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DETECTION OF POLIOVIRUS TYPE 2 IN OYSTERS BY USING CELL CULTURE AND RT-PCR

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ABSTRACT

Shellfish are readily contaminated with viruses present in water containing sewage due to the concentrating effect of filter feeding. Enteroviruses are generally used as a model for the detection of viruses from shellfish due to their public health significance. In the present work, oysters were placed in glass aquaria containing seawater plus unicellular algae. Two experiments were performed: 1) oysters bioaccumulating four different poliovirus type 2 concentrations: 5 x 10^4, 2.5 x 10^4, 5 x 10^3 and 5 x 10^2 PFU/mL during 20h; 2) oyster tissues directly inoculated with 6.0 x 10^5 and 1.0 x 10^5 PFU/mL. After viruses seeding, tissue samples were processed by an adsorption-elution-precipitation method. Positive controls were performed by seeding 6.0 x 10^5 PFU/mL of poliovirus type 2 directly on the final oyster tissue extracts. Oyster extracts were assayed for viruses recovery by plaque assay, RT-PCR and integrated cell culture-PCR methodologies (ICC/PCR). The last one was based on the inoculation of the samples onto VERO cell monolayer followed by RT-PCR analysis of the infected cell fluid. In the first experiment (20h bioaccumulation) until 5 x 10^3 PFU were detected after 24 and 48h growth on VERO cells. Direct RT-PCR and ICC/PCR were able to detect 3 and 0.04 PFU of poliovirus, respectively, when bioaccumulation assay was used. When direct tissue virus seeding was performed, the plaque assays showed that polioviruses were recovered in all tested concentrations. Based on these results, it is possible to conclude that viable polioviruses can be detected in oysters after bioaccumulation and these techniques can be directly applied for monitoring virus contamination in environmental samples.

Key words: oysters, poliovirus, bioaccumulation, cell culture, RT-PCR

INTRODUCTION

In many countries, the sanitary control of shellfish is still based on the detection of fecal coliforms as indicators of fecal pollution. However, these indicators are believed to have limited predictive value for pathogens such as enteric viruses (15,16,17). According to Huss *et al.* (10) the various seafood can be assined to a risk category in terms of health hazards by using a + (plus) to indicate a potential risk related to the hazard characteristics. Molluscan shellfish to be eaten raw are very risk products. Actually, there is no correlation between the number of human viruses present in the water and the number of fecal coliforms and it is largely known that human viruses are present in sewage-contaminated water, since they are excreted in large amounts in human feces. More than 100 different types of viruses can be found in human waste and are potentially transmitted through contaminated water. These viruses are more resistant to environmental conditions and sewage treatment processes, including chlorination and UV radiation, than many of the sewage-associated bacteria (11,28). In laboratory studies, enteric viruses have been reported to survive for 2 to 130 days in seawater (12). The enteric viruses such hepatitis A virus (HAV), human caliciviruses (HuCV), rotaviruses (RV) and polioviruses (PV) have been detected in

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shellfish considered safe for consumption on the basis of bacteriological standards (3,14,24). These viruses, even at low concentrations, can cause illness when ingested. These diseases include paralysis, meningitis, respiratory diseases, epidemic vomiting and diarrhea, myocarditis, infectious hepatitis, etc (20).

In relation to enteroviruses contamination, many of them are vaccine-related polioviruses that can be isolated and quantified by plaque assay (plaque forming units) in cell culture. Concerning to polioviruses, three different species of the Enterovirus genus (1, 2 and 3) are the causative agent of poliomyelitis. These viruses consist of icosahedric particles composed of 60 copies of each of four capsid proteins, VP1, VP2, VP3 and VP4 surrounding the viral genome, which is composed by a single-stranded RNA of positive polarity of about 7,500 nucleotides (27). There is a campaign for global eradication of poliomyelitis and the mass vaccination of children with the OPV vaccine (Sabin 1, 2 and 3) had dramatically reduced the number of poliomyelitis cases caused by wild polioviruses (7). These vaccines are live attenuated and can replicate in the gastrointestinal tract, inducing local intestinal as well as long-lived systemic immunity. One of the disadvantages of the mass vaccination is the dissemination of live attenuated polioviruses in the environment, which may contaminate susceptible individuals (such immunodefficient ones) and cause poliomyelitis. The fecal contamination by OPV-derived polioviruses detected in aerosols from wastewater treatment plants could potentially be another source of virus contamination for humans (18,19). The fecal contamination of natural waters with OPV-derived polioviruses might also allow the viruses to disseminate and contaminate large areas (6). The contamination of the environment, which might be a source of human infections, could have possible implications for eradication of OPV-derived (7). For this reason, an accurate environmental surveillance and assiduous epidemiological investigation is urgently needed (6).

The primary objective of this research was to use RT-PCR and cell culture techniques (separately and integrated) to detect vaccines strains of polioviruses in the environment by using the poliovirus type 2 as a model.

MATERIALS AND METHODS

Virus and cell cultures

Poliovirus type 2 (PV) was propagated in VERO cells (monkey kidney fibroblasts) and assayed for infectivity by the plaque assay (2). Briefly, VERO cells at a density of 2×10^5 cells/mL were grown in 24 well microplates (Corning®, USA) in 199 minimum medium (Sigma - Aldrich, USA) supplemented with 5% Fetal Calf Serum and penicillin G (100 U/mL), streptomycin (100 ug/mL) and amphotericin B (0.025 ug/mL) (all from Invitrogen Life Technologies, USA) during 24h. When the cell monolayers were confluent, a poliovirus suspension was diluted at 1:10 in 199 media without FCS and seeded on these cells (500 mL of virus dilution per well) in order to adsorb. The plates were incubated during 60min at 37ºC under gentle shaking. After this period, the viral inoculum was removed and 1.0 mL of overlay medium (2X concentrated 199 medium and 1.5% carboxymetilcellulose (Sigma - Aldrich, USA) solution, ratio 1:1) was added in each well. After 24h incubation, under 5% CO_2 atmosphere at 37ºC, the overlay medium was removed from the wells by suction and 500 mL of naphthalene black (Sigma - Aldrich, USA) were added in each well (0.1% naphthalene black diluted in 5% acetic acid, pH 2.3-2.5). The cells were stained during 30 min at RT, under gentle shaking, the stain was removed by suction and the plates were air-dried. In order to calculate the infectious titer, the plaques were counted and the titer (PFU/mL) was calculated multiplying the mean number of plaques by the reciprocal of the dilution and the reciprocal of the virus volume added in each well (2).

Bioaccumulation assays

Oysters (*Crassostrea gigas*) with 12-20 g of meat weight were obtained from a cultivated system oyster farm in Florianopolis Island, Santa Catarina State, Brazil. Oyster shells were scrubbed with a stiff brush in running potable water (ca. 0.7 ppm free chlorine). Oysters were then allowed to bioaccumulate PV. The oysters were placed in 60 liters tanks (aquaria) containing 20 liters of seawater. The following experiment was designed: microalgae (*Tetraselmis tetrathele* - 6×10^4 cells/mL) were previously incubated during 30 min with four different poliovirus concentrations (5×10^4, 2.5×10^4, 5×10^3 and 5×10^2 PFU/mL), seeded on the aquaria (12 oysters for each experiment) and incubated during 20h. Each bioaccumulation assay was repeated 5 times in order to study polioviruses recovery by RT-PCR and ICC/RT-PCR. Direct tissue seeding was also performed. Three oysters were considered as one sample, and were injected with 500 µL of poliovirus-infected tissue culture supernatant diluted to contain 6.0×10^5 and 1.0×10^5 PFU/mL. Negative controls consisted on oysters placed on the aquarium without seeding with viruses using the same concentration of microalgae without viruses. Positive controls consisted on the final oyster tissue extract inoculated with 6.0×10^5 PFU/mL. After virus inoculation or bioaccumulation, shells were opened at a hinge with an autoclaved oyster knife and the oyster extracts were prepared as described below (1,3,4,5,22,23).

Oyster extract preparation

The following method for virus extraction from oyster´s extracts was used for the subsequent RT-PCR and cell infection assays (1,3,4,22). Either for the bioaccumulation assay or for the directly inoculated oysters, the flesh of twelve oysters was transferred and homogenized with a shaft blender (Ultra-

Turrax T-25 Ika®, France) at 24,000 rpm for 30s. After, 20 g of this homogenate were transferred to a sterile Schott® bottle of 500 mL of capacity containing 100 mL of pre-chilled (ice bucket) 10% (v/v) tryptose phosphate broth (TPB) (100% TPB contains 20 g tryptose, 2.0 g glucose, 5.0 g NaCl and 2.5 g Na_2HPO_4, pH 9.0) prepared in 0.05M glycine (pH adjusted to 9.0 using 2N NaOH). Tissues were shaken for 30 min on ice in a bench homogenator and then transferred to a 50 mL bottomed and sterile centrifuge tube, and centrifuged at 10,000 X g for 30 min at 4ºC. The pellet was discarded and the pH of supernatant was adjusted to 7.5 using 2M HCl. Polyethylene glycol solution (PEG 6000) (50%, w/v) prepared in 10% TPB was added to a final concentration of 8% (w/v). The mixture was stirred for 2h at 4ºC, and centrifuged at 10,000 X g for 20 min at 4ºC. The supernatant was discarded and the pellet resuspended in 5.0 mL of 0.15 M Na_2HPO_4, pH 9.0. The resuspended pellet was sonicated twice for 30s and transferred to a plastic tube centrifuge and the pH readjusted to 7.4 using 2M HCl.

Cytotoxicity assay

VERO cell monolayers were prepared in 24-well microplates (Corning®, USA) 24 h before the experiment and grown to a density of 1.0 x 10^6 cells/mL. Inoculation was carried out as follows: two fold serial dilutions of unseeded oyster extracts ranging from 1:2 to 1:1,024 were prepared in maintenance medium. Aliquots of 500 µL were then added to the cells previously washed with PBS. All assays were undertaken in triplicate. After 60 min of incubation for adsorption, the oyster extracts were removed and the cells supplied with 1.5 mL of medium supplemented with 2% FCS. The plates were incubated and observed for cytotoxic effects during 24, 48 and 72 h. Each observation has been compared with a negative control (without oyster extract) containing only normal cell monolayer and medium. After each period of incubation, the cellular integrity was confirmed by removing the medium by suction and staining them with 500 µL naphthalene black (Sigma - Aldrich, USA) at 0.1% in 5% acetic acid pH 2.3. After 5 min incubation, the stain was removed by suction and the cells were air dried and observed under an inverted microscope.

Infectivity assays

VERO cell monolayers were prepared in 24-well microplates (Corning®, USA) 24 h before the experiment and grown to a density of 1.0 x 10^6 cells/mL. Inoculation was carried out as follows: two-fold serial of non-cytotoxic (1:64 to 1:1,000) oyster extract dilutions directly spiked with virus and bioaccumulated samples were prepared in maintenance medium. Aliquots of 250 mL were then adsorbed to previously PBS-washed VERO cells. All assays were undertaken in quadruplicate. After 60 min of incubation for virus adsorption, the inoculum was removed and the cells supplied with 1.5 mL serum-free medium.

Viral RNA extraction from oyster extracts (1,3,4,22)

An aliquot (0.5 mL) of oyster extract was used for viral RNA isolation. An equal volume of trichlorotrifluoroethane (Sigma - Aldrich, USA) was added to remove lipids from each aqueous oyster extract, and the suspensions centrifuged at 4,000 X g in a microcentrifuge (Eppendorf®) for 5 min at room temperature. The aqueous phase was transferred to another sterile Eppendorf® tube. Tris-HCl (pH 7.5), EDTA, SDS and proteinase K were added at final concentrations of 10 mM, 5 mM, 0.5% (w/v) and 400 µg/mL, respectively. Samples were then incubated at 37ºC for 30 min. Cetyltrimethylammonium bromide (CTAB) and NaCl were added to final concentrations of 1.3% (w/v) and 0.52 M, respectively, and samples incubated at 56ºC for 30 min. They were subsequently extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous phase was transferred to another microfuge tube and an equal volume of chloroform added. The aqueous phase was then precipitated in 3 vol of chilled 100% ethanol at -20ºC. Resulting pellets were washed once with one vol of chilled 70% (v/v) ethanol, suspended in 500 µL of DEPC treated water (Invitrogen Life Technologies, USA), and stored at -80ºC for RT-PCR assays.

RT-PCR assays

Conventional RT-PCR was performed using random and specific primers for poliovirus reverse transcription and genome amplification. Random hexamers primers were purchased from Invitrogen Life Technologies (USA) and were used for cDNA synthesis. Briefly, the following reaction mixture was prepared: 12.5 µL of DEPC treated water, 1X concentrated M-MLV buffer (Invitrogen Life Technologies, USA), 0.5 mM of dNTP, 50 pmoles of random primer, 0.01 mM DTT, 5U of RNAse inhibitor and 40 U of M-MLV-RT. The reaction was performed at 37ºC during 90min in a final volume of 25 mL. For the PCR, 3.0 µL of cDNA were used. The reaction mix was composed by Tris-HCl 22 mM, pH 8.4, KCl 55 mM, $MgCl_2$ 3 mM and 100 pmoles of the pair of primers Polio R (5´ACGGACACCCAAAGTA-3´) and Polio-L (5´AGCACTTCTGTTTCCC-3´). The PCR program consisted on 40 cycles at 95ºC 1min, 55ºC 1min and 72ºC 1min. Previously to the cycles the cDNA was denatured at 95ºC during 2 min and, at the end of the cycles, a final extension step was performed at 72ºC during 7min. The molecular size of the generated PCR product was 394 bp.

Viral RNA isolation from infected cell supernatant and ICC/PCR assay

An aliquot (0.5 mL) of infected cell supernatant was used for viral RNA isolation. The cell fluid was treated with 1 vol of phenol-chloroform-isoamyl alcohol (25:24:1) and the nucleic acids precipitated with ethanol according to described above for viral RNA isolation from oysters extracts. RT-PCR was performed as described above using 5.0 µL of RNA.

RESULTS AND DISCUSSION

The cytotoxicity of the oyster extracts was evaluated *in vitro* either by direct observation of VERO cell morphology under the microscope or by staining the cells with naphtalene black to evaluate cell damage. The serially diluted oyster extracts were incubated during 24, 48 and 72h in these cells. According to the obtained results, dilutions above 1:32 were not cytotoxic until 72h. So, this dilution of oyster extracts was considered as minimum to evaluate poliovirus recovery from oyster tissues using cell culture (data not shown).

When the PFU assay was used to evaluate PV type 2 recovery in oysters directly inoculated with virus the mean recovery index was 25.3%, after 24h of incubation on VERO cells according to Table 1. Shieh *et al.* (25) found recovery indices of PV type 3 varying from 10 to 76% by PFU assay when two methodologies of virus elution were tested. As shown on Table 1, when polioviruses were inoculated at the end of the oyster extract preparation (representing 100% of inoculated virus), a recovery of 75.5% was observed. This fact can be explained by partial virus inactivation when in contact with the oyster meat extract and this fact was also observed by Herrero *et al.* (9) and Williams and Shay Fout (26). The last authors had observed a reduction of 50% of the PFU when non-cytotoxic mollusk extracts seeded with viruses were added to the cells. According to Table 1, the coeficient of variation observed when virus recovery was analysed by PFU was 54% and similar values were also observed by Lees *et al.* (13) and Shieh *et al*. (25). This fact can be related with the mollusk tissue composition that can vary according to the geographical localization of the oyster farm, season of the year and quality of the effluents discharged into the ocean water.

Bioaccumulation assay mimics very well the natural condition of contamination by filter feeding and so was also used for poliovirus detection in the present work. When the oysters were allowed to bioaccumulate polioviruses in the- aquaria, the virus recovery rate was 11%, after 24h of incubation on VERO cells according to Table 1. Many implications were involved to explain the apparent low recovery index observed when bioaccumulation was used: losses during the tissue extract preparation, virus adsortion to the aquaria walls or even virus elimination in the oyster´s feces. It is very difficult to calculate exactly the number of virus particles that remain adsorbed to the oyster tissues. In general, the viral bioaccumulation behavior was constant during all the experiment´s repeats and this can be observed by the regression analysis applied to two different variables (virus concentration inoculated in the water and concentration of recovered viruses). Cromeans *et al.* (5) also used a bioaccumulation assay to study hepatitis A virus recovery in oysters during 24h of incubation and they observed 10% of virus recovery also using PFU assay.

The artificially seeded oyster tissue extracts obtained after the bioaccumulation of PV type 2 were used for virus detection using RT-PCR and RT-PCR associated with cell culture. The results of RT-PCR were shown on Table 2. According to the obtained results by direct RT-PCR, the detection of PV was positive in 20% of the extracts using 5.0×10^4 PFU (24 PFU in the reaction) and the positivity increased to 60% when 2.5×10^4 PFU (14 PFU in the reaction) and 5.0×10^3 PFU (3 PFU in the reaction) were used. When 5.0×10^2 PFU (0.1 PFU in the reaction) were added, no viruses could be detected by RT-PCR. According to Green and Lewis (8), the PCR can fail to detect viruses on positive samples due to different inhibitor concentrations in each reaction tube. In addition, according to Reynolds *et al.*

Table 1. Recovery of PV by plaque assay from oysters directly inoculated and after bioaccumulation.

		Inoculated virus (PFU/mL oyster extract or sea water)	Recovered virus (PFU/mL oyster extract or sea water)	Recovery percentage (%)
Oysters directly inoculated	C+	6.0×10^5	4.4×10^5	75.5[a]
	1	1.0×10^5	4.1×10^4	31.0[b]
	2	6.0×10^5	1.1×10^5	19.6[b]
Bioaccumulation assay	1	5×10^4	6.0×10^3	10.2[c]
	2	2.5×10^4	3.2×10^3	12.8[c]
	3	5×10^3	5.1×10^2	10.2[c]
	4	5×10^2	26.4[b]	5.2[d]

C+: Positive control (100% of viral recovery);
[a] Mean of recovery percentage from positive control of four different experiments: 75.5 ± 41%. CV = 54%;
[b] Mean of recovery percentage from extracts 1 and 2 of four different experiments = 25.3 ± 5%. CV = 20%;
[c] Mean of recovery percentage from extracts 1, 2 and 3 of five different experiments: 11.07 ± 1%; CV = 8%;
[d] Estimated value based on regression equation: $y = -33.6 + 0.2x$.

Table 2. Detection of PV in bioaccumulated oysters by RT-PCR and by ICC/PCR.

	PV concentration	PFU/volume of reaction (50 µL)	Nº of positive samples	% of positive reactions
RT-PCR	5×10^4	24	1/5	20
	2.5×10^4	14	3/5	60
	5×10^3	3	1/5	60
	5×10^2	0.1[a]	0/5	0
ICC-PCR	5×10^4	0.16	3/3	100
	2.5×10^4	0.11	3/3	100
	5×10^3	0.04	3/3	100
	5×10^2	0.01[b]	0	0

(a): Estimated value based on regression equation: $y = -33.16 + 0.12x$;
(b): Estimated value based on regression equation: $y = -33.16 + 0.12x$.

(21) the low levels of viral contamination in environmental samples decrease the probability to have a viral particle inside a tube of reaction. As seen on Table 2, the incubation of the inoculated oyster extracts during 24h on VERO cells before RT-PCR increased the rate of virus detection to 100%. The sensitivity of the detection obtained was 0.04 PFU of PV type 2. According to Reynolds *et al.* (21), the sensitivity of polioviruses detection in artificially seeded ocean water was 0.1 PFU after 25h of incubation in cell culture and 10 PFU when the period of incubation was lower than 10h. The authors also had worked with viruses very difficult to adapt in cell culture as hepatitis A virus (HAV). After 72h of incubation of HAV isolated from contaminated water, they could detect this virus by RT-PCR even without any discernible cytophatic effect. All the literature cited above had used water samples seeded with viruses to study their recovery and infectivity by cell culture. The present work used oyster extracts and we could also confirm virus viability and infectivity after oyster extracts preparation. In conclusion, we demonstrated that cell culture could be an effective tool to detect enteroviruses contamination in mollusks either using RT-PCR or using PFU assay.

RESUMO

Detecção de poliovírus tipo 2 em ostras através de cultura celular e RT-PCR

Devido ao hábito alimentar filtrante, os moluscos bivalves são contaminados por vírus presentes em águas contaminadas por esgoto. Os enterovírus são geralmente usados como modelos para a detecção de vírus em moluscos bivalves devido a sua importância em saúde pública. No presente estudo, ostras foram colocadas em aquários de vidro contendo água do mar adicionada de algas unicelulares. Dois tipos de experimentos foram realizados: a) ostras bioacumulando quatro diferentes concentrações de poliovírus: 5×10^4, $2{,}5 \times 10^4$, 5×10^3, 5×10^2 PFU/mL durante 20h; b) tecidos de ostras inoculados diretamente com $6{,}0 \times 10^5$ e $1{,}0 \times 10^5$ PFU/mL. Após a semeadura, os tecidos foram processados por um método de adsorção-eluição-precipitação. Controles positivos foram realizados por inoculação de $6{,}0 \times 10^5$ PFU/mL de poliovírus diretamente nos tecidos processados das ostras. Os extratos teciduais foram testados para presença do vírus por ensaios de placa de lise (PFU), RT-PCR e cultura celular integrada ao PCR (ICC/PCR). Este último consistiu na inoculação das amostras sobre monocamadas de células VERO seguida de RT-PCR do fluido celular infeccioso. No primeiro experimento (ensaio de bioacumulação por 20h), foram detectados até 5×10^3 PFU de poliovírus, após 24 e 48h de replicação nas células. Os ensaios de RT-PCR e ICC/PCR foram capazes de detectar 3 e 0,04 PFU de poliovírus, respectivamente nos ensaios de bioacumulação. Quando os extratos teciduais processados foram semeados, os ensaios de placa de lise demonstraram recuperação de vírus infecciosos em todas as concentrações testadas. Pudemos concluir que partículas viáveis de poliovírus podem ser detectadas em ostras após bioacumulação e que estas técnicas podem ser diretamente aplicadas na detecção de vírus em amostras ambientais.

Palavras-chave: ostras, poliovírus, bioacumulação, cultura celular, RT-PCR

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