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Development of a simple and sensitive method to detect enteric viruses from oysters

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Abstract

Development of a rapid method possessing the requisite sensitivity and specificity for virus monitoring is necessary for protection of the shellfish-consuming public. Oysters tissue usually contains virus particles in relatively small concentrations along with various other substances that can interfere with detection steps. Therefore, the critical point concerning the detection of viruses in shellfish tissues resides in the processing of samples. The current study demonstrated the possibility of purifying small amounts of virus particles at the interface of a 10/50% sucrose gradient after a single round of sucrose gradient ultracentrifugation. We could detect HAV and poliovirus simultaneously from oyster tissues by using two different sets of primer. Furthermore, the method showed a high level of virus recovery rate (>95%) as determined by plaque assays of the final samples. Taken the advantages of the simple and sensitive methods, it was possible to detect 2 pfu of HAV in 5 g of oyster digestive tissues within 24h.

Key words – enteric viruses, hepatitis A virus, oyster, RT-PCR, gradient-ultracentrifugation

Introduction

Shellfish are readily contaminated with viruses present in water containing sewage because of the concentrating effect of filter feeding. As such, shellfish particularly oysters and clams, play an important role in the transmission of enteric pathogens, such as the human hepatitis A virus (HAV) and Norwalk virus, because these shellfish are frequently eaten raw [1,3,8-10]. As a result, food- and water-borne gastroenteritis is an important public health concern all over the world. Currently, the sanitary quality control of marketable shellfish is based on an analysis of the level of faecal-pollution bacterial indicators in shellfish or in growing-waters. However, the reliability of these microorganisms as an indicator of viral pollution has been widely questioned [15], emphasised by the occurrence of viral outbreaks associated with the consumption of shellfish supposedly meeting legal bacteriological standards [7].

Accordingly, the development of rapid methods possessing the requisite sensitivity and specificity for virus monitering is necessary to protect the shellfish-consuming public. This is particularly important for the detection of the human viruses HAV and the Norwalk virus, which are not readily grown in cell cultures and most frequently associated with clinically significant shellfish-transmitted

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diseases. The critical point concerning the detection of viruses in shellfish tissues resides in the processing of samples. It requires the manipulation of a large sample volume because of the low copy number of viral nucleic acid usually present. It requires too an efficient elimination of inhibitory substances from shellfish prior to RNA amplification. A variety of methods have been already described for the detection of viral contaminants in shellfish using RT-PCR [2,4,6,11-14]. However, most of these methods include numerous, often fastidious, and time consuming steps for virus release from the shellfish tissue and viral RNA isolation.

In this study, we have developed a procedure which facilitated purification of large amounts of virus particles on the 10-50% interface of sucrose gradients by ultracentrifugation. We could detect HAV and poliovirus simulataeously in oyster by using two different sets of primers in one RT-PCR reaction mixture. Taken the advantages of the simple and sensitive methods, it was possible to detect 0.5 pfu of HAV in 5 g of hepatopancreases within 24hr.

Materials and Methods

Virus and cells

HAV strain HM-175, cytopathic clone 24A, was propagated and assayed by plaque formation in FRhK-4 cells (fetal kidney cells from a rhesus monkey, ATCC CRL-1688) as previously described [13]. A stock virus of 2×10^5 PFU per ml was sonicated and diluted in Dulbecco's mdificatoned eagle medium (DMEM) to achieve the desired virus concentrations.

Oyster contamination

Shucked oysters from the Sourthen areas of Korea were purchased at local markets. Adsorption assays were carried out by addition of viral dilutions, from 2 to 100 PFU of HAV, to 5 g samples of oyster digestive tissues which were then incubated for 1 hr at room temperature.

Virus extraction and purification

Tissue samples collected in 3-4 volumes of a PBS buffer were homogenized on ice using a Potter tissue blender. The homogenized solution was then supplemented with a 1/10 volume of trifluoro-trichloroethane (Sigma) to dissolve the lipid and centrifuged for 15 min at 2,000 ×g to remove any cell debris and nuclei. The supernatant from the first centrifugation was layered onto a sucrose solution composed of two layers with different concentrations, i.e. 10/30%, 10/40%, 10%/50%, 20/40%, and 20/50% (w/w), and centrifuged for 1 hr at 100,000 ×g. The visible band that formed at the boundary of the two layers was removed, diluted four-fold with a PBS buffer, and pelleted by centrifugation fo 30 min at 120,000 ×g. The pellet from the ultracentrifugation was then resuspendend in 200 ml of DEPC-treated water.

RT-PCR

The total RNA was extracted in a one-step method [8] using guanidium-phenol (TRIzol, GIBCO). The cDNA was synthesized by respending the RNA pellet in 25 ml of a reverse transcription (RT) reaction mix containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 20 U of RNasin, 10 mM dithiothreitol, 5.0 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates (dNTPs), 0.1 mg of reverse primer, and 2.5 U of avian myeloblatosis virus reverse transcriptase and incubated for 10 min at 23℃ followed by 30 min at 42℃. The RT mix templates were amplified by the addition of 100 ml of the PCR mix containing (final concentration) 10 mM Tris-HCl (pH8.8), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM (each) dNTP, 50 pmol sense and antisense primers, and 1.25 U of Taq DNA polymerase (Promega). The PCR incubation conditions were 30 cycles of 1 min at 94, 56, and 72℃. An HAV RNA genomic region corresponding to capsid protein-interphase was detected using a antisence primer directed at nt 452-470 (5'gaa tct tcg atg cgt tgc3') and a sense primer directed at nt 267-284 (5'gga tta gcc gca ttc agg g3') (NCBI nucleotide accesion number

J02281). A poliovirus genomic region corresponding to capsid protein was amplified using an antisence primer directed at nt 452-470 (5'gaa tct tcg atg cgt tgc3') and a sense primer directed at nt 267-284 (5'gga tta gcc gca ttc agg g3') (NCBI nucleotide accesion number J02281).

Quantification of PCR product

PCR products were analyzed by electrophoresis in 2% (wt/vol) agarose gels. HAV primer set yields a 375 bp product whereas poliovirus primer set yields a 204 bp product. Band intensity (peak area in arbitrary units) was quantified by scanning densitometry with the QuintiScan software package (Biosoft, Cambridge, United Kingdom).

Results and Discussion

To provide a more simple and effective virus detection method, gradient ultracentrifugation and RT-PCR were combined to purify and detect a small amount of viruses from oyster tissue. The overall virus purification scheme is presented in Fig. 1. Artificial virus contamination was carried out by the addition of the indicated amounts of

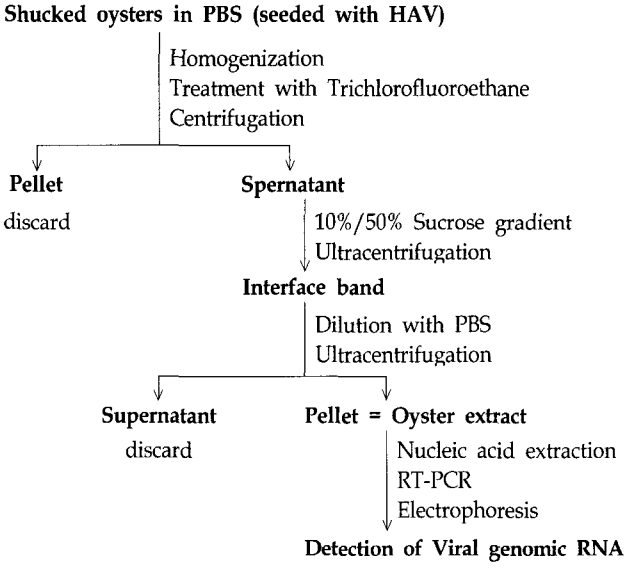

Fig. 1. Outline scheme for extraction, purification, and amplification of viruses from oysters.

HAV to 5 g of oyster digestive tissue which was then incubated for 1 hr at room temperature. Tissue samples collected in 3-4 volumes of PBS buffer were homogenized on ice using a tissue blender. The homogenized solution was then supplemented with a 1/10 volume of trichlorotrifluoroethane (Sigma) to dissolve the lipid and centrifuged for 15 min at 2,000 ×g to remove any cell debris and nuclei.

To determine which sucrose gradient was most effective for concentrating the viruses, the supernatant from the first centrifugation was layered onto sucrose gradients composed of two layers of sucrose solutions with different concentrations, i.e. 10/30%, 10/40%, 10%/50%, 20/40%, and 20/50% (w/w), and centrifuged for 1h at 100,000×g. A visible band of virus particles along with cell debris formed at the interface of the two layers (Fig. 2).

The band was removed, diluted four-fold with PBS buffer, and pelleted by centrifugation for 30 min at 120,000 ×g. The pellet from the ultracentrifugation was then resuspended in 200 ml of DEPC-treated water. Total RNA was extracted and RT-PCR analysis was performed as described in materials and methods. According to the results shown in Fig. 3, the virus particles were most effectively purified at the interface of the 10/50% sucrose gradient. Similar results were also obtained with other enteric viruses, such as polioviruses and adenoviruses

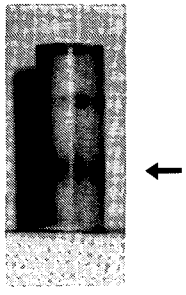

Fig. 2. Purification of virus particles from oyster tissue by sucrose gradient ultracentrifugation.
A visible band of virus particles along with cell debris formed at the interface of sucrose solutions (10/50%) was indicated by an arrow.

Fig. 3. Determination of sucrose gradient for ultracentrifugation to purify viruses from oyster tissue.
The oyster tissue was seeded with 1×10^3 PFU of HAV and purified by sucrose gradient ultracentrifugation. Four different combinations of sucrose concentrations were tested. The results of the agarose gel (2%) electrophoresis of the RT-PCR amplified products from the purified viruses are shown. lane 1, positive control (1×10^3 PFU of HAV); lane 2, 10/30% sucrose gradient; lane 3, 10/40%; lane 4, 10/50%; lane 5, 20/40%; lane 6, 20/50%; lane 7, negative control (10/50% without addition of viruses).

(data not shown). Therefore, this centrifugation condition could be universally used for most viruses present in oyster tissue.

Next, to evaluate the sensitivity of the method, an attempt was made to detect HAV in the finally processed samples after seeding various concentrations of virus into the oyster tissue. According to the results, the lowest limit of virus detection was 0.5 PFU of infectious particles seeded in 5 g of oyster tissue, due to high ratio of HAV particle counts vs. infectious units (Fig. 4). Furthermore, this method exhibited a high virus recovery rate of up to 95% (mean 905) starting from 2×10^3 PFU of viruses, as demonstrated by plaque assay of the final samples.

Since oysters can be contaminated with several different kinds of viruses present in ocean water, an ideal method should be able to detect different kinds of viruses present in oyster tissue. As such, oyster tissue was artificially contaminated with two different viruses, HAV and human poliovirus type I (PV1) and attempts made to detect both viruses from the finally processed samples by duplex RT-PCR. The poliovirus was chosen because it is relatively easy to cultivate and quantitate. According to the results shown in Fig. 5, the genomes of HAV and poliovirus were effectively amplified by a RT-PCR reaction in a single tube. Accordingly it would

Fig. 4. Sensitivity test of the proposed method. The viruses were purified from 5 g of oyster extracts seeded with various titers of HAV by sucrose gradient (10/50%) ultracentrifugation.
The results of the agarose gel (2%) electrophoresis of the RT-PCR amplified products from the purified oyster extracts are shown. The HAV titers added to the oyster tissue were as follows: lane 1, 100 PFU; lane 2, 50 PFU; lane 3, 10 PFU; lane 4, 5 PFU; lane 5, 0.5 PFU; lane 6, 0.2 PFU.

Fig. 5. Duplex RT-PCR to detect HAV and PV1.
Purified virions from oyster tissue contaminated with 1×10^3 PFU of HAV and PV1, respectively, were used for the RT-PCR (lane 4). As controls, 1×10^3 PFU of HAV (lane 1), 1×10^3 PFU of PV1 (lane 2), and a mixture of HAV and PV1 (1×10^3 PFU each) (lane 3) were used for the RT-PCR. Two pairs of primers were used to detect HAV (375 bp) and poliovirus (204 bp).

appear to be possible to detect several viruses simultaneously using the proposed method, if the appropriate sets of primers are included. The band intensity of PCR products from either the control (Fig. 5, lane 2) or virus particles from oyster tissue (Fig. 5, lane 4) was similar, reflecting a high recovery rate of HAV through sucrose gradient ultracentrifugation. On the other hand, the PCR product from PV1 was significantly reduced after contamination into oyster tissue and passing through purification steps (Fig. 5, compare the upper band of lanes 3 and 4). Although the reason for the difference in virus recovery between HAV and PV1 is not clear, one possibility is that PV1 might require more stringent PCR condition for the efficient amplification of the genome.

The present study is part of ongoing research seeking to determine more effective ways of detecting the

presence of virus pathogens in shellfish. In the case of difficult-to-cultivate or nonculturable viruses, the direct detection of viral nucleic acid by PCR offers the best hope of success. However, nucleic acid amplification requires the prior elimination of inhibitory substances from selfish and the manipulation of a large sample because of the low copy number of viral nucleic acid usually present. Therefore, when considering these aspects, the gradient ultracentrifugation-RT-PCR method described in this manuscript may be highly effective.

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초록 : 굴로부터 장바이러스를 검출하기 위한 간단하고 민감한 방법의 개발

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굴 소비자의 보호를 위해서는 매우 민감하고 특이적으로 바이러스를 모니터링할 수 있는 신속진단법의 개발이 필수적이다. 굴 조직은 흔히 비교적 소량의 바이러스와 검출단계를 방해할 수 있는 다른 물질들을 함께 함유한다. 따라서 굴으로부터 바이러스의 검출에서 가장 중요한 과정은 시료의 가공단계이다. 본 연구에 의하면 한번의 sucrose 구배 초원심분리에 의하여 10%와 50% 사이에서 소량의 바이러스를 분리할 수 있음을 제시하였다. 우리는 두 종류의 primer 세트를 이용하여 HAV와 poliovirus를 동시에 굴 조직으로부터 검출할 수 있었다. 또한, 이 방법은 높은(>95%) 바이러스 회수율을 나타내었다. 이 방법은 24시간이내에 5 g의 굴조직으로부터 2 pfu의 HAV를 검출할 수 있을 정도로 신속하고 민감한 검사법이다.