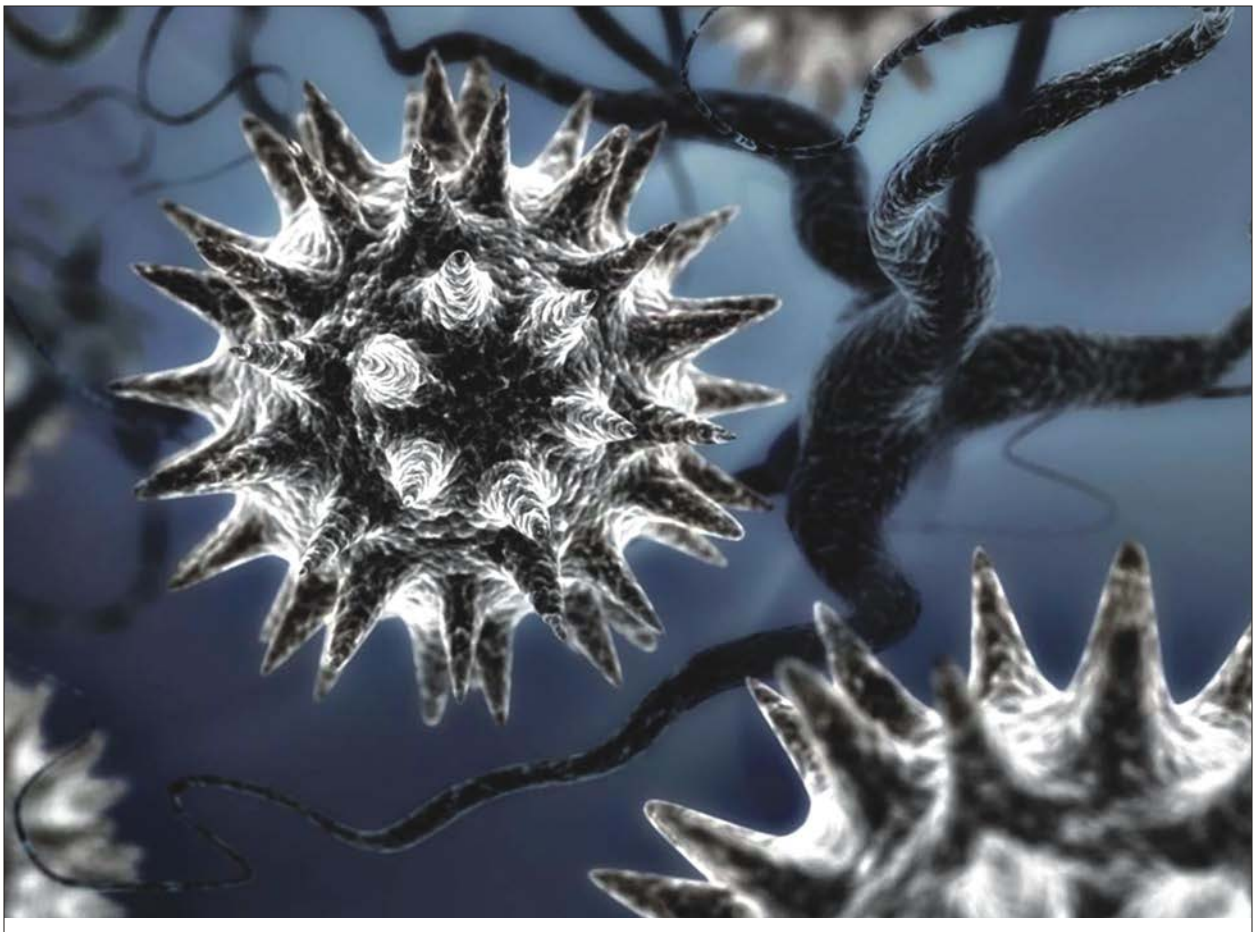

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Contamination of Seafood by Norovirus in India

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INTRODUCTION

Shellfish are known as an important source of food for humans and the disease caused by the consumption of shellfish containing pathogenic viruses of human origin is also well- documented (Lees, 2000). The shellfish concentrate viral particles as a consequence of their feeding process, i.e., the filtering of large volumes of water and, if pathogenic microorganisms are present in the water, the pathogens may accumulate to considerable levels (Burkhardt and Calci, 2000). In accordance with the European Directive on health conditions, the presence of salmonella and Escherichia coli number are routinely used to test for microbiological quality in shellfish. However, as enteric viruses are generally more resistant to inactivation in water sources and are removed slowly from bivalves by depuration processes (De Medici *et al*., 2001). Shrimps grown in sewage contaminated water accumulate pathogenic bacteria and viruses and pose health risk. Thus, shellfish and crustaceans act as potential carriers of viral and bacterial enteric pathogens (Beuret *et al*., 2003). In fact, outbreaks of viral gastroenteritis due to shellfish complying with the relevant European faecal coliform standards have been reported (Lees, 2000). A large number of shellfish-associated outbreaks, indeed, have been attributed to enteric viruses, particularly norovirus (NoV) (Hamano *et al*., 2005). Norwalk virus is the prototype strain of a group of human caliciviruses that are important etiological agents for the epidemic gastroenteritis that are now collectively called Norovirus. Norovirus contains a single-stranded RNA genome of positive polarity that is approximately 7.7 kb in length (Hardy and Estes, 1996). Norovirus appears to be the most common cause of gastroenteritis in humans world-wide and is highly infectious (Glass *et al*., 2000). It is usually spread through the faecal-oral route by the ingestion of contaminated food or water, (Atmar and Estes, 2001) but person-to-person transmission has also been documented (Becker *et al*., 2000). Commercial use of shellfish is an expanding industry, consequently increases the transmission of pathogens associated with shellfish. Noroviruses may enter the ocean or estuaries directly through the discharge of domestic sewage, sewage contaminated rivers and streams, ocean disposal of domestic sewage sludge, malfunctioning boat sewage disposal systems. Monitoring norovirus density in seafood in India should therefore be focused. Hence the present investigation is aimed to detect the noroviruses presence in seafood samples.

MATERIALS AND METHODS

Standard Norovirus Strain

Norovirus positive stool isolates were obtained from Christian Medical College, Vellore, India. The isolates were confirmed by polymerase chain reaction using respective primers

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Table 1: Primer sequences used in RT-PCR evaluation

Primer	Orientation	Target genogroup(s)	Genomic location	DNA sequence
NI	Sense	I	4756-4776	GAATTCCATCGCCCACTGGCT
		II	4492-4512	
E3	Antisense	I	4869-4853	ATCTCATCATCACCATA
		I	4605-4588	

(Table 1) and were used as positive template. PCR reactions were carried out on a Perkin-Elmer Cetus DNA Thermo cycler 2400 (Perkin- Elmer Cetus Corp., USA).

Sample Collection

Twenty three shellfish samples comprising Bivalve molluscan Clams (*Meritrix meritrix*), Crab, Prawns (*Penaeus monodon*) and finfish (*Red snapper, Lutjanus* sp.) were collected from several regions such as Pulicat Lake, Kasimedu, Muttukadu backwaters, Kovalam, Buckingham canal and Ennore creek, Tamil Nadu, India between December 2006 and June 2007. In addition, 27 samples were obtained from seafood vendors. The samples were brought to the laboratory in chilled condition and processed immediately. Live clams were washed with sterile water and opened with a sterile shucking knife to collect the flesh and intravalvular fluid into a sterile beaker. Crab and finfish samples were washed with distilled water and opened with a sterile shucking knife to collect the flesh.

Concentration of Norovirus

Norovirus from seafood samples were concentrated by polyethylene glycol precipitation method (Lewis and Metcalf, 1998). Ten gram of tissue (Fin fish/Clam/Crab/ Prawn) were homogenized with 50 mL of glycine buffer (pH 9.0) and were centrifuged at 5000 rpm for 10 min at 4°C. The pH of the supernatant was adjusted to 7.2 and then mixed with 20% polyethylene glycol 8000 before incubating overnight at 4°C. The incubated supernatant was centrifuged at 6200 rpm for 20 min at 4°C and the pellets were resuspended in 0.15 M Na_2HPO_4 (pH 9.0). The pH was adjusted to 7.2 and stored at -20°C.

Extraction of RNA

RNA was extracted from 200 μL of a PEG suspension by binding to size-fractionated silica particles in the presence of guanidinium isothiocyanate (Boom *et al*., 1990). The RNA was eluted in 49 μL of RNAse free distilled water and 1 μL of RNAsin (Biocorporals, India). RNA was either used directly in RT-PCR or stored at -70°C.

Reverse Transcriptase PCR Evaluation

cDNA Synthesis

cDNA synthesis was carried out using cDNA synthesis kit/Revertaid kit (Biogene UK/Fermentas, USA). Five microliter of purified template was added to 1 μL of 10 mM of random hexamer and 0.3 μM each of dATP, dCTP, dGTP and dTTP in a final reaction volume of 12 μL and incubated at 65°C for 5 min and then snap-cooled. 5X RT buffer (components containing 1× First Strand Buffer® 0.01 M dithiothreitol) 40 units of RNA inhibitor, were added to the chilled primer/template mixture making a final volume of 19 μL. This mixture was incubated at 42°C for 2 min before the addition of 1 μL of Reverse Transcriptase (Fermentas, USA). The reactions were then incubated for 50 min at 42°C followed by 70°C for 15 min.

Conventional RT-PCR

Five microliter cDNA was added to 35 μL of PCR master mix (10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 20 pmol of each primer (NI forward primer and E3 reverse primer), 1 U of *Taq* polymerase). After an initial denaturation at 94°C for 2 min, 30 amplification cycles of 95°C for 1 min, 40°C for 1 min and 72°C for 1 min were performed followed by a final extension of 72°C for 10 min.

Gel Electrophoresis and Southern Hybridization of PCR Products

Twenty microliter of reaction mix of RT-PCR amplicons were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide at 10 V/cm for 1.5 to 2 h and visualized with UV light. The PCR amplifies a 113 bp region of the RNA polymerase gene. Molecular weights were determined by comparison with a 100bp DNA ladder (Genei Bangalore, India). Hybridization of the PCR products was performed for further confirmation. The blot was incubated with anti dioxigenin alkaline phosphatase conjugate antibody solution (made up in Buffer 1) for 30 min at room temperature. The blot was subsequently washed with Buffer 2 twice for 15 min and then with assay buffer for 3 min. Finally, the chemiluminescent substrate 3-(2′-spiroadamantane)-4-methoxy-4-(3″- phosphoryloxy)-phenyl-1,2 dioxentane (AMPPD) (Tropix Inc, Bedford, MA, USA) was added to the assay buffer and the blot was developed within 1 h by exposing filters to X-ray film (Kodak, India) for 40 min at room temperature and chromogenic detection of the hybrids with nitroblue tetrazolium (NBT) and BCIP (5-bromo-4-chloro-3-indolylphosphate) was also performed.

Bioanalyzer

Preparing Gel Dye Mix

DNA dye concentration and DNA gel matrix were allowed to equilibrate at room temperature. Twenty five microliter of dye concentration was added to DNA gel matrix, vortexed and transferred to spin filter and centrifuged at 2240x g for 15 min.

Loading the Gel Dye Mix

The gel dye was allowed to settle at room temperature for 30 min. A new DNA chip was placed on the chip priming station. Nine microliter of gel dye mix was pipetted into the well marked as G and the chip priming station was closed. The plunger was pressed down until it was held by the chip for 60 sec. After 5 sec the plunger was pulled back slowly to 1 mL position. The chip priming station was opened and 9 μL of gel dye was pipetted into the well marked G and 1 μL of ladder was added to the well labeled ladder.

Loading of the Sample

Five microliter of marker was pipetted into all 12 sample wells and in ladder well. One microliter of sample was added into the well. The chip was placed in the Laser Induced Fluorescent instrument (LIF) and the results were interpreted. The Bioanalyzer produces the electropherogram at the end of each run showing the exact base pair (bp), concentration and molarity of each sample against the specific Fluorescent Unit (FU). Hence the exact base pair as well as the purity of the amplified product along with the marker DNA can be monitored.

Norovirus Cloning, Sequencing and Phylogenetic Analysis

Since, water may contain multiple virus strains (Lodder *et al*., 1999) each norovirus-positive RT-PCR product was cloned and selected for sequence analysis. PCR products were purified with purification kit (QIAGEN, Germany). The purified PCR products were cloned using the TOPO TA Cloning® system (Invitrogen, India) according to the manufacturer's instructions. After transformation, minimum of five positive colonies were

selected. Selected colonies were grown in LB medium containing 50-100 μg mL^{-1} ampicillin for overnight. Plasmid DNAs were isolated and were sequenced to confirm the presence of the insert using automatic sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems, MWG, Bangalore, India). Sequences were compared with other sequences in genbank using Basic Local Alignment Search Tool (BLAST) family of programs on the World Wide Web service of National Centre for Biotechnology Information (NCBI), USA (http://www.ncbi.nlm.nih.gov). Multiple sequence alignment was performed using Tree view.

RESULTS AND DISCUSSION

Detection of Norovirus by Conventional RT-PCR

Diarhea is one of the commonest diseases amongst children and accounts for the highest cause of infant mortality worldwide, resulting in a significant public health impact, particularly in developing countries (Kilic *et al*., 2007). Gastroenteritis disease account for an estimated 12,600 child deaths each day in Asia, Africa and Latin America. Etiological agents associated with acute diarrhea in tropical countries include viruses, bacteria and parasites (Bonkoungou *et al*., 2008; Djeneba *et al*., 2007). Without a doubt, countries that have inadequate infrastructure to support a growing population, coupled with poor wastewater treatment facilities and improper sanitation mechanisms or systems, will always be prone to gastroenteritis infection (Rustam *et al*., 2006).

Anthropogenic environmental effects increasingly result in the release of faecal pathogens to estuarine and marine environments. Pathogen sources include boat waste discharge, floods that flush wastes into these waters, or the release of untreated or improperly-treated sewage (Richards, 2001). The shellfish samples collected from Kovalam, Buckingham Canal, Muttukadu, Pulicate lake, Royapuram market, Washermanpet market, Kasimedu market and Tondiarpet market were positive for norovirus. Figure 1 shows the agarose gel photograph of norovirus detected from the seafood samples by RT-PCR. From the results, it was found that out of 50 samples, 12 samples were positive for norovirus and remaining 38 samples were negative for norovirus. Prevalence of the norovirus was noticed among 16% of shellfish samples collected directly from the sampling sites and 8% of shellfish samples collected from seafood vendors. However, finfish samples were negative for norovirus. Literature survey clearly indicates that clams are sedentary filter feeding organisms obtain food by filtering a large volume of water and in the process they accumulate particulate matter including pathogenic bacteria and viruses (Sobsey and Jaykus, 1991). While both pathogenic viruses and bacteria are bioconcentrated, viruses are known to persist within bivalve tissues well beyond the 2 or 3 day period used to depurate bacterial contamination (Kingsley and Richards, 2003). Thus, the microbial contamination level in their tissues is considerably greater than in the surrounding waters (Rippey, 1994). Fecal viruses are more resistant to sewage treatment processes to the extent that even proper sewage treatment may not completely inactivate all sewage-associated viruses (Tree *et al*., 2005). Crabs and Prawns acquire enteric virus through their feeding habitat (Di Girolamo *et al*., 1972) and these enteric viruses can survive for fairly long time in water column and in association with particulate matter and accumulating sediments, these environments serve as reservoirs of infective viral particles (Girones *et al*., 1989; Callahan *et al*., 1995; Gantzer *et al*., 1998; Formiga-Cruz *et al*., 2002). Shrimps grown in sewage contaminated water may accumulate pathogenic bacteria and viruses and may pose health risk. Thus, shellfish and crustaceans act as potential carriers of viral and bacterial enteric pathogens (Beuret *et al*., 2003).

Foodborne transmission of NoV is typically due to contamination caused by people handling the food rather than by contaminated raw materials however, not in the case of

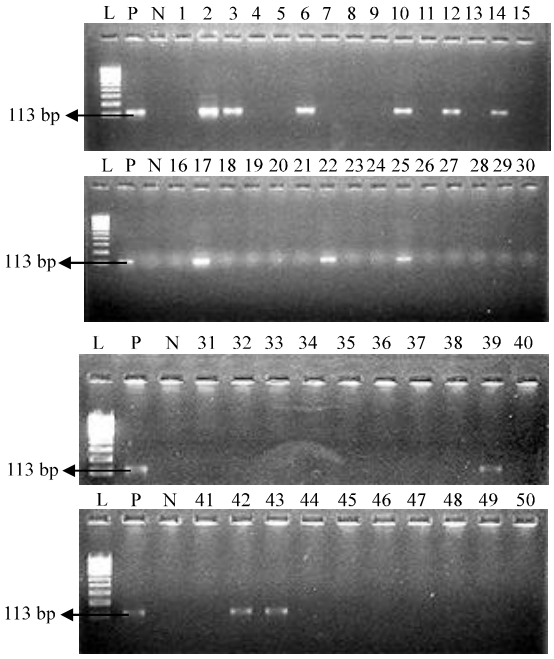

Fig. 1: Detection of Norovirus by RT- PCR in Seafood samples. Lane L: 100 bp marker, Lane P: Positive control, Lane N: Negative control, Lane 2, 3, 6, 10, 12, 14, 39, 42 and 43: Positive for norovirus, Lane 1, 4, 5, 7, 8, 9, 11, 13,15, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 44, 45, 46, 47, 48, 49 and 50: Negative for norovirus

shellfish. Several outbreaks of gastroenteritis due to NoV have been associated with consumption of shellfish grown in faecally contaminated waters (Lees, 2000). Mammalian viruses are extremely specific for both species and tissue, the source of human norovirus virus contamination of shellfish harvesting beds is always due to human faecal pollution.

Detection of Norovirus using Southern Transfer Hybridization of PCR Products

PCR products of 50 seafood samples were also analyzed by southern hybridization technique. The RNA extracted from the seafood samples were amplified using the norovirus specific primers NI/E3, blotted on a nylon membrane and probed using the digoxigenin labelled norovirus probe. Figure 2 shows the southern hybridization of the PCR products amplified from seafood samples. Analysis of the PCR products by hybridization using the specific probes showed that 12 samples were positive for norovirus and remaining 38 samples were negative for norovirus. Hence the results of southern hybridization confirm the results of agarose gel electrophoresis (Table 2).

Detection of Norovirus by Bioanalyzer

The amplified PCR products were subjected to Bioanalyzer to know the exact molecular size and purity of amplified product. The molecular size of the PCR products of NI and E3 primers was 113 bp. Figure 3 shows the representative seafood samples detected by bioanalyzer to detect the exact base pair (bp) of the amplified PCR product. Figure 4 represents the electropherogram of marker DNA ranging from 15 to 1,500 bp while Fig. 5a and b is the electropherogram run for the positive and negative control of the standard norovirus

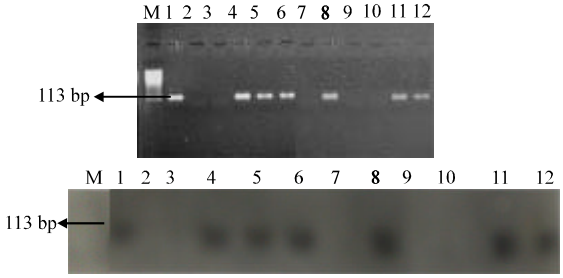

Fig. 2: Southern hybridization of the PCR products amplified from seafood samples. M: 100 bp DNA marker, 1: Positive control, 2: Negative control and 3 to 12: Seafood samples

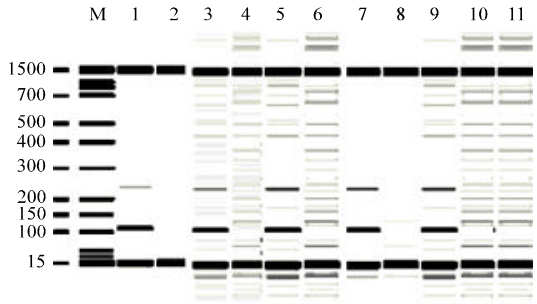

Fig. 3: Representative seafood samples detected by bioanalyzer

Table 2: Results of the detection of Norovirus in Sea food samples collected from various sampling sites

Sampling site	RT-PCR	S hybridi zation	Sampling site	RT-PCR	S. hybridi zation
Pulicat lake crab	-	-	Royapuram market prawn	-	-
Pulicat lake clam	+	+	Saidapet market prawn	-	-
Pulicat lake prawn	+	+	Saidapet market crab	-	-
Pulicat fin fish	-	-	Saidapet market finfish	-	-
Kasimedu crab	-	-	Chrompet market prawn	-	-
Kasimedu prawn	+	+	Chrompet market crab	-	-
Kasimedu finfish	-	-	Chrompet market finfish	-	-
Muttukadu crab	-	-	Tambaram sanatorium market crab	-	-
Muttukadu prawn	-	-	Tambaram sanatorium market prawn	-	-
Muttukadu clam	+	+	Tambaram sanatorium market finfish	-	-
Muttukadu finfish	-	-	Chinthadripet market clam	-	-
Kovalam crab	+	+	Chinthadripet market crab	-	-
Kovalam prawn	-	-	Chinthadripet market prawn	-	-
Kovalam clam	+	+	Tondiarpet market prawn	+	+
Kovalam finfish	-	-	Tondiarpet market crab	-	-
Buckingham canal crab	-	-	Tondiarpet market finfish	-	-
Buckingham canal clam	+	+	Washermanpet market crab	+	+
Buckingham canal prawn	-	-	Washermanpet market prawn	+	+
Buckingham canal fin fish	-	-	Washermanpet market finfish	-	-
Ennore creek clam	-	-	Besant Nagar market crab	-	-
Ennore creek crab	-	-	Besant Nagar market prawn	-	-
Ennore creek prawn	+	+	Besant Nagar market finfish	-	-
Ennore creek finfish	-	-	Triplicane market crab	-	-
Royapuram market finfish	-	-	Triplicane market prawn	-	-
Royapuram market crab	+	+	Triplicane market finfish	-	-

+: Positive for norovirus, -: Negative for norovirus

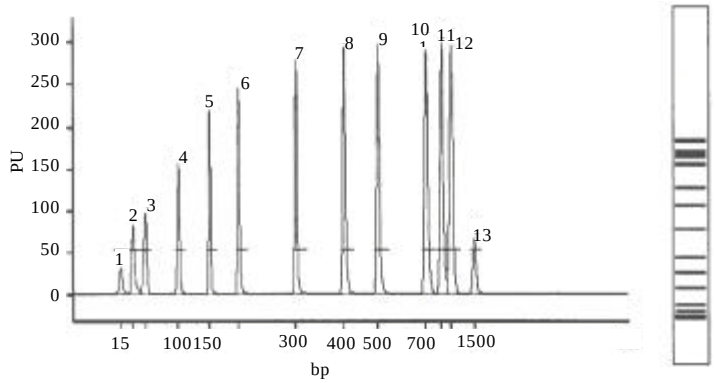

Fig. 4: Electropherogram of marker DNA

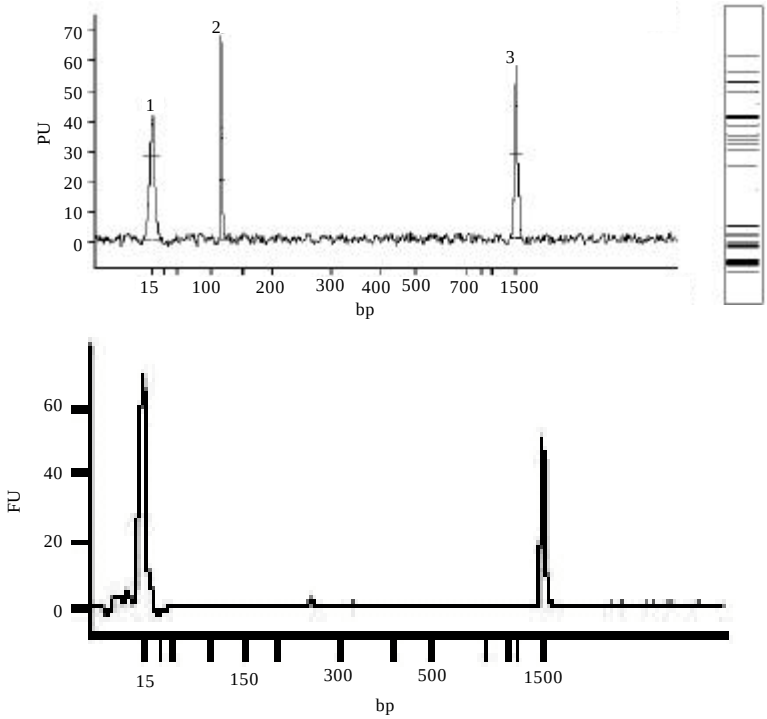

Fig. 5: Electropherogram of (a) positive and (b) negative control

used for the entire study. Electropherogram of the shellfish samples detected the exact molecular size of PCR products of Crab, Clam and Prawn as 116 (Fig. 6), 117 (Fig. 7) and 114 bp (Fig. 8, 9), respectively. Finfish samples collected from different sites show negative for norovirus Fig. 10.

PCR Amplicon Cloning

Representative RT-PCR amplicons for NoV generated from shellfish samples were cloned using a TA cloning system [TOPO®, Invitrogen, UK] as previously described

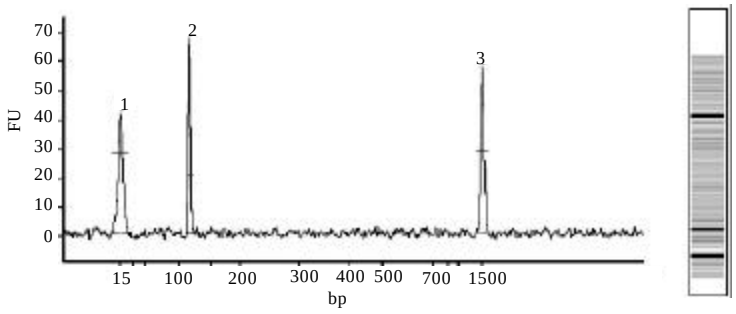

Fig. 6: Electropherogram for Crab sample collected from Kovalam

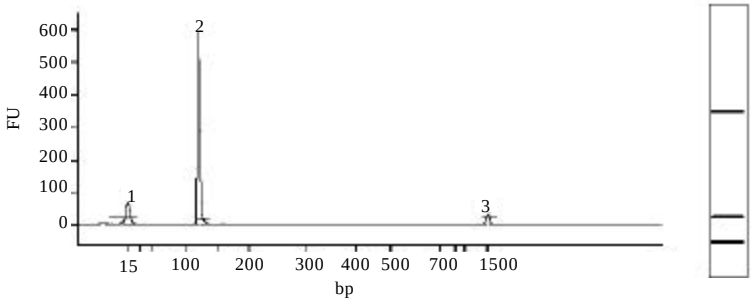

Fig. 7: Electropherogram for Clam sample collected from Pulicat lake

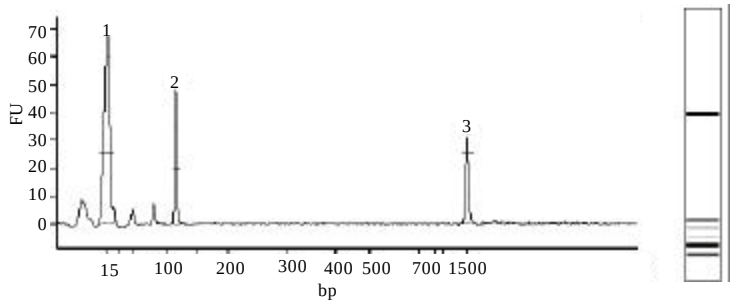

Fig. 8: Electropherogram for Prawn sample collected from Ennore creek

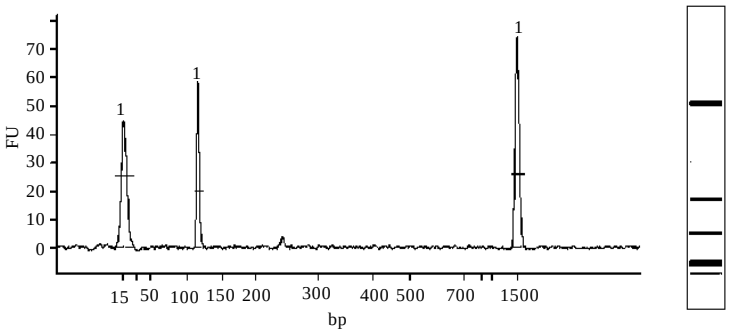

Fig. 9: Electropheogram for Prawn sample collected from Tondiarpet market

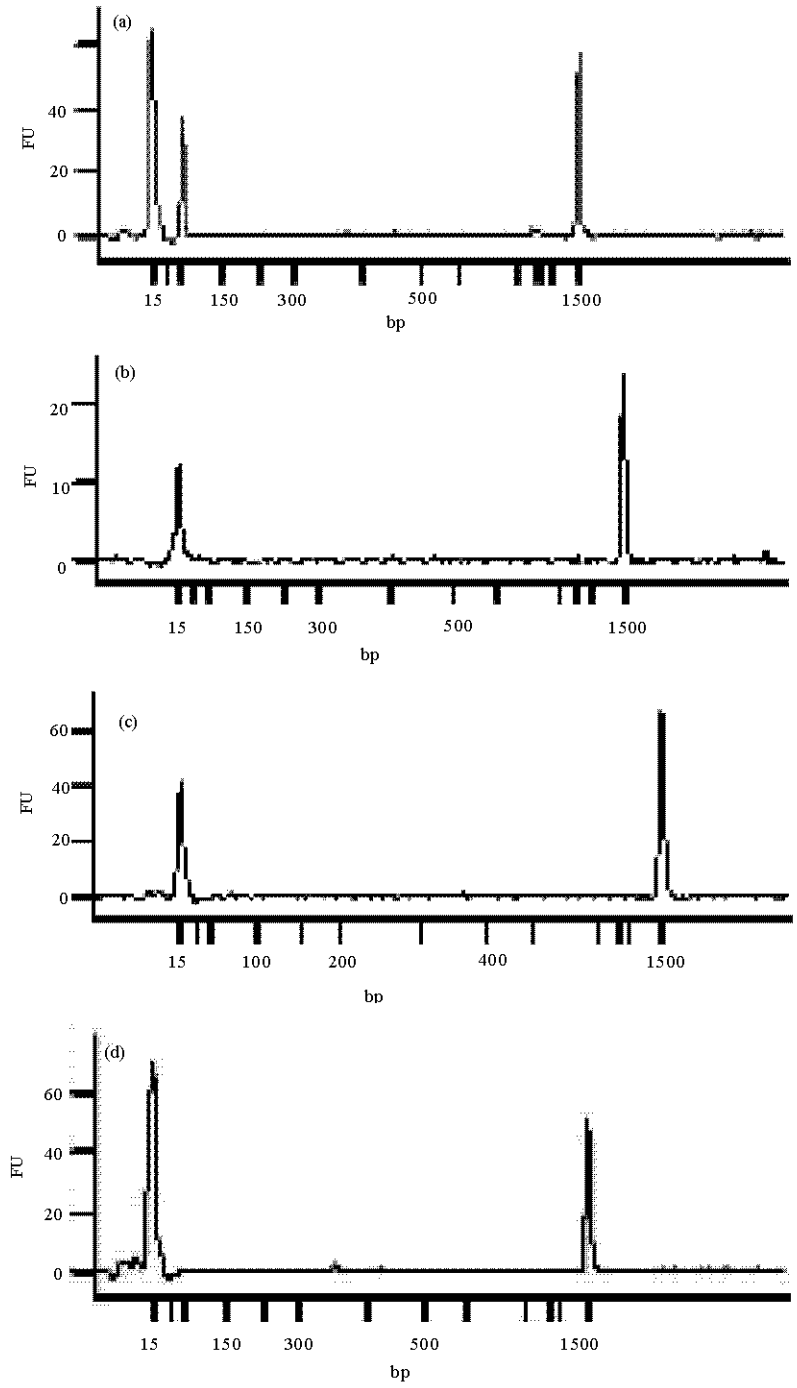

Fig. 10: Electropherogram for the detection of norovirus from finfish collected at (a) Pulicat lake, (b) Kovalam, (c) Muttukadu and (d) Kasimedu

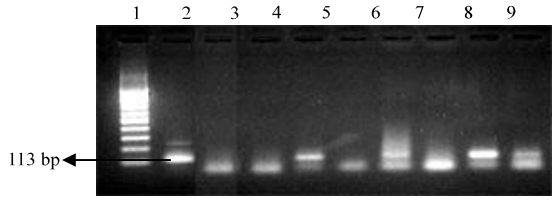

Fig. 11: Agarose gel profile of the PCR amplified products from the transformants. Lane 1: DNA marker, Lane 2: positive control (Norovirus), Lane 3: negative control and Lane 4 to 9: different transformants

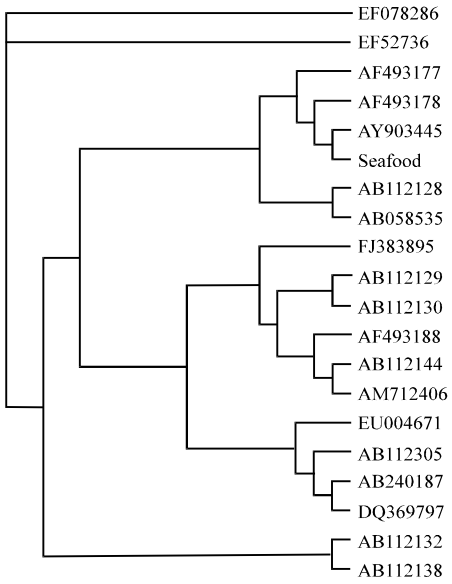

Fig. 12: Phylogenetic tree illustrating the genetic relationship of norovirus isolates from seafood samples of India

(Leoni *et al*., 2003). Duplicate plates of the transformant were made and the colonies were picked and processed directly for PCR. The PCR was performed using the insert specific primers NI and E3. Figure 11 shows the agarose gel profile of the PCR amplified products from the transformants. The expected PCR products of 113 bp specific for NI and E3 primers were obtained which confirms the successful transformation of norovirus.

Nucleotide Sequencing

Plasmid DNAs were isolated and were sequenced to confirm the presence of the insert. NoV amplicon (100 bp segment) was characterized for genogroup, genotypes and genetic relationship with the reference strains based on their capsid regions classification scheme (Altschul *et al*., 1997). Their partial nucleotide sequences were compared to each other as well as to reference NoV strains available in the DDBJ DNA/GenBank database by BLAST. The nucleotide sequence of the 5' ends of the NoV capsid gene was determined by direct sequencing with the amplified fragments. All of the NoV sequences showed highest identity of 77-94% and were classified into genogroup I. The Phylogenetic tree illustrating the genetic relationship of norovirus isolates from seafood samples of India is shown in Fig. 12.

Sequenced NoVs were very similar at the amino acid level and were most closely related to the Hu/NLV/GI/684/US, Hu/NV/I/Hualien/LWT/2003/TW, Hu/NLV/GI/464/US, NV/Saitama T83GI/02/JP, NV/Saitama T67GI/02/JP, NV/Saitama T62GI/02/JP, NV/Saitama T61 GI/02/JP, NV/Saitama T59GI/02/JP, strain.

CONCLUSION

Among the 50 samples analyzed, 24% of shellfish samples were contaminated by norovirus. Norovirus is known to be environmentally resistant and retains pathological effect for a prolonged period. In the present study the detection of viral RNA in seafood samples indicates the viral particle is still intact since naked RNA is rapidly degraded in the environment. Therefore, detection of viral RNA in environmental samples confirms that it is potentially infectious. Shellfish can act as vehicles of enteric viruses, when present in polluted aquatic environment. Therefore, virological monitoring of the seafood could prevent the outbreak of gastroenteritis in India.

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