Asia-Pacific. p. 75.

Nascimento SMM, Vieira RHSF, Theophilo GND, Rodrigues DP, Vieira GHF (2001). *Vibrio vulnificus* as a health hazard for shrimp consumers. Revista do Instituto de Medicina Tropical de Sao Paulo. 43: 263- 266.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor. Laboratory Press, NY.

Sudheesh PS, Xu HS (2001). Pathogenicity of *V. parahaemolyticus* in tiger prawn *Penaeus monodon* Fabricius: possible role of extracellular proteases. Aquaculture. 196: 37-46.

Turner JW, Good B, Cole D, Erin KL (2009). Plankton composition and environmental factors contribute to Vibrio seasonality. The ISME Journal. 3: 1082-1092.

Wong HC, Liu SH, Ku LW, Lee IY, Wang TK, Lee YS, Lee CL, Kuo LP, Shih DYC (2000). Characterization of *Vibrio parahaemolyticus* isolates obtained from foodborne illness outbreaks during 1992 through 1995 in Taiwan. J. Food. Prot. 63: 900-906.