

Comparison of three methods for concentration of rotavirus from artificially spiked shellfish samples

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Received: 01-03-2014, **Revised:** 03-06-2014, **Accepted:** 07-06-2014, **Published online:** 06-07-2014

doi: 10.14202/vetworld.2014.463-466 **How to cite this article:** Mohan V, Rawat S, Lokesh KM, Mohan HV, Avinash Reddy D, Kumar A and Bhilegaonkar KN (2014) Comparison of three methods for concentration of rotavirus from artificially spiked shellfish samples, *Veterinary World* 7(7): 463-466.

Abstract

Background: Shellfish are a nutritious food source whose consumption and commercial value have risen dramatically worldwide. Shellfish being filter feeders concentrate particulate matters including microorganisms such as pathogenic bacteria and viruses and thus constitute a major public health concern. Effective preliminary sample treatment steps such as concentration of virus from shellfish are essential before RNA/DNA isolation for final PCR accuracy and reproducibility due to presence of PCR inhibitors in shellfish.

Aim: The current study was done to compare three methods for concentration of rotavirus from shellfish samples.

Materials and Methods: Shellfish samples artificially spiked with tenfold serial dilutions of known concentration of rotavirus were subjected to three different concentration methods namely; proteinase K treatment, precipitation with polyethylene glycol 8000 and use of lysis buffer. RNA was isolated from the concentrated samples using phenol chloroform method. Rota viral RNA was detected using RT-PCR.

Results: Concentration of virus using proteinase K and lysis buffer yielded better result than concentration by PEG 8000 in samples with lowest concentration of virus. Among these two methods proteinase K treatment was superior as it showed better amplification of the highest dilution (10^7) used.

Conclusion: Treatment with proteinase K was better than other two methods as it could detect the viral RNA in all three tenfold serial dilutions.

Keywords: concentration, lysis buffer, polyethylene glycol 8000, proteinase K, RT-PCR, shellfish.

Introduction

Shellfish is cheap and highly proteinaceous food whose consumption has increased globally. Shellfish by virtue of their filter feeding habit can concentrate pathogenic organisms such as enteric viruses and bacteria present in contaminated water [1]. 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, although not all have been clearly linked with documented disease outbreaks [2, 3]. Faecal indicator bacteria routinely used to assess sanitary quality of water is successful only in prevention of shellfish borne infection of bacterial origin and have limited predictive value for pathogenic viruses [4]. Hence there is need for methods that can be used for direct virological examination of samples in order to assess the health risk due to enteric viruses.

Identification of enteric virus from shellfish is difficult due to the low concentration of virus and the presence of substances liable to interfere with enzyme system used for amplification [5,6]. The main inhibitors in shellfish are acid polysaccharides [7]. Effective preliminary sample treatment steps such as concentration of virus from shellfish are therefore essential before RNA/DNA isolation for final PCR accuracy and reproducibility [8].

The aim of the study is to compare three methods namely Proteinase K treatment [9, 10], precipitation with polyethylene glycol 8000 [11], and use of lysis buffer [12] for concentration of rotavirus from shellfish after artificially spiking the shellfish with known concentration of faecal sample containing rotavirus.

Materials and Methods

Processing of samples: The shellfish were 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

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virus RNA. Three methods were used for the concentration of viral RNA from the shellfishes and are discussed below:

Proteinase K method: A volume of 1 ml of proteinase K (100 µg/ml) (Fermentas) was added to the centrifuge tubes containing 1g homogenized shellfish tissue. It was then kept in shaking incubator at 180 rpm at a temperature of 37°C for 1 h. Proteinase K was inactivated by incubating in 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.

Lysis buffer method: Nearly 1g homogenized shellfish tissue was taken in a 15ml centrifuge tube and equal volume of lysis buffer (3M Sodium Acetate (Sarabhai M Chemicals Ltd, India) and 10% SDS (Sisco research Laboratories Ltd), pH 5.4) was added. It was then kept in shaking incubator at 180 rpm for 1 h. The supernatant collected after centrifugation at 3000 rpm was stored at -20°C.

Polyethylene glycol method: A volume of 5ml of Glycine buffer (1 M Glycine (sd-Fine Chem. Ltd, India) and 1M Sodium chloride (Sisco research Laboratories Ltd, India), pH 9) was added to 1 g of the homogenized shellfish tissue and centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was collected and pH adjusted to 7.2, mixed with 20% polyethylene glycol 8000 (PEG8000) (Sisco research Laboratories Ltd) and incubated overnight at 4°C. The incubated supernatant was centrifuged at 6200 rpm for 20 min at 4°C and the pellet obtained was resuspended in 0.15 M Na₂HPO₄ (Sisco research Laboratories). The final pH was adjusted to 7.2 and the suspension stored at -20°C.

Spiking of shellfish with rotavirus positive faecal sample: To evaluate the effectiveness of the various concentration methods spiking study was done using rotavirus positive faecal sample having a copy number of $9.3 \times 10^3/100$ ng as control. Approximately 100 mg of faecal sample was taken in an Eppendorf tube and the volume was made to 1 ml with PBS. Tenfold serial of the faecal sample were made with each dilutions consisting of copy numbers of $9.3 \times 10^9/ml$, $9.3 \times 10^8/ml$ and $9.3 \times 10^7/ml$. A total of 9 homogenized shellfish samples were taken and separated to three sets, each set having three shellfish samples. The three sets of dilutions were added to three sets of shellfish such that each set of shellfish samples receive three different dilutions of faecal samples. The samples were kept undisturbed for about 5 h. The three set of shellfish samples were subjected to the three different concentration methods described above.

Isolation of RNA by phenol: chloroform: isoamyl alcohol method: An aliquot of 1 ml of supernatant was treated with 0.1 volumes of 10% of sodium dodecyl sulphate (SDS) and incubated for 1 h at 56°C in 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, and then the mixture was centrifuged again at 12,000 g for 10 min and upper clear aqueous phase transferred to fresh micro centrifuge 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.

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), 1.5 µl DMSO, 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 5x 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 thermo cycler 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 10x 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 thermalcycler 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

The results of the evaluation of three viral concentration and extraction methods are presented in Figure-1. Concentration of virus using proteinase K and lysis buffer yielded better result than concentration by PEG 8000 in samples with lowest concentration of virus. Virus could be detected by RT-PCR in all the three dilutions (10^7 to 10^9 viral particles/ml) in samples

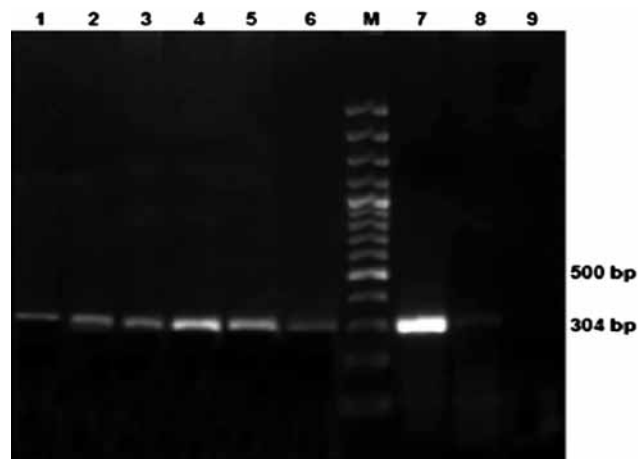


Figure-1: Concentration of rotavirus by various methods; Lane M: 100 bp plus ladder, Lane 1-3 proteinase K concentration method (10 to the power 9, 10 to the power 8 and 10 to the power 7 viral particles/ml), Lane 4-6 concentration by lysis buffer (10 to the power 9, 10 to the power 8 and 10 to the power 7 viral particles/ml), Lane 7-9 concentration by PEG 8000 (10 to the power 9, 10 to the power 8 and 10 to the power 7 viral particles/ml)

concentrated by proteinase K and lysis buffer method. Among these two methods proteinase K treatment was better as it showed better amplification of the highest dilution (10^7) used. However in PEG method, virus could be detected in only two dilutions.

Discussion

The development of nucleic acid amplification methods to detect viral contamination in shellfish is complicated by the following two factors: (i) they have low viral titers, and (ii) they contain compounds that interfere with enzymatic nucleic acid amplification reactions. Elimination of inhibitors is difficult to evaluate and depending on the time of the year and shellfish life, different compounds may be present [13]. To detect viruses using PCR, it is important that the samples of nucleic acids from the original specimens be as pure as possible. The purification stage is particularly important when the virus concerned is an RNA virus, for which a reverse transcription (RT) stage is necessary. Indeed, the high susceptibility of reverse transcriptase to interfering or inhibitory substances is a major limiting factor in amplification reactions [14, 15]. The three concentration and extraction methods evaluated in the study were proteinase K method, lysis buffer method and poly-ethylene glycol precipitation. In our study extraction and concentration by proteinase K showed the best result followed by lysis buffer method and PEG. Similar results were obtained by Tibollo and coworkers [16]. In their study concentration by proteinase K was found to be faster and sensitive when compared to PEG. The European Committee for Standardization (CEN) has approved the proteinase K method as it is simpler and has shown adequate results. Proteinase K treatment have shown a more efficacious removal of PCR inhibiting substances in another study by Di Pasquale and coworkers [17].

Conclusion

Among the three methods compared, use of proteinase K was found to be the best method for

concentration of rotavirus from shellfish samples. Concentration of virus by proteinase K could detect virus in all the three serial dilutions of virus and is also easier to perform when compared to precipitation with PEG 8000.

Author's contributions

This study was a part of VM's M.V.Sc. thesis under the guidance of KNB. KNB and SR 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.

Acknowledgements

The authors are thankful to the Director, Indian Veterinary Research Institute, Izatnagar for providing necessary facilities to carry out this work. The research was funded by ICAR, India.

Competing interests

The authors declare that they have no competing interests.

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