

Detection of *Vibrio cholerae* by Polymerase Chain Reaction Method

NIRMALA THAMPURAN and P.K SURENDRAN

Central Institute of Fisheries Technology,
P.O. Matsyapuri Cochin - 682029, India

The food safety and quality control in the seafood processing industry necessitates rapid detection of pathogens. Most of the pathogens occur in the food in small numbers only so that detection becomes very difficult. Some of the pathogens like *Vibrio cholerae* also exist in the so called "viable but non-culturable" state in the environment so that ordinary biochemical protocol becomes unsuccessful to detect their presence. Molecular methods such as polymerase chain reaction (PCR) has the advantage over the existing biochemical detection method in detecting very small numbers of stressed cells of these pathogens and that too in a relatively short time. A PCR method is described to detect *Vibrio cholerae* in fish by amplifying a 564 bp fragment of the CTX gene.

Key words : Polymerase chain reaction, seafood safety, *Vibrio cholerae*

Recent ecological studies conducted all over the world have shown that *Vibrio cholerae* is part of the aquatic flora (Colwell & Spira, 1992) and hence there is a very good chance of them being detected in shrimp from cultured or wild sources. *V. cholerae* is also found to persist in coastal waters and marine shrimp. A major problem faced by the seafood exporting countries in the recent years is the contamination of the consignments by bacterial pathogens such as *V. cholerae* and *Salmonella* spp. and the subsequent rejection and destruction of the material by the importing nations. In many instances they escape detection in the exporting countries while they are being detected in the importing countries because of the difference in the sensitivity of the method adopted in these countries. A method based on Polymerase Chain Reaction, called PCR is widely used to detect *V. cholerae* (Fields *et al.*, 1992).

These methods are designed to detect the toxigenic potential of *Vibrio cholerae* by detecting the gene responsible for toxin production. This is important in the present context since it is known that not only *V. cholerae* O1 but certain non-O1 strains can also cause cholera. *V. cholerae* O139 is

an important group of the non O1 category (Albert, 1994) and has received much attention nowadays. Similarly, many of the environmental strains of *V. cholerae* O1 could be non toxigenic. Sakasaki & Donovan (1984) noted that 60 to 70% of *V. cholerae* O1 from seafood and environment could be non-toxigenic. Such strains will not be a health hazard, but conventional test may detect them leading to false alarm and rejection.

It has also been reported that the environmental strains of non-O1 *V. cholerae* can show cross reaction with polyvalent antisera (Shimada *et al.*, 1987) leading to a false positive result. Above all, it has been postulated that the viable but non-culturable (VBNC) state exist for *V. cholerae* and such cells escape detection by cultural methods while it can be detected by PCR methods. The pathogenic potential of VBNC state cells has been confirmed by human volunteer studies (Colwell & Huq, 1994) Further, PCR test is very rapid while cultural method requires a minimum of 5 days. Results of a preliminary study conducted to chalk out the methodology and to compare the two techniques are reported in this paper.

Materials and Methods

The experiment was carried out in three steps. Step 1 was the standardization of the procedure. Known O1 and non-O1 strains were used in this step. These strains were from the culture collection of the CIFT. In addition to the vibrio O1 and non-O1 strains, other bacterial strains of *Aeromonas hydrophila* and other *Vibrio* spp. were also studied. Cells from growth on TCBS plates were transferred to sterile distilled water (1 ml) in microfuge tubes and lysed by heating at 100° C for 10 min. The lysed cells were subjected to PCR by the following protocol.

The method adopted was basically that proposed by Karunasagar & Karunasagar (1995) with some modifications. Primers and other biochemicals used in the study were from Bangalore Genei Pvt. Ltd. (Bangalore)

Primer 1 - 5'CGG GCA GAT TCT AGA CCT CCT G 3'

Primer 2 - 5'CGA TGA TCT TGG AGC ATT CCC AC 3'

This encodes for a specific region in the cholera toxin A gene of 564 bp fragment. For PCR amplification, the reaction mixture was set with a pre-incubation at 95°C for 5 min. and then 30 cycles with 94°C for 1min., 60°C for 1min and 72°C for 1min. Final incubation was at 72°C for 10 min in a

thermocycler (PTC-150 Minicycler™, USA). The product from PCR was separated by electrophoresis on agarose gel and examined under UV light under DNA Transilluminator (Genei, Bangalore) for bands corresponding to 564bp using ethidium bromide as tracking dye for visualization.

The step II was designed to assess the inoculum level required for the test. Fresh prawn (25 g) were spiked with known numbers of the cells of *V. cholerae* O1 at 5 inoculum levels so as to get a cell concentration of 0.5, 5, 50 and 500 cells.ml⁻¹ of APW enrichment broth. One ml aliquots were withdrawn from the 4 test broths immediately after inoculation and after 6 and 24 h incubation periods into microfuge tubes and centrifuged at 500 rpm for 5 min. The supernatant was separated and centrifuged at 10000 rpm for 10 min at 4°C. The pellet was resuspended in 100ml of deionised water and lysed as already mentioned. The lysate was subjected to PCR as done in step I.

In step III, the fish and shellfish samples received by the laboratory from factories and suspected for presence of *V. cholerae* were included in the study. A total of 20 samples were analyzed. 25 g of the material was homogenized with 225 of APW in 500 ml flask and incubated at 37°C. After 6 h and 24 h, 1 ml aliquots samples were withdrawn in microfuge tubes and centrifuged at 500 rpm for 5 min. The supernatant was separated and centrifuged at 10000 rpm for 10 min at 4°C. The pellet derived after 6 h and 24 h were pooled and finally resuspended in 100 ml of deionised water and lysed. The lysate was subjected to PCR as done in previous steps. The product of the PCR was separated as described earlier for visualization.

Results and Discussion

The results of the PCR done using known O1 and non O1 strains of *Vibrio cholerae* are presented in Table 1.

Table 1. PCR using known O1 and non-O1 *Vibrio cholerae* strains

Sample	Result
O1 strains	+
Non O1 strains	-
<i>Vibrio</i> sp.	-
<i>Aeromonas hydrophila</i>	-

It was seen that the O1 strain alone gave expected result and a specific band corresponding to 564 was noted (Fig. 1). On the other hand, all the non-O1 strains gave negative test. Other bacterial strains also gave negative test result.

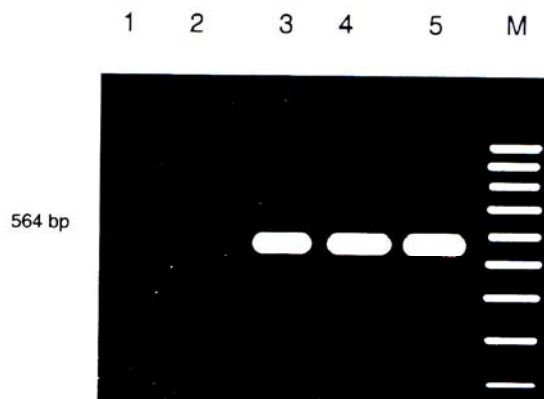


Fig. 1. Detection of ctx A gene of *Vibrio cholerae* Lane 1: Negative control; Lane 2: *Vibrio cholerae* strain negative for ctx A gene Lanes 3,4,5: *Vibrio cholerae* strain positive for ctx A gene

In Table 2, the results of the inoculation studies using pure cells in fish muscle are presented. At low inoculum level, none of the samples gave positive result. The samples taken immediately after inoculation at 0 h also showed a negative result. But cells with initial numbers of >5, 50 and 500 cells when inoculated gave positive results after enrichment for 6 h and 24 h. The negative test could be either due to the low inoculum level at 0 h or due to interference from the fish muscle particles in APW. Side by side with the PCR test, presence of *V. cholerae* were also determined by standard biochemical procedure (US FDA, 1995). The 6 h incubation was better than the 24 h, as the detection became difficult after overgrowth of other microorganisms.

The samples included in step III were frozen shrimp, fish and crab of commerce meant for export. In this case, all the samples were found to be negative for *V. cholerae* by PCR. The biochemical method also failed to detect any *V. cholerae* in these samples. Karunasagar *et al.* (1995; 1998) reported that sensitivity of PCR performed directly using seafood homogenate was 10^4 cells.ml⁻¹ but the sensitivity could be improved to less than 10 cells.g⁻¹ by enrichment in APW. The present results are comparable to their observation.

Table 2. Effect of cell number and incubation period on detection

		PCR	Biochemical
0.5 cells.ml ⁻¹	0 h	-	-
	6 h	-	-
	24 h	-	-
5 cells.ml ⁻¹	0 h	-	-
	6 h	+	-
	24 h	+	-
50 cells.ml ⁻¹	0 h	-	-
	6 h	+	+
	24 h	+	+
500 cells.ml ⁻¹	0 h	-	-
	6 h	+	+
	24 h	+	+

To conclude, it can be said that all strains of *V. cholerae* encountered in the environment are not toxigenic and hence pathogenic. Since the test is primarily aimed at detecting the toxigenic gene, strains belonging to O1 and O139 that are involved in the epidemic cholera can be detected by the PCR method. The non-toxigenic strains of *V. cholerae* are present in nature and they are harmless so far as human infection is concerned. In seafood safety, the essential step should be to detect these pathogenic strains in a very short period. Conventional biochemical method requires at least 5 days for confirmation and hence if food safety is the main concern one should go for PCR method as it can give accurate results in relatively short duration. Since PCR method detects the target gene sequence for toxicity, it fails to distinguish live cells from dead ones as is done in the culture method and this may create a false alarm causing massive loss to the producer.

The authors are thankful to Director, Central Institute of Fisheries Technology, for the kind permission to publish this paper.

References

- Albert, J.M. (1994) *J. Clin. Microbiol.* **32**, 2345
- Colwell, R.R. and Huq, A. (1994) in *Vibrio cholerae* and cholera. (Wachsmuth, K.I., Blake, P.A and Olsvik, O., Eds.), p. 117, American Society for Microbiologists, Washington DC

- Colwell, R.R. and Spira, W.M. (1992) in *The Ecology of Vibrio cholerae in Cholera* (Barua, D. and Greenough, W.B., Eds.), p. 107, Plenum Medical Books Co., New York
- Fields, P.I., Popovic, T., Wacksmuth, K. and Olsvik, O. (1992) *J. Clin. Microbiol.* **30**, 2118
- Karunasagar, I., Sugumar, K.G., Karunasagar, I. and Reilly, A. (1995) *Mol. Mar. Biol. Biotechnol.* **4**, 365
- Karunasagar, I. and Karunasagar, I. (1998) in *Advances and Priorities in Fishery Technology*, (Balachandran, K.K., Iyer, T.S.G., Madhavan, P., Joseph, J., Perigreen, P.A., Raghunath, M.R. and Varghese, M.D., Eds), p. 369, Society of Fisheries Technology (India), Cochin
- Sakasaki, R. and Donovan T.J. (1984) *Methods in Microbiology* **16**, 271
- Shimada, T., Sakasaki, R. and Oue, M. (1987) *J. Appl. Microbiol.* **62**, 452
- US FDA (1995) *Bacteriological Analytical Manual* 8thedn. AOAC International, MD, USA