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Role of Enrichment Period for PCR Detection of Salmonella in Seafood

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This communication presents the results of a study initiated to understand the role of enrichment period for detection of Salmonella by PCR assay. A total of 159 fresh and raw seafood (fish, shrimp, clam, crab, mussel, edible oyster, cuttlefish, and lobster) samples were analyzed for presence of Salmonella with the 0, 6, 12, 24, 36, and 48 h enrichment periods prior to PCR assay. The assay was performed with Salmonella specific PCR that amplifies a 284 bp invA gene fragment. Results obtained from PCR assay were compared with conventional culture method. The sensitivity of PCR assay was confirmed in spiked seafood samples with Salmonella cells in the range 2 to $2x10^6$cfu/25 g. Inhibition of seafood matrix on PCR was also determined. All seafood samples detected negative for Salmonella at 0 h PCR, whereas, 35.2% seafood samples found to be positive for Salmonella by 24 h PCR assay. Thereafter, incremental increase in enrichment period did not significantly enhance the detection efficiency of PCR assay. A total of 57/159 seafood samples were positive by PCR assay, whereas, 37/159 were positive by culture method. The sensitivity of PCR assay was found to be at 2 cfu/25 g level with the exception of cuttlefish samples. Detection limit of Salmonella dead cells was $3x10^6$cfu/250 ml in fish homogenate by 24 h enrichment followed by PCR assay. The 24 h enrichment preceeding the PCR assay can be used as an alternative and rapid technique for detection of Salmonella that gives greater efficiency and sensitivity as compared to the conventional method. Present study highlighted the remote possibility of Salmonella dead cells encountering as false positive in natural contaminated seafood samples. This PCR assay can be used in the routine analysis of seafood samples and would be an ideal step for implementation of seafood safety measure at harvest and post-harvest level.

Keywords : Salmonella, seafood, PCR, contamination, enrichment

Salmonella serovars are causative agents of the largest number of enteric infections to humans. Raw food and ready to serve products are the main routes of Salmonella transmission. The incidence of seafood borne salmonellosis has been reported all over the world. The U.S. Centre for Disease Control and Prevention has estimated 7.4% of imported and 1.3% of domestic seafood samples as contaminated with non-typhoidal Salmonella (Heinitz et al., 2000). Though, there is relatively high prevalence of Salmonella contamination in developing countries including India, due to poor documentation system, most of the outbreaks go unreported. Iyer & Shrivastava (1989), Nambiar & Iyer (1991) and Hatha & Lakshmanperumalasamy (1997) reported the prevalence of Salmonella in Indian seafood. The presence of Salmonella in fish and fishery products has also been reported from Thailand (Rattagool, 1990), Japan (Saheki et al., 1989) and Spain (Martinez-Urtaza et al., 2003).

Generally, Salmonella in food or seafood is isolated by conventional culture method and it involves pre-enrichment in lactose broth or buffered peptone water followed by selective enrichment, by differential plating and finally confirmation by routine biochemical and serological assays (Andrews & Hammack, 2001). This method is time

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consuming and requires five to seven days to confirm the results and hence has difficulty in analyzing large number of seafood samples. More recently, PCR based molecular technique has emerged as an alternative method for detectionof Salmonella in naturally contaminated foods. In most of the studies, PCR detection assay for Salmonella has been carried out with artificially contaminated samples (Lin & Tsen, 1999; Li & Mustapha, 2002; Wang & Yeh, 2002). In spiked conditions, cells are found to be in active and viable state and hence are easily detected by PCR method. In natural contaminated environment, as the cells are exposed to different stress factors, more time is required for multiplication. There are reports that bacteria present in food samples have reduced viability due to prolonged exposure to unfavorable conditions such as high salt concentrations, unfavourable pH, and exposure to sunlight (Gouws et al., 1998). Thus, incorporation of an enrichment period prior to PCR assay plays an important role in detection process of Salmonella serovars, particularly in food, in addition to other factors like sample DNA preparation, assay conditions and food matrix. These factors have been well demonstrated (Soumet et al., 1994; Lantz et al., 1998; Malorny et al., 2003a). Detection of Salmonella by PCR from meat and poultry foods after different enrichment periods has been reported (Fach et al., 1999; Ferretti et al., 2001; Chiu et al., 2005). However, no comprehensive studies have been carried out by involving different seafoods and assay parameters. The present study was performed to determine the optimum enrichment period required for the detection of Salmonella from seafood by PCR assay. The study also covered the sensitivity, inhibition and detection of dead Salmonella cells by PCR assay with different enrichment periods.

Materials and Methods

A total of 159 fresh seafood samples consisting of fish (n=47), shrimp (n=31), clam (n=21), crab (n=16), mussel (n=15), edible oyster (n=12), cuttlefish (n=10), and lobster (n=7) were collected from fish markets at Cochin (India). Seafood samples were analyzed for presence of Salmonella by PCR assay with 0, 6, 12, 24, 36 and 48 h enrichment periods.

Enrichment and PCR Assay for Salmonella in seafood

Each 25 g sample of seafood was homogenized with 225 ml buffered peptone water/lactose broth (Difco, USA) in a stomacher 400 (Seward Medicals, UK) for 30 seconds and subjected to incubation at 37ºC. Subsequently, template DNA was prepared from 1 ml of enriched seafood after incubation at 0, 6, 12, 24, 36 and 48 h. The sample DNA was extracted by boiling the cells with 250 ml of TE (Tris EDTA) buffer [10 mM Tris. HCl, 1mM EDTA (pH 8.0)] for 10 min, thereafter, cooled in ice and finally cell lysate was collected by centrifugation for 10000 g for 5 min at 4ºC. An aliquot of 5 µl DNA lysate was used as a template DNA for the assay and Salmonella specific invA gene primers were used for the assay (Rahn et al., 1992). A 25 µl of PCR mixture contained 0.4 mM concentration of each primer, 200 mM of dNTP (Finnzyme, Finland), 1X reaction buffer (20 mM Tris.HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$), 1U of Taq polymerase (Dynazyme II, Finland) and 5 µl of sample DNA to each PCR tube. DNA amplification was carried out in Mastercycler personal (Eppendorf, Germany) with an initial denaturation at 95ºC for 2 min followed by 35 cycles of 95ºC for 30 sec, 64ºC for 30 sec and 72ºC for 30 sec. A final extension of 5 min was employed at 72ºC. The amplified products and their size was determined by electrophoresis on 2% agarose gel. Gel images were captured by using gel documentation system (Alpha Innotech Corporation, USA). Each PCR assay was performed with a suitable negative (Escherichia coli) and positive (Salmonella enterica subsp. enterica serovar Typhimurium, ATCC 23564) controls. All seafood samples were analyzed for presence

of Salmonella by USFDA culture method with the help of key biochemical and serological tests (Andrews & Hammack, 2001).

Sensitivity of PCR assay

About 25 g each of fish, mussel, crab, oyster, clam, and cuttlefish samples, previously confirmed negative for Salmonella, were spiked with Salmonella Typhi, Salmonella Typhimurium, Samonella Enteritidis, Salmonella Mbandaka, Salmonella Bareilly, and Salmonella Breanderup, respectively, in the range of 2 to 10^6 cfu/250 ml. The PCR assay was carried out with 0, 6, 12, and 24 h enrichment period. The assay was performed in duplicate and count of Salmonella cells spiked into the samples was estimated by plating on xylose lysine desoxycholate agar (Oxoid, UK).

PCR inhibition assay

To assess the interference of seafood matrices during the PCR assay, a control assay was performed with Salmonella Typhimurium spiked into 250 ml Lactose broth so as to get cell concentrations ranging from 2 to 10^6 cfu/250 ml. PCR assay was carried out with 1 ml of Salmonella culture after 24 h of incubation and the results compared with spiked seafood samples. Samples which showed positive result with control, but negative with seafood were rated as inhibitory matrix.

Detection limit for dead cells of Salmonella

Fish samples, previously confirmed negative for Salmonella by both PCR and culture methods were used for detection of dead cells in PCR assay. Fish sample (25 g) was homogenized with 225 ml of buffered peptone water. Heatkilled Salmonella Typhimurium was mixed with fish homogenate at the range of $3x10^2$, 10^3, 10^4, 10^5, 10^6 and 10^7 cfu/250 ml and incubated at 37°C. Similarly, PCR assay was carried out with 5 ml of template DNA extracted from 1 ml of fish homogenate at intervals of 0, 6, 12, 24, 36 and 48 h and assayed in duplicate.

Results and Discussion

Detection of Salmonella serovars in seafood with the incorporation of an enrichment step prior to PCR assay has showed variable results with different enrichment periods. There was a substantial increase in Salmonella in seafood with an incremental increase in enrichment period. Without enrichment (0 h), PCR results showed all seafood samples negative for Salmonella. At 6 h enrichment, fish, shrimp, crab, clam, mussel, edible oyster and lobster samples were positive for Salmonella and, overall 20.1% of seafoods were found to be

Table 1. Detection of Salmonella in naturally contaminated seafood by PCR assay at different enrichment period

	tasted	0 h	6 h	12 h	24 h
Fish	47	0	10 (21.2)*	13 (27.6)	17 (36.1)
Shrimp	31	0	6 (19.3)	8 (25.8)	11 (35.4)
Clam	21	0	6 (28.5)	7 (33.3)	9 (42.8)
Crab	16	0	2 (12.5)	3 (18.7)	5 (31.2)
Mussel	15	0	4 (26.6)	6 (40.0)	6 (40.0)
Oyster	12	0	2 (16.6)	3 (25.0)	5 (41.6)
Cuttlefish	10	0	0	2 (20.0)	2 (20.0)
Lobster	7	0	1(14.2)	1 (14.2)	1 (14.2)
Total	159	0	32 (20.1)	43 (27.0)	56 (35.2)

* Figures in parenthesis denote percentage

positive for Salmonella. Further increase in enrichment periods by 12 and 24 h, showed sharp rise of 27% and 35.2% detection of Salmonella respectively (Table 1). The subsequent 12 h enrichment period showed insignificant improvement (<1%) in the assay and finally, 48 h enrichment had no further contribution towards the performance of PCR assay. A total of 35.2% seafood samples were detected positive for Salmonella by 24 h enrichment followed by PCR assay, whereas, culture method had 23.2% positive detection for Salmonella (Table 2). A comparison between PCR assay and culture method revealed that 63.5% of seafood samples were negative for Salmonella by both methods and 11.9% of seafood samples were positive by the PCR alone (Table 2). A representative of Salmonella positive results obtained from seafood is shown in Fig. 1.

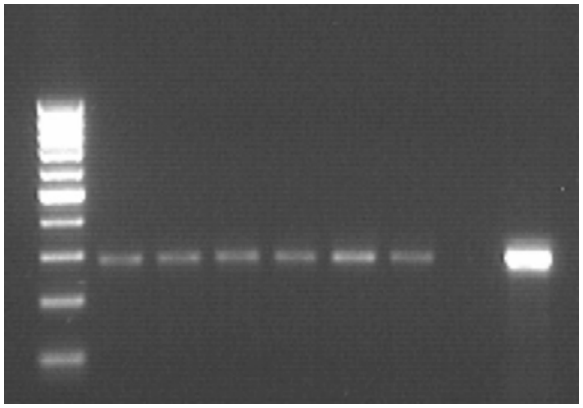

Fig. 1. Representative positives from Salmonella in naturally contaminated seafood by 24 h PCR assay. Lanes 1 to 6 contain positive from clam, crab, mussel, oyster, cuttlefish, and lobster, respectively, lane 7: Escherichia coli (negative control), lane 8: Salmonella Typhimurium ATCC 23564 (positive control), M: 100 bp DNA ladder

Homogenates of fish, mussel, crab, oyster, clam, and cuttlefish were spiked with different Salmonella serovars ranging from 2 cfu to 10^6 cfu/250 ml. Result showed the presence of Salmonella specific amplicon of 284 bp by 24 h enrichment followed by PCR in all dilutions of fish homogenates (Fig. 2). Similar results were obtained with spiked mussel, crab, clam, and oyster samples with the exception of cuttlefish. The results obtained from spiked samples were compared with control assay of Salmonella (without seafood) and visible inhibition was observed in PCR assay with cephalopods spiked with cells $\leq 10^2$cfu/250 ml (Fig. 3, 4).

Detection of Salmonella dead cells was carried out with heat killed (20 min, 100°C) Salmonella Typhimurium in fish homogenates. PCR assay for dead cells in fish homogenates spiked with $3x10^2$ and $3x10^4$ cfu/250 ml did not detect Salmonella at 0, 6, 12, 24, 36, and 48 h of incubation. Dead Salmonella cells inoculated at the level of $3x10^7$ cfu/250 ml were detected by PCR at all enrichment

Table 2. PCR assay in comparison with culture method (USFDA)

Sample Type	No. of sample	(PCR/ CM) [a]			
		+/+	-/-	-/+	+/-
Fish	47	9	30	0	8
Shrimp	31	8	20	0	3
Clam	21	8	12	0	1
Crab	16	4	11	0	1
Mussel	15	3	9	0	3
Oyster	12	3	7	0	2
Cuttlefish	10	2	6	2	0
Lobster	7	0	6	0	1
Total	159	37	101	2	19

[a] +/+ ; 24 h PCR positive/culture method (CM) positive, -/-; 24 h PCR negative/culture method (CM) negative.

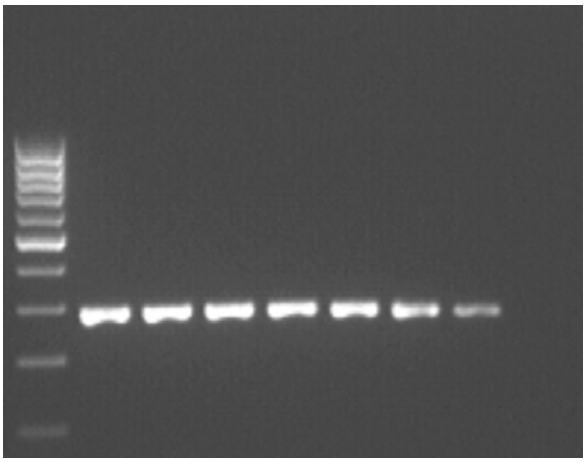

Fig. 2. PCR sensitivity assay in fish samples, Lanes 1 to 8: homogenate inoculated with Salmonella Typhi at different levels, $2x10^6$, 10^5, 10^4, 10^3, 10^2, 20, 2, 0 cfu /250 ml, M:100 bp DNA ladder

levels, whereas, PCR assays showed positive result for fish homogenate inoculated with dead cells $3x10^5$ cfu/250 ml at 0 h only and $3x10^6$ cfu/250 ml detected up to 0, 6, 12, 24 h of incubation (Table 3).

Detection of Salmonella serovars from seafood by PCR assay with the incorporation of enrichment step has played significant role in the detection assay. Present study attempts to reveal the impact of different enrichment period for detection of Salmonella with the application of different incubation period in seafood samples prior to PCR assay. The assay exhibited that involvement of 24 h enrichment period prior to PCR was an optimum step for detection of Salmonella in seafood. The enrichment step demonstrated that at zero hour PCR i.e. immediately after homogenization of the sample, all seafood samples were detected negative for Salmonella even though, Salmonella was present in many samples. Detection efficiency of PCR assay increased tremendously with the incremental increase in enrichment period and by end of 24 h enrichment, 35.2% seafood samples were positive for Salmonella. A further 24 h increase in incubation has not augmented the performance of assay, indicating that 24 h enrichment period was found to be the most suitable enrichment period for detection of Salmonella in seafood. The observation of PCR results was compared with conventional culture method and confirmed that PCR assay was found to be far superior to culture method for detection of Salmonella in seafood. The main advantage of PCR assay in seafood is the ability to screen large number of samples within 24 to 28 h, while the culture method needs 5-7 days to complete the analysis. Hence, development of PCR based methods is more desirable for rapid screening of Salmonella in seafood without compromising the specificity and sensitivity of the assay. Present study showed considerable difference in number of samples obtained positive for Salmonella by culture and PCR method. Results were in concurrence with similar studies where 10-15% superior results could be obtained by PCR assay as compared to the culture method (Fratamico, 2003;

Table 3. Detection of Salmonella dead cells (heat killed) in fish homogenate

Sl. No.	Fish Homogenate Inoculated (cfu/250ml)	PCR Result [a] at					
		0 h	6 h	12 h	24 h	36 h	48 h
1	$3x10^2$	-	-	-	-	-	-
2	$3x10^3$	-	-	-	-	-	-
3	$3x10^4$	-	-	-	-	-	-
4	$3x10^5$	+[b]	-	-	-	-	-
5	$3x10^6$	+	+	+	+	-	-
6	$3x10^7$	+	+	+	+	+	+

[a] Duplicate, [b] Weak positive

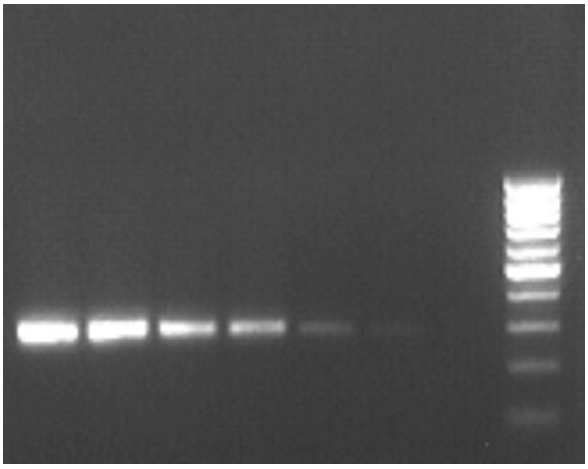

Fig. 3. PCR inhibition assay in cuttlefish samples, Lanes 1 to 7: homogenate inoculated with Salmonella Breanderup at different levels, $2x10^6$, 10^5, 10^4, 10^3, 10^2, 20, 2 cfu/250 ml, M: 100 bp DNA ladder

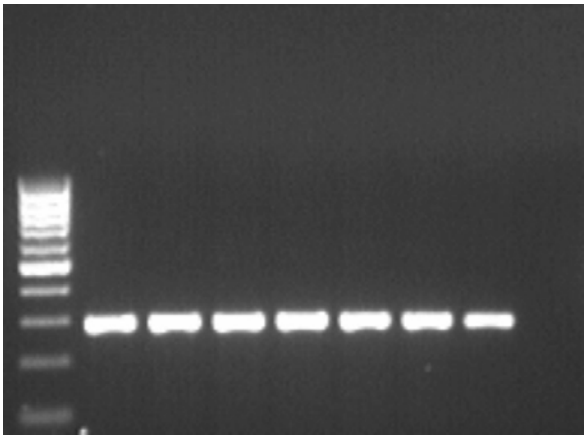

Fig. 4. PCR inhibition (control) assay without seafood, Lanes 1 to 8: buffered peptone water inoculated with Salmonella Breanderup at different levels, $2x10^6$, 10^5, 10^4, 10^3, 10^2, 20, 2, 0 cfu/250 ml, M: 100 bp DNA ladder

Lofstrom et al., 2004). The results obtained in this study was found to be quite diverse to the results reported by Shabarinath et al. (2007) in which 52% and 20% of the seafood samples from Mangalore (India) were positive for Salmonella by PCR and culture method, respectively. An earlier study reported the effect of pre enrichment on the sensitivity and specificity of PCR for Salmonella in raw poultry and demonstrated 100% similarity in PCR and conventional assay (Mint et al., 2006). In naturally contaminated samples, Salmonella are generally low in number, as proved in this study, thus, it is suggested that DNA preparation need to be carried out with mild extraction buffers (Soumet et al., 1994). The PCR amplicons obtained from naturally contaminated seafood were found to be comparatively feeble than spiked samples (Fig. 1). This could be due to the fact that in spiked samples, fresh and actively growing Salmonella cells were introduced, whereas in the naturally contaminated seafood samples cells may be stressed due to unfavourable conditions of food. The PCR assay was highly specific and gave 284 bp amplicon with all Salmonella serovars tested. Present results were in concurrence with an earlier report in which invA of 284 bp primer could detect almost all Salmonella serovars without any nonspecific product in Salmonella related strains (Malorny et al., 2003).

The degree of sensitivity of PCR assay in the artificially contaminated samples was significant and Salmonella was detected from homogenate inoculated with 2 cfu/250 ml. In all seafood samples tested, except in cuttlefish, the level of sensitivity was comparable. There was apparent inhibition in the PCR assay with artificially contaminated cuttlefish samples and this observation was further consolidated with PCR assay in naturally contaminated cuttlefish samples. Results revealed that PCR was more sensitive in fish, shrimp, clam, crab, oyster and lobster samples. However, in case of cuttlefish, culture method was found to be superior to PCR (Table 2). These observations suggested that sensitivity of PCR assay was dependent on nature of the food material and inhibitory effect must be ascertained well before the application of the assay in routine analysis.

The most disadvantageous aspect regarding PCR assay is that it may not reveal the nature of target DNA, whether it has originated from live or dead cells. However, researchers may be interested to know that the amplified product was originated from

live Salmonella cells. A positive result in PCR with presence of dead cells may cause a false alarm to the consumers. Hence, persistence of the dead Salmonella DNA in seafood was evaluated by PCR to understand the level at which dead cells could be detected in seafood. Present study demonstrated that dead cell $<10^5$ cfu/250 ml did not affect the PCR results and was not detected by PCR at 6 to 48 h of incubation. These observations further revealed that dead cells at the range $3x10^6$ cfu/250 ml were detected at all levels of incubation and suggested that dead Salmonella cells in this range would influence the PCR results. However, in natural conditions, it may be impossible to obtain dead cells at the range $3x10^6$ cfu/250 ml in seafood samples. Results were in compliance with the study elsewhere, which showed dead Salmonella cells (10^6 cfu/25g) in food samples with a PCR based commercial kit after 18 h enrichment period (Fach et al., 1999). The persistence of dead Salmonella DNA from an inoculum ($2x10^6$ Salmonella/ ml) in seawater was carried out by PCR assay and DNA was detected up to 4 days of incubation at 20°C (Dupray et al., 1997).

Present study demonstrated that the incorporation of an enrichment step prior to PCR plays vital role in the successful detection of Salmonella from seafood. The optimum enrichment period has not only increased the cell count by virtue of multiplication but also diminished the impact of Salmonella dead cells posing intimidation as a false positive for viable Salmonella in contaminated seafood. Accelerated and more sensitive PCR method for the detection of Salmonella serovars in seafood would be an ideal technique for seafood industry to have quick quality checks. The study recommends 24 h incubation for reliable and rapid detection of Salmonella in seafood. Relatively shorter enrichment periods are to be developed using combinations of different media supplement and growth stimulus factors.

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