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RESEARCH ARTICLE

Isolation and identification of *Vibrio* species by multiplex PCR and investigation of its prevention by using Indian spices

■ **AASHISH TIWARI, UMA SHANKAR AND DIVYA SAHNI**

SUMMARY

The present study focuses the incidence of *Vibrio* spp. in water bodies which are used by people in surroundings for their daily needs. *Vibrio* constitute of both pathogenic and non-pathogenic species, thus, for differentiating between them a rapid method named multiplex PCR assay has been for the study. The method used was to first determine the physical and chemical constituents of all the 15 water samples (pond water) collected from different regions of Uttar Pradesh. Isolation of bacteria was done on selective media (TCBS) which showed both green and yellow colonies. These colonies were cultured separately on non selective media. All the pure cultures obtained were thus, used to extract their DNA using Phenol chloroform method. DNA was then visualized on agarose gel and quantified using double beam spectrophotometer. Samples with higher yield of DNA were used for amplification using species specific primers by multiplex PCR. The results were documented and thus, study derived the information that most of the waterbodies showed presence of pathogenic *Vibrio* species while few of them showed presence of non-pathogenic *Vibrio* spp.

Key Words : mPCR, *Vibrio*, Chemical parameters, Water, Amplification, Species specific

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The *Vibrio* genus encompasses gram negative bacteria species indigenous of marine and estuarine waters. To date, one hundred and thirty species of *Vibrio*s have been described and twelve were classified as human pathogens implicated mostly in food or water-borne diseases (Thompson *et al.*, 2006). Several species of *Vibrio* are pathogens.Most disease-causing strains are associated with gastroenteritis, but can also infect open wounds and cause septicemia. They can be carried by numerous marine animals, such as crabs or prawns, and have been known to cause fatal infections in humans during exposure. Pathogenic *Vibrio* species include*V. cholerae* (the causative agent of cholera),*V. parahaemolyticus*, and *V. vulnificus*. *V. cholerae* is generally transmitted

MEMBERS OF THE RESEARCH FORUM

Author to be contacted :
UMA SHANKAR, Division of Biotechnology, Cyto Gene Research and Development, LUCKNOW (U.P.) INDIA
Email: publication.cytogene@gmail.com

Address of the Co-authors:
AASHISH TIWARI, Department of Microbiology, Sai Nath University, RANCHI (JHARKHAND) INDIA

DIVYA SAHNI, ICAR Central Soil Salinity Research Institute Regional Research Station, LUCKNOW (U.P.) INDIA

by contaminated water.Pathogenic*Vibrio* species can cause foodborne illness (infection), usually associated with eating undercooked seafood. The pathogenic features can be linked to quorum sensing where bacteria are able to express its virulence factor via its signalling molecules (Tan *et al*., 2014). It is well established that *Vibrio cholerae*, the causative agent of cholera, is autochthonous to the aquatic environment globally and is not confined to cholera endemic areas (Kenyon and Piexoto, 1984; Louis and Russek-Cohen, 2003 and Schuster and Tyzik, 2011). The simultaneous detection of several pathogens with a multiplex PCR (m-PCR) approach would be relatively rapid and cost-effective. An m-PCR assay for the simultaneous detection of Aeromonas salmonicida, Yersinia ruckeri, and Flavobacterium psychrophilum has been described recently (Cerro *et al.,* 2002).

Several authors have suggested the use of molecular methods, for example, amplification of specific sequences of the microbial genome by PCR, as a viable alternative to the traditionally used morphological and biochemical methods (Silva, 2003; Baron, 2004; Becker, 2005; Cremonesi, 2005 and Yang *et al*, 2007). One of the methodologies that has been widely used in the last few years for bacterial identification is Multiplex PCR (mPCR), in which more than one pair of primers are used in the same reaction allowing the simultaneous amplification of several DNA sequences (Tamarapu *et al*, 2001).

Therefore, it is possible to identify more than one bacterial species in the same PCR reaction affording a broader as well as faster analysis of the presence of pathogenic bacteria in foods (Tamarapu *et al*, 2001). In the search for quicker and more efficient methods for detection and identification of food pathogens, it was proposed the development and the evaluation of specificity and detection limits of a mPCR for the identification of the three CPS species, *S. aureus, S. intermedius, and S. hyicus*using primers specific for the nuc gene sequence, which codes for the production of a thermostable endonuclease enzyme (thermonuclease or TNase).

Multiplex PCR-based detection is a popular and effective method to distinguish closely related bacterial species such as *Vibrio*-species (Edwards and Gibbs 1994 and Haldar *et al*., 2010). This is carried out either through the use of different gene specific primers to detect various strains of a particular species of *Vibrio* (Rodkhum *et al*., 2006) or through the use of a single gene-specific primer set to differentiate *Vibrio*s (Haldar *et al.,* 2010).

The paucity of clean water for domestic use has led to the increase in the number of deaths in both the urban and rural parts of developing economies. Deaths due to water related diseases in India are in the range of nearly 80 per cent. Lack of water, sanitation, and hygiene results in the loss of 0.4 million lives while air pollution contributes to the death of 0.52 million people annually in India (WHO, 2007). Environmental factors contribute to 60 years of ill-health per 1,000 population in India compared to 54 in Russia, 37 in Brazil, and 34 in China. The socio-economic costs of water pollution are extremely high: 1.5 million children less than 5 years die each year due to water related diseases; 200 million person days. Water related diseases plague many Indians. The availability of fresh and good quality drinking water to all Indians remains a concern.

Water is one of the most important compounds to the ecosystem. Better quality of water described by its physical, chemical and biological characteristics. But some correlation was possible among these parameters and the significant one would be useful to indicate quality of water. Due to increased human population, industrialization, use of fertilizers in agriculture and man-made activity, the natural aquatic resources are causing heavy and varied pollution in aquatic environment leading to water quality and depletion of aquatic biota. It is therefore, necessary that the quality of drinking water should be checked at regular time interval because due to use of contaminated drinking water, human population suffers from a variety of water borne diseases (Manjare *et al.,* 2010).

MATERIAL AND METHODS

Sample collection :

Water samples from 15 different locations were taken for the study. The samples were collected from different regions of Uttar Pradesh (India). The water used for the work has their direct contact with people in surrounding for their day to day use. The water samples were collected in sterilized air tight plastic vials. The time between sample collection and analysis was approximately 24 hours.

Physico-chemical parameters analysis of water :

The water samples from different regions were

collected in plastic bottles and immediately brought in Laboratory for the determination of various physico-chemical parameters like temperature, pH, Free CO_2, Chlorides, Calcium, Magnesium, Potassium were estimated in the laboratory by using standard methods as prescribed by APHA (1985); Trivedy and Goel (1986) and Kodarkar (1982).

Isolation of *Vibrio* spp. from water samples :

The samples were spread onto the selective media of Thiosulfate citrate bile salts sucrose (TCBS) agar plates and incubated at 37°C for 48 hours. The colony pigment, morphology and other culture characteristics were observed and analyzed. The discrete colonies obtained on TCBS media were then streaked on fresh plates of Tryptone Soya Agar (TSA) media. The bacterial culture was maintained on the slants of TSA media during the entire period of study. Subculturing was done between gaps of three days.

Broth preparation for DNA extraction :

Nutrient broth media was prepared in a 100 ml Erlenmeyer flask, sterilized under standard conditions of 121°C, 15psi for 15-20 minutes. A loop of culture was inoculated into the broth and incubated at 37°C for 24 hours for all the isolated cultures separately.

Bacterial genomic DNA isolation and its spectrophotometric determination :

Cell suspension grown for 24 hrs at 37°C was used for genomic DNA isolation for each isolate. Fresh pure bacterial cells were treated with TE Buffer (10Mm Tris HCl and 1mM EDTA) at pH-8 and 10 per cent SDS. Aqueous layer of DNA separated using phenol, chloroform and isoamyl alcohol (25:24:1). Proteinase K treatment removed all the unwanted proteins from nucleic acid. Storage of isolated DNA was done in TE Buffer at 4°C for the further study. The isolated DNA quantity was determined by analyzing the absorbance at 260 nm using UV-Visible Spectrophotometer.

Amplification and identification of *Vibrio* species using multiplex PCR :

Identification of *Vibrio* species present in each sample done by using species specific primers for five different species of *Vibrio* (*V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus, V. mimicus and V. alginolyticus*) simultaneously. They are targeted at a species-specific tox gene region of the *Vibrio*. Table A lists the primers used for the amplification of these genes (Singh *et al*., 2015).

Table A : List of primers used for amplification in thermal cycler

Universal forward	
VM_F	CAGGTTTGYTGCACGGCGAAGA 5'
Reverse primer	
V. cholera (V_C)	AGCAGCTTATGACCAATAACGCC 5'
V. parahaemolyticus (V_P)	TGCGAAGAAAGGCTCATCAGAG 5'
V. vulnificus (V_V)	GTACGAAATTCTGACCGATCAA 5'
V.mimicus (V_M)	TCTTGAAGAAGCGGTTCGTGCA 5'
V.alginolyticus (V_A)	GATCGAAGTRCCRACACTMGGA 5'

Specific and sensitive amplification of target gene sequences by m-PCR are dependent on a number of key parameters like annealing temperature, primer concentration, Mg2+ concentration, extension time, and the amount and quality of Taq polymerase used (Henegariu *et al*., 1997). The PCR reactions were performed using a 20µl reaction mixture containing 10X Taq DNA Polymerase buffer with 2mM $MgCl_2$, 2.5 mM dNTPs, 5µM primers, Taq DNA polymerase, template DNA and sterile distilled water. The PCR amplified bands were analysed on 1.2 per cent agarose gel with 1X TAE buffer.

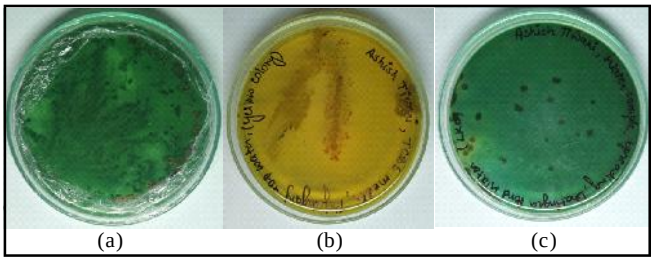
RESULTS AND DISCUSSION

The water samples were analyzed for their physical and chemical parameters using standard methods. The physical parameters included the pH of the samples which ranged from 6.8 to 8.3 compared with standards of WHO, 1993 *i.e.* (pH 6.5-8.5). The highest was seen for BKT and Barabanki samples whereas lowest for Gudamba thana sample. For temperature, maximum value was recorded at 26.2°C (Fajulaganj) and minimum of 21°C (Ajamgarh). Various chemical parameters were tested that include calcium, magnesium, sodium, potassium, chlorides, carbonates and bicarbonates. The average value for Calcium (Ca) was observed to be 2.16 meq/lit showing minimum value of 1.4 meq/lit (Kukrail) and maximum value recorded was 3.2 meq/lit (Bsawan Porwa). Chloride is the indicator of contamination with animal and human waste. Chloride is a common constituent of all natural water and is generally not classified as harmful constituent (Chutia and Sarma, 2009). The chloride contents varied from 3.5 meq/lit (Gudamba thana, deva and BKT) to 8.0 meq/lit (Aliganj) which indicates pollution status of water body. The value of the magnesium showed an average value of 1.3 meq/

lit which fluctuates from a minimum value of 0.8 meq/lit (Fajulaganj and Kukrail) to 1.5 meq/lit (Ajamgarh, Gudamba thana, Aliganj, Deva, Chinhat, Bsawan Purwa and Dhatingra).Sodium level in different water samples showed a range between 0.19 meq/l (Jankipuram) – 0.51 meq/lit (Raitha). Another chemical component from the water sample is of potassium which showed a range of 0.044 meq/lit (Jankipuram) to 0.162 meq/lit (Raitha).

The water samples from different regions were spread onto TCBS agar media to isolate the bacteria present in it. Two different types of colony were observed *i.e.*, green or yellow or both. *V. cholerae* and its biotype ferment sucrose, this results in a pH shift and production of yellow-brown colonies. According to Fishbein, *et al.* (1974) *V. parahaemolyticus* produces light bluish colonies. Cultures grown on TCBS if not examined immediately after removal from the incubator the yellow colonies of *Vibrio* spp. (e.g., *V. cholerae*) may revert to a green colour when left at room temperature (Furniss *et al.*, 1978; Murray, 2011; Forbes, 2007 and Koneman, 2006). The colour colony observed in the study for all the samples are listed in the table given Table 2.

Out of 15 samples, 11 samples showed green colonies when plated onto TCBS media thus, could have presence of *V. parahaemolyticus, V. vulnificus and V. mimicus*. Only 5 samples showed yellow colonies which indicates that it could have presence of *V. cholera and V.alginolyticus.* Water sample from Dhatingra (pond) had both green and yellow colonies after the incubation period. All the colonies were identified based on their morphological characteristics but no biochemical tests



(a) (b) (c)

Fig. 1 : TCBS agar media spreading with water sample (200 µl) and incubating at 37^{0}C for 48 hrs (a) Location: Adil Nagar water sample; Colony colour-Green colour (b) Location: Fajulaganj pond water; Colony colour-Yellow colour(c) Location: Dhatingra; Colony colour-Green colour

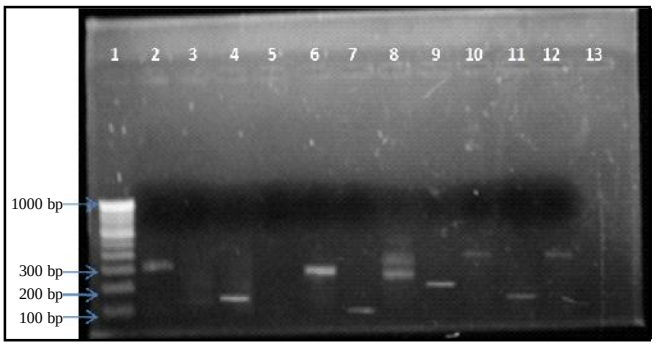


Fig. 2 : Multiplex PCR of water sample using 5 species specific primers with bacterial samples isolated from different water samples (a) A 1.2 per cent agarose gel, showing amplified band in lane 2-13, stained in ethidium bromide and visualized under UV light (b) Lane 1:Marker, Lane 2-13- Amplified PCR products from different water amples of varied locations. Presence of amplified bands shows presence of *Vibrio* species in these water specie (c) Sizes of the amplicons for five *Vibrio* species: V_A – 144bp , V_V - 412 bp , V_C – 375 bp , V_M – 177 bp, V_P – 96 bp.

Table 1: Chemical parameters of the water samples

Sr. No.	Location	Ca (meq/lit)	Mg (meq/lit)	Na (meq/lit)	K (meq/lit)	CO_3	HCO_3 (meq/lit)	Cl	pH	Temp (°C)
1.	Ajamgarh	2	1.5	0.41	0.054	0.15	3	7.5	7.3	21
2.	Jankipuram	1.8	1.2	0.19	0.044	0.25	5	6	6.9	24.5
3.	Gudamba thana	2.3	1.5	0.61	0.059	0.1	2	3.5	6.8	23
4.	Fajulaganj	1.9	0.8	0.19	0.054	0.15	3	6.5	7.2	26.2
5.	Adil Nagar	2.2	1.2	0.43	0.041	0.25	5	7.5	7.0	24
6.	Aliganj	2.5	1.5	0.35	0.083	0.2	4	8	8.1	24
7.	Barabanki	1.6	1	0.37	0.093	0.15	3	6.5	8.3	22
8.	Deva	2	1.5	0.26	0.15	0.2	4	3.5	7.1	23
9.	Chinhat	2.2	1.5	0.42	0.142	0.1	2	4	7.1	25.8
10.	Kukrail (PW)	2.1	1.2	0.37	0.149	0.1	2	5.5	8.2	26.1
11.	Kukrail (PW)	1.4	0.8	0.29	0.107	0.15	3	6.5	8.2	23.1
12.	BKT(PW)	3	1.8	0.33	0.099	0.1	2	3.5	8.3	22
13.	Bsawan Purwa (PW)	3.2	1.5	0.371	0.117	0.2	4	4	8.0	24
14.	Raitha (PW)	1.8	1	0.51	0.162	0.15	3	5.5	6.9	24
15.	Dhatingra (PW)	2.5	1.5	0.26	0.112	0.25	5	7	7.0	25

were performed for identification of *Vibrio* at species level. Instead, a more m- effective tool for the rapid and specific detection of bacterial species was used *i.e.*, mPCR assay.

The natural occurrence of *Vibrio*s in marine and estuarine environment has been reported by Varnam and Evans (1991). Incidence of *Vibrio*s in marine-caught seafoods including shrimp has been reported by Adeleye *et al*. (2010), while Boinapally and Jiang (2007) showed that *Vibrio*s are also found in pond-reared shrimp. In the present survey, 5 different *Vibrio* species were studied and thus, their presence and absence in various water bodies. These water bodies are used by people living in surroundings for their day to day work. If pathogenic bacteria species inhabitant in these water bodies, they could prove fatal to people.

The study shows presence or absences of five *Vibrio*s species residing these water bodies. Table 3 shows these presence or absence based on the amplified bands observed on the electrophoresis gel. The five species specific primers were used for amplification and identification. Primers used were V_C for *V.cholerae;* V_A for V. *alginolyticus;* V_M *for V. mimicus,* V_P *for V. parahaemolyticus, and* V_V *for V. vulnificus.*

In the present study no water sample showed complete absence of *Vibrio* species, as all the water

Table 2 : Appearance of the colonies observed on TCBS agar plates spread with water samples

Sr. No.	Sample name	Location	Green colony	Yellow colony
1.	Ajamgarh	Gorakhpur	√	□
2.	Jankipuram	Lucknow	√	□
3.	Gudamba thana	Lucknow	√	□
4.	Fajulaganj	Lucknow	□	√
5.	Adil Nagar	Lucknow	√	□
6.	Aliganj	Lucknow	√	□
7.	Barabanki	Barabanki	□	√
8.	Deva	Lucknow	□	√
9.	Chinhat	Lucknow	□	√
10.	Kukrail (Pond water)	Lucknow	√	□
11.	Kathauta (Pond water)	Lucknow	√	□
12.	Bakshi Ka Talaab (Pond water)	Lucknow	√	□
13.	Bsawan Purwa (Pond water)	Lucknow	√	□
14.	Raitha (Pond water)	Lucknow	√	□
15.	Dhatingra (Pond water)	Lucknow	√	√

Table 3 : Amplified band fragments observed on the Agarose gel after mPCR amplification

Sr. No.	Sample name	Location	V_C	V_A	V_P	V_M	V_V
1.	Ajamgarh	Gorakhpur	□	□		√	□
2.	Jankipuram	Lucknow	□	□	□	□	√
3.	Gudamba thana	Lucknow	□	□	√	□	√
4.	Fajulaganj	Lucknow	□	√	□	□	□
5.	Adil Nagar	Lucknow	√	□	□	√	□
6.	Aliganj	Lucknow	□	□	□	□	□
7.	Barabanki	Barabanki	□	√	□	□	□
8.	Deva	Lucknow	□	√	□	□	□
9.	Chinhat	Lucknow	□	√	□	□	□
10.	Kukrail (Pond water)	Lucknow	□	□	√	□	□
11.	Kathauta (Pond water)	Lucknow	□	□	□	□	√
12.	Bakshi Ka Talaab (Pond water)	Lucknow	□	□	√	□	□
13.	Bsawan Purwa (Pond water)	Lucknow	□	□	□	√	□
14.	Raitha (Pond water)	Lucknow	□	□	√	□	□
15.	Dhatingra (Pond water)	Lucknow	√	√	□	□	□

samples showed growth on the selective media (TCBS). Some samples showed few colonies while others showed loads of colonies (green, yellow or both green and yellow). This may be due to the increase in bacterial count with increasing detritus organic load at the pond bottom (Sujatha, 2007 and Kannapiran *et al*., 2009). According to Karunasagar *et al*. (1992), bacterial population in shrimp tissues, pond water and bottom mud increases because of accumulation of metabolites and unused feed. Mary (1977); Chen *et al*. (1989); Chen (1992) and Dalmin *et al*. (1997 and 2002) also attest increase in bacterial load in water and sediment with increase in organic load. The study found that three pathogenic species of *Vibrio i.e., V. cholera, V. parahaemolyticus* and *V. vulnificus* were seen in slightly higher percentage as compared to the non pathogenic ones. Similar results were seen in Gopala *et al*. (2005) study where PCR technique was used to amplify toxR gene and identify the *Vibrio* species, the occurrence of various *Vibrio* species in water, sediment and shrimp samples from multiple shrimp farm environments from the east and west coast of India was studied. This study confirms the presence of majorly *V. parahaemolyticus* in aquaculture environments. Similarity was seen with our results as *V. parahaemolyticus* was the species which was identified at maximum (4) region water samples, followed by *V. vulnificus* (3) and lastly by *V. cholera* (2).

However, five *Vibrio* spp., namely, V. *alginolyticus, V. cholera, V. mimicus, V. parahaemolyticus, and V. vulnificus* were isolated from water samples from 15 different regions of Lucknow. The study thus, helped to identify a higher occurrence of pathogenic *Vibrio* species in these water bodies and a moderate occurrence of non pathogenic *Vibrio* species in few regions. Since, mPCR is a specific and rapid method for identification of bacteria; it could be used for detection of pathogenic species in water which are consumed by people. It will help determine whether a water body meets the sanitary condition to extend which is suitable for people using it. The traditional method which uses biochemical tests for species level identification of microbes is time taking, thus, mPCR could be a better alternative.

Conclusion :

The mPCR assay is a rapid process for identification of bacterial species in the targeted sample. A huge number of samples could be analysed simultaneously for directly determining the identity of bacteria based on the certain PCR-amplified DNA fragment. This study targets 15 different regions of Uttar Pradesh (majorly Lucknow) for collecting the water samples. These water samples have been tested for their physico-chemical properties which help to determine the quality status of the water and compared to the standards given by WHO. The study also conducts an analysis of presence or absence of five *Vibrio* spp., namely, V. *alginolyticus, V. cholera, V. mimicus, V. parahaemolyticus and V. vulnificus* in these water bodies. Multiplex PCR method was used for identification based on the amplified PCR product which contains species-specific sequences to determine the identity of the bacteria.

REFERENCES

Adeleye, I.A., Daniels, F.V. and Enyinnia, V.A. (2010). Characterization and pathogenicity of *Vibrio* spp. contaminating seafoods in Lagos, Nigeria. *Internet. J. Food Safety,* **12** : 1-9.

APHA (1985). *Standard methods for examination of water and wastewater*, 20th Ed., American Public Health Association, Washington, D.C.,U.S.A.

Baron, F. (2004). Development of a PCR test to differentiate between *Staphylococcus* aureus and*Staphylococcus* intermedius. *J. Food Protect.,* **67**(10) : 2302-2305.

Becker, K. (2005). Thermonuclease gene as a target for specific identification of *Staphylococcus* intermedius isolates: Use of a PCR-DNA enzyme immunoassay. *Diagnostic Microbiol. & Infectious Dis.,* **51**(4) : 237-244.

Boinapally, K. and Jiang, X. (2007). Comparing antibiotic resistance in commensal and pathogenic bacteria isolated from wildcaught South Carolina shrimps vs. farm-raised imported shrimps. *Canadian J. Microbiol.,* **53**(7) : 919-924.

Chen, D. (1992). An overview of the disease situation, diagnostic techniques, treatments and preventives used on shrimp farms in China. In, W. Fulks and K.L. Main, (eds.), *Diseases of cultured penaeid shrimp inAsia and the United States*, The Oceanic Institute, Honolulu, pp.47-55.

Chen, J.C., Tu, C.C. and Yang, W.S. (1989). Acute toxicity of ammonia to larval Penaeus japonicus. *J. Fish. Soc.Taiwan,* **16** : 261-270.

Chutia, Jayanta and Sarma, Siba Prasad (2009). Relative content of chloride and sulphate in drinking water samples in different localities of Dhakuakhana Sub division of lakhimpur District of Assam. *Internat. J. Chemical Sci.,* **7**(3) : 2087-2095.

Cremonesi, P. (2005). Development of a multiplex PCR assay for the identification of Staphylococcus aureus enterotoxigenic strains isolated from milk and dairy products. *Molecular & Cellular Probes*, **19**(5) : 299-305.

Dalmin, G., Purushothaman, A., Kannapiran, E., Sankar, G. and Srinivasan, K. (1997). Microbiology of a shrimp pond with reference to total heterotrophic, coliform and beneficial bacteria. Proc. Natn. Sem. Water Quality Issues in Aquaculture Systems, 18- 19 Dec., 1996, (R. Santanam, V. Ramadhas and P. Gopalakrishnan, eds.), Tamil Nadu Vet. andAnim. Sci. Univ., Tuticorin, pp. 9-14.

Dalmin, G., Purushothaman, A. and Kathiresan, K. (2002). Distribution of total heterotrophic bacteria (THB) and *Vibrio* parahaemolyticus in shrimp culture ecosystem. *Indian J. Fish.*, **49** : 247-253.

Del Cerro, A., Marquez, I. and Guijarro, J.A. (2002). Simultaneous detection of *Aeromonas salmonicida*, *Flavobacterium psychrophilum*, and *Yersinia ruckeri*, three major fish pathogens, by multiplex PCR. *Appl. Environ. Microbiol.*, **68** : 5177-5180.

Edwards, M.C. and Gibbs, R.A. (1994). Multiplex PCR: advantages, development and applications. *Genome Res.*, **3** : (S65- S75).

Fishbein, M., Wentz, B. and Landry, W.L. (1974). *Vibrio* parahaemolyticus isolates in the U.S.: 1696-1972. In: International Symposium of *Vibrio* parahaemolyticus. Fujino, T., G. Sakaguchi, R Sakazaki, *et al.* (eds.), Saikon, Tokyo, pp. 53-58.

Forbes, B.A. (2007). *Bailey and Scott's Diagnostic Microbiology*, (12th ed.) C.V. Mosby Company, St. Louis, MO.

Furniss, A.L., Lee, J.V. and Donovan, T.J. (1978). The *Vibrio*. Public Health Laboratory Science. Monograph series No. 11. London. pp. 3-57.

Gopala, Shubha, Ottaa, Subhendu, K., Kumar, Sanath, Karunasagar, Indrani, Nishibuchib, Matsuaki and Karunasagar, Iddya (2005). The occurrence of *Vibrio* species in tropical shrimp culture environments; implications for food safety. *Internat. J. Food Microbiol.*, **102** : 151-159.

Haldar, S., Neogi, S.B., Kogure, K., Chatterjee, S., Chowdhury, N., Hinenoya, A., Asakura, M. and Yamasaki, S. (2010). Development of a haemolysin gene-based multiplex PCR for simultaneous detection of *Vibrio* campbellii, *Vibrio* harveyi and *Vibrio* parahaemolyticus. *Lett. Appl. Microbiol.*, **50** : 146-152.

Henegariu, O., Heerema, N.A., Dlouhy, S.R., Vance, G.H. and Vogt, P.H. (1997). Multiplex PCR: critical parameters and step-by-step protocol. *Bio Tech.*, **23**(3) : 504-511.

Kannapiran, E., Ravindran, J., Chandrasekar, R. and Kalaiarasi, A. (2009). Studies on luminous, *Vibrio* harveyi associated with shrimp culture system rearing Penaeus monodon. *J. Environ. Biol.*, **30** : 791-795.

Karunasagar, I., Ismail, S.M., Amarnath, H.V. and Karunasagar, I. (1992). Bacterial load of tropical shrimp and marine sediment. *FAO Fish. Suppl. Rep.*, **470** : 1-8.

Kenyon, J.E. and Piexoto, D.R. (1984). Seasonal variations of *Vibrio* cholerae (non-O1) isolated from California coastal waters. *Appl. Environ. Microbiol.*, **47**(6) : 1243-1245.

Kodarkar, M.S. (1992). Methodology for water analysis, physico-chemical, Biological and Microbiological Indian Association of Aquatic Biologists Hyderabad; Pub., **2** : pp. 50.

Koneman, E.W. (2006). *Colour atlas and textbook of diagnostic microbiology*, (6th Ed.) J.B. Lippincott Company, Philadelphia, PA.

Louis, V. and Russek-Cohen E. (2003). Predictability of *Vibrio* cholerae in Chesapeake Bay. *Appl. Environ. Microbiol.*, **69**(5) : 2773-2785.

Manjare, S.A., Vhanalakar, S.A. and Muley, D.V. (2010). Analysis of water quality using physico-chemical parameters Tamdalge tank in Kolhapur district, Maharashtra. *Internat. J. Advanced Biotechnol. & Res.*, **1**(2) : 115-119.

Mary, P.P. (1977). Studies on the Gastrointestinal Microflora of the Mullet, Liza dussumeri (Valenciennes) (Mugiliformes, Teleostei). Ph.D. Thesis, Annamalai University, Annamalainagar, T.N. (INDIA).

Murray, P.R. (2011). *Manual of clinical microbiology*, (10th Ed.) American Society for Microbiology, Washington, D.C., U.S.A.

Rodkhum, C., Hirono, I., Crosa, J.H. and Aoki, T. (2006). Multiplex PCR for simultaneous detection of five virulence hemolysin genes in *Vibrio* anguillarum. *J. Microbiol. Methods.*, **65** : 612-618.

Sanjoy, Banerjee, Mei ChenOoi, Mohamed Shariff and Helena Khatoon (2012). Antibiotic resistant salmonella and *Vibrio* associated with farmed litopenaeus vannamei. *Scientific World J.*, Volume, Article ID 130136, **6** pages.

Schuster, B.M. and Tyzik, AL. (2011) Ecology and genetic structure of a northern temperate *Vibrio* cholerae population related to toxigenic isolates. *Appl. Environ. Microbiol.*, **77**(21) : 7568-7575.

Silva, W.P. (2003). Identification of Staphylococcus aureus, S. intermedius, and S. hyicus by PCR amplification of coa and nuc genes. *Brazilian J. Microbiol.*, **34**(1): 125-127.

Singh, A.R.P., Hooda, M. and Singh, M. (2015) Rapid identification of human pathogenic *Vibrio* species in fresh water using multiplex PCR. *Internat. J. Engg. Res. & Technol.*, **4**(7) : 1045-1049.

Sujatha, V. (2007). Studies on the Tetragenococcus halophilus and its potential probiotic activity on pathogenic bacteria associated with aquaculture systems. Ph.D. Thesis, University Madras, CHENNAI, INDIA.

Tamarapu, S., Mckillip, J.L. and Drake, M. (2001). Development of a multiplex polymerase chain reaction assay for detection and differentiation of Staphylococcus aureus in dairy products. *J. Food Protect.*, **64**(5) : 664-668.

Tan, W.S., Yunos, M., Yusrina, N., Pui-Wan, T., NurIzzati, M. Tan-Guan-Sheng , A., Wai-Fong, Y. and Kok-Gan,C. (2014). Characterization of a marine bacterium *Vibrio* brasiliensis T33 Producing N-acyl Homoserine Lactone Quorum Sensing Molecules. *Sensors*, **14** (7): 12104-12113.

Thompson, F.L., Austin, B. and Swings, J. (2006). *The biology of Vibrio*. Washington DC :ASM Press. DC. doi: 10.1128/9781555815714.ch2.

Trivedy, R.K. and Goel, P.K. (1986). *Chemical and biological methods for water pollution studies*, Environmental Publication, Karad, M.S. (INDIA).

Varnam, A.H. and Evans, M.G. (1991). *Food Borne Pathogens*, Wolfe Publishing, LONDON, UNITED KINGDOM.

World Health Organization [WHO] (2007). Guidelines for drinking water quality, Incorporation First Addendum, Volume 1, Recommendations, (3rd Ed.), WHO, GENEVA, SWITZERLAND.

Yang, Y., Su, X., Yuan, Y., Kang, C., Li, Y., Zhang, W. and Zhong, X. (2007). Detection of *Staphylococcus aureus* in dairy products by Polymerase chain reaction assay. *Agril. Sci. China*, **6**(7) : 857-862.