Global Veterinaria 8 (5): 525-531, 2012
ISSN 1992-6197

Specific Detection of Pathogenic *Vibrio species* in Shellfish by Using Multiplex Polymerase Chain Reaction

Reham A. Amin and Amani M. Salem

Department of Food Control, Fac. Vet. Med., Benha University, Egypt

Abstract: The purpose of this study was to investigate the prevalence of *Vibrio spp.* in shellfish using the traditional culture and multiplex PCR methods. This study was conducted on 40 samples of shrimp and crab (20 samples of each). The prevalence of *Vibrio parahemoluticus* in the examined shrimp and crab samples was found to be 20 and 30% by traditional culture method and 70 and 50% by m-PCR method, respectively, while the prevalence of *Vibrio mimicus* in the examined shrimp and crab samples was found to be 25 and 5% by m-PCR method, respectively and failed to be detected in all the examined samples by culture method. *Vibrio vulnificus* and *Vibrio cholera*e failed to be detected in all the examined samples by both methods. The m-PCR procedure, which used five primers, produced specific amplicons of the expected sizes from mixed populations of *Vibrio spp.* in shellfish samples and from pure cultures. Overall, results of the present study indicated that the m-PCR is a potential technique for simple, rapid and reliable detection of the major *Vibrio spp.* for routine monitoring and risk assessment of shellfish.

Key words: Crab · Shrimp · *Vibrio* Species · M-PCR

INTRODUCTION

Today world is witnessing the resurgence in the consumption of shellfish due to the new awareness about its low cholesterol, fat content, important source of vitamins, minerals, polyunsaturated fatty acids of the n-3 family (PUFA n-3) and high-quality animal protein content that make it an important component in the human diet worldwide**.** Additionally, shellfish, as a food component, is characteristically tender, easily digested, additive-free and minimally processed. These characteristics make them a product that almost completely fulfils the demands of consumers and the market's demand in terms of consistent quality, off-season product availability and controlled sizes [1].

The international market demand for shrimp, one of the most popular seafood in the world, continues to grow rapidly. According to FAO [2], shrimp (both fresh and frozen) contributes about 6.03% of the world's total seafood export. Also, shrimp accounts for about 20% of the value of exported fishery products over the past 20 years**.** Hence, shrimp can be considered as a product with high commercial value in the seafood trade. The increase in population density has increased the vulnerability of shellfish growing and harvesting areas through shellfish exposure to human and industrial contaminants [3].

Crabs and shrimps have been reported to be the sources of outbreaks [4]. Members of the family *Vibrinoceae*; *Vibrio parahemolyticus*, *Vibrio mimicus*, *Vibrio cholera* and *Vibrio vulnificus,* which are common inhabitants of aquatic environment and are associated with various marine organisms such as molluscs, shrimps, crabs, clams and oysters [6]. They are targeted as causative agents of human disease due to the consumption of shellfish**.** *Vibrio parahemoluticus* is one of the pathogens causing fish and human diseases, but it is considered as a human pathogen more than a fish pathogen [9]. Instances of food poisoning related to *V. parahemoluticus* may be due to the habit of consuming raw or semi-cooked seafood and shellfish or to post-process contamination of foods with this organism**.** Although the illness is self-limited, the infection may cause septicemia that is life-threatening to immunocompromised people as well as prolonged steroid use.

Corresponding Author: Reham A. Amin, Department of Food Control, Fac. Vet. Med., Benha University, Egypt.

Vibrio mimicus contaminated raw food has been linked to the development of gastroenteritis**.** *Vibrio mimicus* is closely related to *V. cholera*e and so it can be difficult to differentiate the two species because they share many phenotypic characteristics**.** *Vibrio mimicus* can also carry the cholera toxin gene as well as other virulence-associated genes that are used to identify *V. cholera* [12].

Vibrio cholera, which exists worldwide, is called non-O1/O139 *V. cholera*. It lives in fresh or salt water and can cause disease when water comes into contact with an open wound, or following ingestion of raw or undercooked seafood including raw oysters and crabs or through fecal contaminated food or water. *Vibrio cholera* produces cholera toxin (CT), which leads to voluminous secretory diarrhea that disseminates the organism back into the water reservoir [13].

Vibrio vulnificus is transmitted to human through contact with skin, mucosa or wounds exposed to marine water and consumption of certain cooked or raw seafood, particularly shrimp, oysters and clams, more frequently in the summer**.** *Vibrio vulnificus* infections are associated with three distinct clinical syndromes: primary septicemia, gastroenteritis and wound infection [13].

Nowadays there is a great amount of microbiological techniques based on cultivation, isolation of pure cultures and serotyping of these microorganisms [15]. Identification and detection of *Vibrio spp.* through conventional culture and biochemical test methods is labor intensive, monospecific and time consuming. Additional drawbacks of conventional methods are that the interpretation of results requires specialized training that may not be available to all laboratories and the variability in biochemical characteristics within species [16]. Moreover, serotyping is expensive, as well as, a certain amount of isolates is untypable and cannot distinguish all isolates that originate from various regions or sources, because of the limited availability of antisera [17].

Molecular methods that utilize the PCR and nucleotide sequence determination overcome many of the limitations of phenotypic methods**.** Most importantly, molecular methods are comparatively easy, efficient**,** laborsaving, of high discriminative and reproducible abilities**,** accurate, less time consuming, identify multiple species in one assay which would be a useful tool for clinical laboratories and would enhance *Vibrio* surveillance and diagnosis [18].

Numerous studies have been published on m-PCR detection of food borne pathogens including *Vibrio spp.* Some complications remain unresolved, e.g. precise differentiation of *Vibrio parahemolyticus* from closely related species, simultaneous detection of all the species in the same sample, etc [16]. Hence, it is necessary to develop m-PCR protocols which fit the local situation. Keeping in view the above points the present study was envisaged to isolate and identify the *Vibrio* in species level in marine shrimps and crabs by conventional method, develop a multiplex PCR assay for the simultaneous identification of the four most commonly encountered *Vibrio spp.* (*V. parahemolyticus*, *V. mimicus*, *V. cholerae* and *V. vulnificus*) in the same seafood samples and compare the efficiency (accuracy) of conventional and multiplex PCR methods.

MATERIALS AND METHODS

Food Sample Preparation: A total of forty samples of shrimps and crabs (20 samples of each) obtained from various estuarine areas of Egypt were collected during September, October and November 2011. All samples were transported to the laboratory refrigerated at 4°C or on ice and were subsequently analyzed with the described methodology [20].

Identification: The isolated *Vibrio spp.* were confirmed by Gram staining, motility, cytochrome oxidase, Voges Proskauer test, halophilic characteristics (0%, 3%, 6%, 8% and 10%), colony morphology on bovine blood agar and TCBS agar and by API 20E (BioMérieux, Marcy l'Etoile, France) [21].

DNA Extraction: Confirmation of phenotypic identification of isolates was performed by PCR on DNA extracted from single colonies as previously reported [22]. Aliquots (1.0 ml) of incubated food sample (homogenate) were centrifuged at 9000 x g for 3 min. in a Hermle 2160M centrifuge. The pellet was then resuspended in 500 µl of TE buffer (pH 7.8) and vortexed. All the samples were finally lysed for 10 min. in a boiling water bath. Then the lysate was cooled in an ice bath. Aliquot (2 µl) of the extract was used as the template for PCR amplification [23].

PCR Primers: Oligonucleotides primers, ranging from 20- to 24-mers, were selected from either the FDA's BAM standard methods**.** The primer sets used in this study for m-PCR, their corresponding gene targets and size of expected amplification products were shown in Table (1).

Table 1: Sequence of primers and final concentration and product size for each of five primer pairs in the multiplex PCR

Target species	Primer	Sequence (5 to 3)	Concn (µM)	Amplicon size (bp)
V. cholerae	*Vc.sodB*-F	AAG ACC TCA ACT GGC GGT A		
	Vc.sodB-R	GAA GTG TTA GTG ATC GCC AGA GT	0.5	248
V. mimicus	*Vm.sodB*-F	CAT TCG GTT CTT TCG CTG AT		
	Vm.sodB-R2	GAA GTG TTA GTG ATT GCT AGA GAT	0.75	121
V. parahaemolyticus	*Vp.flaE*-79F	GCA GCT GAT CAA AAC GTT GAG T		
	Vp.flaE-34R	ATT ATC GAT CGT GCC ACT CAC	1.0	897
V. vulnificus	*Vv.hsp*-326F	GTC TTA AAG CGG TTG CTG C		
	Vv.hsp-697R	CGC TTC AAG TGC TGG TAG AAG	0.25	410
All *Vibrio spp.*	*V.*16S-700F	CGG TGA AAT GCG TAG AGA T		
	*V.*16S-325R	TTA CTA GCG ATT CCG AGT TC	0.05	663

Table 2: Comparison of m-PCR method and cultural methods for detection of *Vibrio species* (n =20)

	Shrimp				Crab			
	Positive Samples				Positive Samples			
	m-PCR		Culture		m-PCR		Culture	
Vibrio species	No.	%	No.	%	No.	%	No.	%
VP	14	70	4	20	10	50	6	30
VM	5	25	-	-	1	5	-	-
VV	-	-	-	-	-	-	-	-
VC	-	-	-	-	-	-	-	-

Assessment of Multiplex PCR Specificity and Limit of Detection (LOD) for *Vibrio species* [24]: In this first place, the specificity of each primer set was assessed individually. Once verified the correct functioning of each set, all primers were evaluated together by multiplex PCR.

Development of the Multiplex Assay: Optimization was performed for each primer pair individually and then all primers in combination. Final primer concentrations were adjusted to give approximately equal signals for each gene fragment. Each reaction contained 2 units of AmpiTaq Gold (Applied Biosystems, Foster City, CA) and final concentrations of each deoxynucleoside triphosphate (dNTP) and $MgCl_2$ of 0.2 mM and 1.5 mM, respectively and primer concentrations ranged from 0.05 to 1 µM (Table 1). Thus, a typical 20-µl reaction mixture contained 1.5 µl crude lysate, 0.2 µl of AmpliTaq Gold, 2 µl dNTP stock (2 mM each), 2 µl 10x buffer, 1.2 µl MgCl2 stock (25 mM), 2.9 µl double-distilled water and the various amounts of each 10 µM stock of the primers: VC primers, 0.5 µl each and 16S rRNA primers, 0.1 µl each. The thermal cycling profile was as follows: a 15-min soak at 93°C followed by 35 cycles of 92°C for 40 s, 57°C for 1 min and 72°C for 1.5 min and a final soak at 72°C for 7 min.

Visualization of PCR Products in Agarose Gels and Fragment Analysis [24]: PCR amplicons were visualized on agarose gels. For results evaluation the PCR products obtained from the multiplex reactions were loaded on 2% agarose gels (*Sigma*) containing 5 µg/ml of ethidium bromide (*Sigma*) in TBE buffer and migrated in the Wide Mini-Sub™ Cell GT electrophoresis system (*Bio-Rad*) for 1hr at 100 V. The molecular marker 100-1500 bp DNA ladder (*Dominion, MBL*) was loaded. In the case of agarose gel analysis, 5 µl of PCR product were loaded by lane. The result for each isolate was determined by comparison to those for control isolates of *Vibrio parahemolyticus*, *Vibrio mimicus*, *Vibrio cholerae* and *Vibrio vulnificus*.

RESULTS AND DISCUSSION

The prevalence of *V. parahemolyticus* in the examined shrimp and crab samples was found to be (4) 20% and (6) 30% by culture method and (14) 70% and (10) 50% by m-PCR method, respectively, while the prevalence of *V. mimicus* in the examined shrimp and crab samples was found to be (5) 25% and (1) 5% by m-PCR method, respectively, although it failed to be detected in all the examined shellfish samples by culture method. *V. vulnificus* and *V. cholera*e failed to be detected in all the examined shellfish samples by both the culture and m-PCR methods (Table 2).

Hosseini *et al*. [25] found only *V. parahemolyticus* in 2.1% of the examined shrimp samples by PCR**.** Yang *et al*. [26] had previously reported that 14.9% of frozen and iced seafood samples were contaminated with *V. parahemolyticus*. Noorlis *et al*. [27] stated that by using the MPN-PCR technique, about 24% of freshwater fish samples were found to harbor *V. parahemolyticus* and has been identified as a potential reservoir for this pathogen.

The high prevalence of *Vibrio spp.* in the examined samples could be due to temperature abuse**.** The short generation time of 12 minutes for *V. parahemolyticus* permits the organisms to accumulate in millions in a few hours. Temperature abuse may be due to improper storage or a long holding time on the display rack at the retail level without proper temperature control [27].

From previous studies and according to literature, the prevalence of pathogenic vibrios appears to be influenced by two main physicochemical environmental factors**.** Firstly, temperature has a marked influence on the occurrence of vibrios. The seasonal variation and cycle are considered to correlate with water temperature that is a major factor affecting the abundance of *V. parahemolyticus* and resulting in the emergence of more virulent serotypes and thereby increases opportunities for outbreaks of food borne illnesses, which are a cause for concern for the seafood industry [28]. Secondly, seawater salinity exerts a strong influence on the survival of *Vibrio spp.* Low salinity may favor *V. vulnificus* growth in shellfish**,** while *V. parahemolyticus* tolerates higher salinity values [30].

The development of a suitable methodology permitting sufficiently rapid, sensitive and correct monitorization of *Vibrio* spp. in shell fish always represents interest since it would facilitate greatly their sanitary control and lower the incidence of associated food borne infections [24]. For these reasons, in the past few years, several biomolecular techniques, able to identify these bacteria were proposed.

Vibrio parahemolyticus was confirmed by PCR at 897 bp chromosomal locus specific for this species. *Vibrio mimicus* was confirmed by PCR at 121 bp chromosomal locus specific for this species. Fourteen (70%) and 10 (50%) isolates from the examined shrimp and crab samples, respectively, were confirmed to be *Vibrio parahemolyticus* by the *toxR* using m-PCR. Five (25%) and 1 (5%) isolates from the examined shrimp and crab samples, respectively, were confirmed to be *Vibrio mimicus* by using m-PCR (Fig. 1).

The sensitivity of the m-PCR was higher than that observed by conventional isolation procedures**.** This method allows also the detection of species which may enter in a viable but non-culturable (VBNC) state n response to adverse environmental conditions. VBNC cells no longer grow on conventional media, but may retain their pathogenicity [31]. Most members *Vibrio spp.* are halophilic and the addition of NaCl is often required for enzymatic activity; however, the concentration of NaCl can affect the biochemical profile and lead to erroneous identification with at least one system (API 20E) [34]. For these reasons, more specific, rapid and sensitive molecular methods for *Vibrio* species identification are needed.

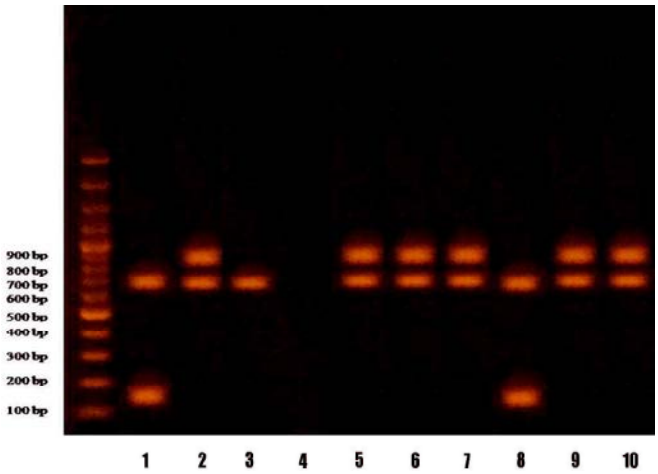

Fig. 1: Agarose gel electrophoresis showing results of the multiplex PCR. Lane1 = *V. mimicus* positive control; lane 2 = *V. parahemolyticus* positive control; lane 3 = *Vibrio spp.* positive control; lane 4 = blank; lane 5 to lane 7 = V. *parahemolyticus* positive samples; lane 8 = *V. mimicus* positive sample; lane 9 and lane 10 = V. *parahemolyticus* positive samples

However, comprehensive detection of all of the aforementioned pathogenic vibrios by the targeting of at least 10 genes would be unreliable by conventional PCR. Discrimination of potentially virulent strains by PCR demands the targeting of multiple genes. The multiplex PCR developed in this study has reliably identified *V. parahemolyticus* and *V. mimicus* strains**.** Panicker *et al*. [28] used three multiplex PCR assays for detection of these species, each targeting one species and several toxigenic genes, however, *V. parahemolyticus*, *V. cholerae* and *V. vulnificus* can co-exist in coastal environment, diseased animal, seafood or aquaculture**.** Nhung *et al*. [40] proposed a *dnaJ* gene-based multiplex PCR but did not verify their method with mixed population of all the three target species in same sample and less difference among amplicon sizes was problematic. Tarr *et al.* [16] targeted *sodB, flaE* and *hsp* genes for detection of *V. cholera*, *V. parahemolyticus* and *V. vulnificus*, respectively, by multiplex PCR but also did not verify their method with mixed population of target species.

On the contrary, the multiplex PCR using well-designed primers of this study has good discrimination capacity without ambiguity of false-positive results from non target species. Besides, our PCR assay has shown good efficacy with mixed DNA templates containing all target species and the species-specific primer sets produced amplicons of various sizes that can be easily distinguishable by electrophoresis. Besides, our assay is highly advantageous because it could simultaneously detect the four target species which are a public safety concern in foods, even if there is 100-fold variation in cell number [23]. However, to circumvent the detection problem for low *Vibrio* population or cells in stress condition in environmental or food samples, an enrichment step is recommended [41]. Additionally, the advantages of our multiplex PCR are that conserved housekeeping genes were used as a source of markers so that the targeted gene were present in all isolates of a species and multiple species can be discriminated with a single PCR assay [28].

Based on the above mentioned study, it was concluded that the novel multiplex PCR assay developed in this study was more specific, sensitive, rapid and accurate method for the detection of *V. parahemolyticus* and *V. mimicus* in shrimps and crabs than the traditional culture method. Rapid, reliable and comprehensive detection of multigene amplicons from pathogenic vibrios with required specificity and sensitivity is important for rapid assessment of suspected food poisoning cases and steady supply of microbiologically safe seafood products in order to guard consumer health and protect the shellfish industry from financial loss.

ACKNOWLEDGEMENTS

The authors would like to express gratitude to Central Diagnostic and Research Laboratory, Faculty of Veterinary Medicine, Kafrelsheikh University and Dr. Ashraf, M. Ahmed, Assistant Professor of Microbiology and director of Central Diagnostic and Research Laboratory, Faculty of Veterinary Medicine, Kafrelsheikh University, for kindly providing laboratory facilities and for technical assistance.

REFERENCES

1. Gillet, R., 2008. Global study of shrimp fisheries. FAO Fisheries Technical Paper. No. 475. Rome, FAO, pp: 331.
2. FAO (Food and Agriculture Organization), 2009. Fisheries topics. Statistics and information. FAO Fisheries and Aquaculture Department, Rome. Available at ww.fao.org/ fishery/ topic/ 2017/ en,assessed December 25, 2009. (Online).
3. Shumway, S.E. and G.E. Rodrick, 2009. Shellfish safety and quality. Cambridge, UK: Woodhead Publishing Limited, pp: 608.
4. Daniels, N.A., L. MacKinnon, R. Bishop, *et al*. 2000. *Vibrio* parahemolyticus infections in the United States, 1973-1998. J. Infect. Dis., 181: 1661-1666.
5. Murchie, L.W., M. Cruz-Romero, J.P. Kerry, M. Linton, M.F. Patterson, M. Smiddy, *et al*. 2005. High pressure processing of shellfish: a review of microbiological and other quality aspects. Innovative Food Science and Emerging Technologies, 6: 257-270.
6. Thompson, F.L., T. Iida and J. Swings, 2004. Biodiversity of vibrios. Microbial. Mol. Biol. Rev., 68: 403-431.
7. Shehane, S. D. and R. K. Sizemore, 2002. Isolation and preliminary characterization of bacteriocins produced by *Vibrio* vulnificus. J. Appl. Microbiol., 92: 322-328.
8. Commission Regulation, 2005. Commission Regulation (EC), 22.12.2005. No. 2073/2005 of 15 de November 2005 on microbiological criteria for foodstuffs. Official J. the European Union, pp: 1-26.

9. IFAS (Institute of Food and Agricultural Sciences), 2009. Preventing food borne and non food borne illness: V. parahemolyticus. This document is FSHN09-01, one of a series of the Food Science and Human Nutrition Department, Florida Cooperative Extensio Service, Institute of Food and Agricultural Sciences, University of Florida. Original publication date: July 2009. visit the EDIS Web site at http://edis.ifas.ufl.edu.
10. Snoussi, M., E. Noumi, D. Usia, L.A. Sechi, S. Zanetti and A. Bakhrouf, 2008. Distribution of some virulence related-properties of *Vibrio* alginolyticus strains isolated from Mediterranean seawter (Bay of Khenis, Tunisia): investigation of eight *Vibrio* cholera virulence genes. World J. Microbiol., 24: 2133-2141.
11. Mizuno, T., S.Z. Sultan, Y. Kaneko, T. Yoshimura, Y. Maehara, H. Nakao, T. Tsuchiya, S. Shinoda and S. Miyoshi, 2009. Modulation of *Vibrio* mimicus hemolysins through limited proteolysis by an endogenous metalloprotease. FEBS J., 276: 825-834.
12. Boyd, E.F., K.E. Moyer, L. Shi and M.K. Walder, 2000. Infectious CTX$^{\Phi}$ and the *vibrio* pathogenicty island prophage in *Vibrio* mimicus: evidence for recent horizontal transfer between V. mimicus and V. cholera. Infect. Immun, 68: 1507-1513.
13. Botana, L.M., 2008. Seafood and freshwater toxins: Pharmacology, physiology and detection, 2nd ed. New York: CRC Press.
14. Luan, X., J. Chen, Y. Liu, Y. Li, J. Jia, R. Liu and X.H. Zhang, 2008. Rapid quantitative detection of *Vibrio* parahemolyticus in seafood by MPN-PCR. Current Microbiol., 57: 218-221.
15. Kong, R.Y.C., S.K.Y. Lee, T.W.F. Law, S.H.W. Law and R.S.S. Wu, 2002. Rapid detection of six types of bacterial pathogens in marine waters by multiplex PCR. J. Water Res., 36: 2802-2812.
16. Tarr, C.L., J.S. Patel, N.D. Puhr, E.G. Sowers, C.A. Bopp and N.A. Strockbine, 2007. Identification of *Vibrio* isolates by a multiplex PCR assay and rpoB sequence determination. J. Clin. Microbiol., 45: 134-140. doi: 10.1128/JCM.01544-06.
17. Zhao, F., D. Zhou, H. Cao, L. Ma and Y. Jiang, 2011. Distribution, serological and molecular characterization of *Vibrio* parahemolyticus from shellfish in the eastern coast of China. J. Food Cont., 22: 1095-1100.
18. Gugliandolo, C., V. Lentini, M.T. Fera, E. La Camera and T.L. Maugeri, 2009. Water quality and ecological status of the Alcantara River estuary (Italy). New Microbiologica, 32: 77-87.
19. Velusamy, V., K. Arshak, O. Korostynska, K. Oliwa and C. Adley, 2010. An overview of foodborne pathogen detection: In the perspective of biosensors. Biotechnology Advances, 28: 232-254.
20. International Organization for Standardization "ISO" 8914 1990. Microbiology-General Guidance on Methods for the detection of V. parahemolyticus. ISO, Geneva, Switzerland.
21. Neogi, S.B., N. Chowdhury, M. Asakura, A. Hinenoya, S. Haldar, S.M. Saidi, K. Kogure, R.J. Lara and S. Yamasaki, 2010. A highly sensitive and specific multiplex PCR assay for simultaneous detection of *Vibrio* cholera, *Vibrio* parahemolyticus and *Vibrio* vulnificus. Letters in Appl. Microbiol., 51: 293-300.
22. Gugliandolo, C., G.P. Irrera, V. Lentini and T.L. Maugeri, 2008. Pathogenic *Vibrio*, Aeromonas and Arcobacter spp. associated with copepods in the Straits of Messina (Italy). Mar. Poll. Bull., 56: 600-606.
23. Lei, I.F., P. Roffey, C. Blanchard and K. Gu, 2008. Development of a multiplex PCR method for the detection of six common foodborne pathogens. J. Food and Drug Analysis, 16: 37-43.
24. Espeñeira, M., M. Atanassova, J.M. Vieites and F.J. Santaclara, 2010. Validation of a method for the detection of five species, serogroup, biotypes and virulence factors by multiplex PCR in fish and seafood. J. Food Microbiol., 27: 122-131.
25. Hosseini, H., A.M. Cheraghali, R. Yalfani and V. Razavilar, 2004. Incidence of *Vibrio spp*. In shrimp caught off the south coast of Iran. J. Food Cont., 15: 187-190.
26. Yang, Z., X. Jiao, X. Zhou, G. Cao, W. Fang and R. Gu, 2008. Isolation and molecular characterization of *Vibrio* parahemolyticus from fresh, low temperature preserved, dried and salted seafood products in two coastal areas of eastern China. Int. Food Microbiol., 125: 279-285.
27. Noorlis, A., F.M. Ghazali, Y.K. Cheah, T.C. Tuan Zainazor, J. Ponniah, R. Tunung, J.Y.H. Tang, M. Nishibuchi, Y. Nakaguchi and R. Son, 2011. Prevalence and quantification of *Vibrio* species and V. parahemolyticus in freshwater fish at hypermarket level. Int. Food Res. J., 18: 673-679.
28. Panicker, G., D.R. Call, M.J. Krug and A.K. Bej, 2004. Detection of pathogenic *Vibrio spp*. In shellfish by using multiplex PCR and DNA microarrays. J. Appl. Environ. Microbiol., 70: 7436-7444.

29. Wittman, R.J. and G.J. Flick, 1995. Microbial contamination of shellfish: Prevalence, risk to human health and control strategies. Ann. Rev. Pub. Health, 16: 123-140.
30. Wright, A.C., V. Garrido, G. Debuex, M. Farrell-Evans, A.A. Mudbidri and W.S. Otwell, 2007. Evaluation of postharvest processed oysters by using PCR-based most probable number enumeration of *Vibrio* vulnificus bacteria. J. Appl. Environ. Microbiol., 73: 7477-7481.
31. Maugeri, T.L., M. Carbone, M.T. Fera and C. Gugliandolo, 2006. Detection and differentiation of *Vibrio* vulnificus in seawater and plankton of a coastal zone of the Mediterranean Sea. Res. Microbiol., 157: 194-200.
32. Bauer, A. and L.M. Rørvik, 2007. A novel multiplex PCR for the identification of *Vibrio* parahemolyticus, *Vibrio* cholera and *Vibrio* vulnificus. Lett. Appl. Microbiol., 45: 371-375.
33. Kwok, A.Y., J.T. Wilson, M. Coulthart, L.K. Ng, L. Mutharia and A.W. Chow, 2002. Phylogenetic study and identification of human pathogenic *Vibrio* species based on partial hsp60 gene sequences. Can. J. Microbiol., 48: 903-910. doi:10.0039/w02-089.
34. Martinez-Urtaza, J., A. Lozano-Leon, A. Vina-Feas, J. de Novoa and O. Garcia-Martin, 2006. Differences in the API 20E biochemical patterns of clinical and environmental *Vibrio* parahemolyticus isolates. FEMS Microbiol. Lett., 255: 75-81.
35. Kim, Y.B., J. Okuda, C. Matsumoto, N. Takahashi, S. Hashimoto and M. Nishibuchi, 1999. Identification of *Vibrio* parahemolyticus strains at the species level by PCR targeted to the toxR gene. J. Clinical Microbiol., 37: 1173-1177.
36. Karunasagar, I., G. Sugumar, I. Karunasagar and A. Reilly, 1997. Rapid detection of *Vibrio* cholera contamination of seafood by polymerase chain reaction. Molecular Marine Biology and Biotechnology, 4: 365-368.
37. Dileep, V., H.S. Kumar, Y. Kumar, M. Nishibuchi, I. Karunasagar and I. Karunasagar, 2003. Application of polymerase chain reaction for detection of *Vibrio* parahemolyticus associated with tropical seafoods and coastal environment. Letters in Appl. Microbiol., 36: 423-427.
38. Takahashi, H., Y. Hara-Kudo, J. Miyasaka, S. Kumagai and H. Konuma, 2005. Development of a quantitative real time polymerase chain reaction targeted to the toxR for the detection of *Vibrio* vulnificus. J. Microbiol. Methods, 61: 77-85.
39. Fukushima, H., Y. Tsunomori and R. Seki, 2003. Duplex real – time SYBR green PCR assays for detection of 17 species of food – or waterborne pathogens in stools. J. Clin. Microbiol., 41: 5134-5146.
40. Nhung, P.H., K. Ohkusu, J. Miyasaka, X.S. Sun and T. Ezaki, 2007. Rapid and scientific identification of 5 human pathogenic *Vibrio* species by multiplex polymerase chain reaction targeted to dna J. gene. Diagn. Microbiol. Infect. Dis., 59: 271-275.
41. Blanco-Abad, V., J. Ansede-Bermejo, A. Rodriguez-Castro and J. Martinez-Urtaza, 2009. Evaluation of different procedures for the optimized detection of *Vibrio* parahemolyticus in mussels and environmental samples. Int. J. Food Microbiol., 129: 229-236.