

Prevalence of *Rotavirus* in shellfish from Southern Kerala

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Abstract

Aim: To study the prevalence of *Rotavirus* in shellfish from Southern Kerala.

Materials and Methods: The shellfish samples after processing was concentrated using proteinase K. RNA was isolated from the concentrated samples using phenol chloroform method. Rota viral RNA was detected using reverse transcription-polymerase chain reaction.

Results: A low prevalence of 2.5% (5/200) was obtained during the study. *Rotavirus* was detected in 2 out of 60 brown mussels (3.33%), 2 out of 70 yellow clams (2.86%) and 1 out of 70 black clams (1.43%).

Conclusion: Low prevalence of *Rotavirus* was obtained in our study. A more extensive study need to be conducted to estimate the prevalence of enteric virus in shellfish.

Keywords: concentration, proteinase K, reverse transcription-polymerase chain reaction, *Rotavirus*, shellfish.

Introduction

Globally, diarrhoeal diseases account for about one in six deaths among children younger than 5 years of age [1]. In India 2.3 million annual deaths are reported among children of which 334,000 are attributable to diarrhoeal diseases [1-3]. In developed and developing world, *Rotavirus* constitutes the foremost cause of severe diarrhea in children [3]. *Rotavirus* infection is responsible for 90,000-153,000 child deaths in India each year [4].

More than 140 viruses are present in human faeces [5] which can contaminate water bodies including shellfish growing areas. These enteric viruses cause a wide spectrum of illnesses in man including hepatitis, gastroenteritis, meningitis, fever, rash, conjunctivitis, and may be diabetes or severe acute respiratory syndrome. Shellfish which are important source of food for humans can concentrate these viruses due to their filter feeding process [6]. These poses a public health threat as shellfish are often consumed raw or improperly cooked. A number of human enteric viruses including *Norovirus*, Aichi, *Rotavirus*, *Enterovirus*, adenovirus, astrovirus, *Sapovirus*, hepatitis A and hepatitis E virus have been detected in shellfish, but only a few reports have been linked to shellfish borne outbreaks [7,8]. Though surveillance studies indicate the presence of *rotaviruses* in bivalve

shellfish and aquatic water bodies [9-11] no shellfish borne outbreaks related to rota virus has been reported which may be due to absence of symptoms in adult population who are primary consumers of seafood rather than children.

The main objective of the study was to assess the prevalence of *Rotavirus* in shellfish from Kerala as previous reports has indicated the high incidence of *Rotavirus* in children from Kerala for which shellfish consumption may be a possible source.

Materials and Methods

Ethical approval

All the procedures have been carried out in accordance with the guidelines laid down by the Institutional Ethics Committee and in accordance with local laws and regulations.

Samples

A total of 200 shellfish samples were collected from October 2011 to February 2012. Samples were collected from various parts of Kollam and Trivandrum districts in Kerala. Three species of shellfish (brown mussel, yellow clam and black clam) were collected during the period (Table-1). Yellow clams were obtained from several retail markets in Kollam and were from Ashtamudi Lake. Black clams were collected directly from T S Canal in Kollam. Brown mussels were collected from various retail markets in Trivandrum. The samples were collected in sterile plastic bags, properly labelled and immediately transferred to the laboratory under chilled conditions and stored at -20°C under further processing.

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Table-1: Shellfish samples.*

Species	Source	Numbers
Brown mussels	Kovalam	20
	Vizhingam	20
	Poovar	20
Yellow clams	Vaadi market	40
	Thevalli	30
Black clams	T S Canal	70
	Total	200

*Collected from various parts of Kollam and Trivandrum districts in Kerala

Processing of samples

The shellfish was washed in running tap water. The shells were shucked. The digestive diverticulum were cut into small pieces, homogenized with hand pestle and transferred to a 15 ml centrifuge tube for concentration and extraction of virus RNA.

Concentration of virus

A volume of 1 ml of proteinase K (100 µg/ml) (Fermentas) was added to the centrifuge tubes containing 1 g homogenized shellfish tissue. It was then kept in shaking the incubator at 180 rpm at a temperature of 37°C for 1 h. Proteinase K was inactivated by incubating in the water bath at 65°C for 15 min. The supernatant was collected after centrifugation at 3000 rpm for 5 min and stored at -20°C.

Extraction of RNA from concentrated samples

An aliquot of 1 ml of supernatant was treated with 0.1 volumes of 10% of sodium dodecyl sulphate and incubated for 1 h at 56°C in the water bath. To this equal volume of phenol-chloroform-isoamyl alcohol mixture in a ratio of 25:24:1 was added. It was vortexed and centrifuged at 12,000 g for 15 min. at 4°C. The upper aqueous layer was carefully transferred to another tube without disturbing the interphase. It was mixed with equal volume of chloroform-isoamyl alcohol (24:1) and vortexed, then the mixture was centrifuged again at 12,000 g for 10 min and upper clear aqueous phase transferred to fresh microcentrifuge tube. To the aqueous phase 0.1 volume of 3 M sodium acetate and equal volume of isopropanol were added and mixed gently. The samples were kept at -20°C overnight for RNA precipitation. The RNA was pelleted by centrifugation at 10,000 g for 15 min. The pellet obtained was washed with 1 ml of 70% chilled ethanol to remove excess salts by centrifuging it at 10,000 g for 5 min. The pellet was air dried and suspended in 20 µl nuclease free water or RNA storing solution (Amresco), heated at 56°C for 5-10 min. to dissolve the pellet and stored at -20°C.

Reverse transcription-polymerase chain reaction (RT-PCR) for detection of *Rotavirus*

Preparation of cDNA

PCR tubes (0.2 ml) were taken on ice and reaction mix was prepared as follows- 1 µl each of forward and reverse primer (rota 1 and rota 2) (Table-2), 1.5 µl dimethyl sulfoxide, 5.5 µl NFW and 5 µl of

dsRNA for single reaction. The tubes were spun and boiled at 95°C for 5 min in a thermocycler (Eppendorf, Germany) and then snap chilled on ice. Following master mixture was separately prepared and dispensed in tubes containing RNA and primers. Master mixture was prepared by adding 2.5 µl of ×5 RT buffer, 2.0 µl of 0.1 M dithiothreitol, 0.4 mM dNTP, 0.5 µl RNAase inhibitor (40 U/µl), 1 µl of M-MuLV RT (20 U/µl) and 4 µl NFW (Fermentas). After brief centrifugation, the tubes were loaded in thermocycler under the following conditions of 37°C for 60 min and 65°C for 10 min.

PCR assay

PCR mixture containing the following components was prepared- 2.5 µl of ×10 PCR buffer without MgCl₂ (Fermentas), 1.5 mM MgCl₂ (Fermentas), 0.2 mM dNTP (Fermentas), 1 µl each of forward and reverse primers (10 pmol/µl)(GCC), 0.2 µl *Taq* DNA polymerase (5 U/µl)(Fermentas) and 13.3 µl NFW (Fermentas). To the PCR mixture 5 µl of cDNA was added. The tubes were briefly centrifuged and loaded on to thermal cycler, and the cycling conditions were set as follows. After an initial denaturation of 5 min at 94°C, 30 cycles each of 1 min denaturation at 94°C, annealing of 1 min at 55°C and 2 min extension at 72°C was carried out followed by a final extension for 7 min at 72°C. The PCR products were stored at -20°C until further use. PCR product was visualized using agarose gel electrophoresis.

Results

Of the total 200 shellfish samples screened by RT-PCR for the presence of *Rotavirus* five samples were positive with an overall prevalence of 2.5%. (Figure-1) *Rotavirus* was detected in 2 out of 60 brown mussels (3.33%), 2 out of 70 yellow clams (2.86%) and 1 out of 70 black clams (1.43%). *Rotavirus* could be detected only in brown mussel collected from Vizhingam, yellow clam from Vaadi market and black clam from T S Canal as depicted in the Table-3.

Discussion

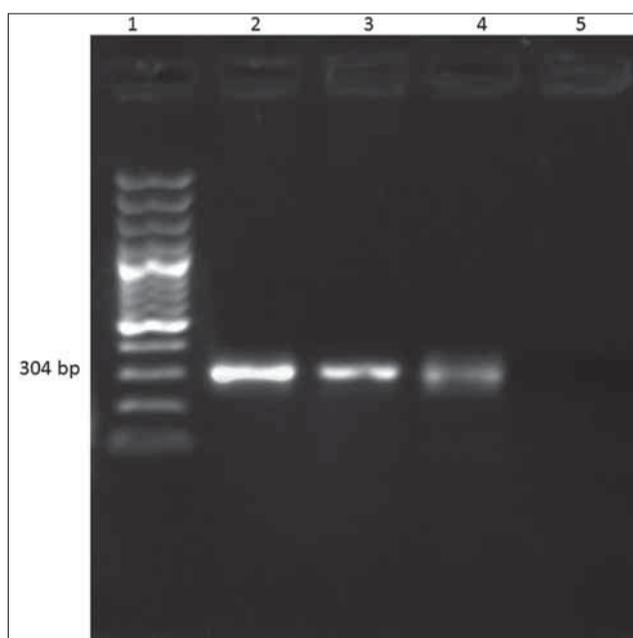
Rotaviruses are the main etiological agent of viral gastroenteritis in infants and young children. Many viruses transmitted by the faecal-oral route are widely prevalent in the community, and infected individuals can shed millions of virus particles in their faeces. Concentrations of 3×10^5 - 5×10^{11} viral particles/g of stool have been reported for *Rotavirus* [13] while the infective dose is as low as 10-100 virus particles [14]. Shedding of large quantities of virus in stool begins 2 days before the onset of diarrhoea and last for up to 10 days after onset of symptoms. Enteric virus including *Rotavirus* can thus contaminate shellfish growing areas from a number of sources including septic tank leakages, boat discharges, overflows and spills from sewage treatment plants seepage from sewage reticulation networks and accidental contamination after heavy rainfall.

Table-2: Primers used in the study.

Virus	Gene	Primer name	Primer sequence	Product size	Reference
Rota virus	Vp7	Rota 1	5'GATCCGAATGTTGTGTAAATCCAAT3'	304 bp	[12]
		Rota 2	5'AATTCGCTACGTTTCTCTTGG3'		

Table-3: Prevalence of Rotavirus in shellfish by RT-PCR.

Species	Source	Number of screened	Number of positive
Brown mussel	Kovalam	20	Nil
	Vizhingam	20	2
	Poovar	20	Nil
Yellow clams	Vaadi market	40	2
	Thevalli	30	Nil
Black clam	T S Canal	70	1
		200	5

**Figure-1:** Shellfish samples positive for *rotavirus*. Lane 1: 100 bp ladder. Lane 2: Positive control. Lane 3-4: Positive shellfish samples. Lane 5: Negative control.

Enteric viruses in contrast to enteric bacteria persist in shellfish for an extended period which will have a significant impact on public health. Viruses are mainly concentrated in the pancreatic tissue, also called digestive diverticula. Virus accumulation in shellfish occurs by different mechanisms including mechanical entrapment and ionic bonding [6]. Virus accumulation in shellfish depends on factors such as water temperature, mucus production, glycogen content of the connective tissue, and gonadal development [15].

The low prevalence of *Rotavirus* was observed in the present study. The low prevalence observed in our study may be due to presence of PCR inhibitors that are normally present in shellfish or may be due to low concentration of *Rotavirus* present in shellfish. Although no reports are available in India regarding the prevalence of *Rotavirus* in shellfish, a high prevalence of *Rotavirus* in children from Kerala

has been reported [16]. Similar results were obtained in a study in China where in 3.33% of the shellfish samples were positive for *Rotavirus* [17]. *Rotavirus* has been reported in shellfish from other parts of the world. Prevalence as high as 52% have been reported in a 3 year-long study in southern France [18] and as low as 0.5% in Puglia in Italy [19] have been reported.

Conclusion

A low prevalence of 2.5% of rotaviral RNA could be detected in our study. Only limited studies have been done in India in this area and more extensive studies have to be undertaken to understand the true prevalence of enteric virus in shellfish in India. Shellfish consumption may thus be regarded as a two-faced issue, on the one hand as a delicious food providing nutrient, and on the other, a matter of concern as shellfish is a common cause for infections such as gastroenteritis or hepatitis.

Authors' Contributions

This study was a part of VM's M.V.Sc. thesis under the guidance of KNB. KNB designed the experiment, sample collection was done by VM and DAR. Experiment was performed by VM and assisted by SR, KML, HVM under guidance of KNB. Manuscript preparation was supervised, reviewed and edited by KNB and AK. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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