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DETECTION OF HUMAN ENTEROVIRUS AND ADENOVIRUS IN SHELLFISH COLLECTED IN MOROCCO MEDITERRANEAN COAST

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ABSTRACT

The aim of this study was the screening for the presence of enteric human virus in shellfish (clam and cockle) collected from two production area in Moroccan Mediterranean coast. Between October 2006 and April 2008, forty four samples were collected and tested for viral contamination using cell culture (HEp-2 and Vero cells) and integrated cell culture PCR. Overall, 88.6 % of all analysed samples were contaminated by at least one of the studied viruses, Adenovirus was detected in 52.3 % of the samples and Enterovirus in 36.3%. The presence of viruses in shellfish production area can represent a potential health risk by causing gastroenteritis. The procedure used in this study may be a tool for monitoring shellfish viral contamination in Morocco.

Keywords: Enterovirus, adenovirus, cell culture, ICC-PCR, shellfish

INTRODUCTION

Human enteric viruses are excreted in large numbers in feces and sewage. This constitutes a major public health threat as bivalve shellfish are filter-feeders, they act as natural biofilters in the seawater and can thus readily and efficiently bioconcentrate human pathogens from faecally contaminated waters. More than 140 enteric viruses are found in human faeces and infected individuals excrete large numbers of these viruses in stool and urine (**Leclerc *et al*., 2000**). As a result, consumption of virus-contaminated shellfish represents a significant health threat to shellfish consumer; as well as an economic threat to the sea food industry. Several incidents of shellfish associated viral gastroenteritis have been reported (**Lees, 2000**). More than a billion people rely on seafood as their main source of animal protein and contaminated seafood is a frequent etiology of diseases contracted from the ocean (**Fleming *et al*., 2006**).
Commercial use of shellfish has become an expanding industry, which may increase the transmission of pathogens associated with shellfish consumption. The annual production of the mollusk in Morocco exceeded 1636 tons (**FAO, 2007**), and more than half of this quantity is exported to European Union countries.
Further the sanitary survey of shellfish and water in Morocco is for predicating on bacterial and physico-chemical parameters. The presence of fecal indicators of bacteria is routinely used for microbiological quality of shellfish. However, the bacteria are not reliable indicators of the presence of enteric viruses in bivalves (**Formiga-Cruz *et al*., 2003**). Noroviruses, Adenoviruses (AdV), enteroviruses (EV) and Hepatitis A virus are more resistant to inactivation in water sources and more slowly removed from shellfish by depuration (**Hernroth and Allard, 2007; Ueki *et al*., 2007**).
Human AdV and EV are non-enveloped enteric viruses belonging to Picornaviridae and Adenoviridae families and they can be cultivated in cell culture. The EV ordinarily causes mild or even unapparent infections. However, in rare cases, they can be associated with serious illnesses, such as endocarditis, myocarditis, encephalomyelitis, and meningitis in infants (**Bendig *et al*., 2001; Kim *et al*., 2001; Legay *et al*., 2002; Ward, 1978**). Human AdV are considered as a cause of gastroenteritis in children (**Allard *et al*., 2001; Rigotto *et al*., 2011**). These viruses can establish latent and persistent infections with viral shedding for weeks (**Yates *et al*., 2006**) and the viruses can be excreted even if diarrhea is not present (**Fox *et al*., 1969; Spigland *et al*., 1966**).
Conventional cell culture methods have limited sensitivity for the detection of viruses and molecular procedures including integrated cell culture PCR (ICC-PCR) (**Pinto *et al*., 1995**) are currently being tested.

The aim of our study was to screen for human AdV and EV in shellfish grown in Moroccan Mediterranean coast by combining the cell culture and ICC–PCR procedures for virus detection in bivalve shellfish (clams and cockles).

MATERIAL AND METHODS

Sampling

From October 2006 to April 2008, samples of two bivalve molluscan shellfish (Acanthocardia tuberculatum and Callista chione) were collected in two different zones in the Mediterranean coast of Morocco (Martil city: Zone 2 and M'diq city: Zone 1). The samples were shipped to laboratory on the same day, in chilled condition and processed immediately.

Shellfish processing

Each sample was washed, scrubbed under running tap water and opened with a sterile shucking knife to collect hepatopancreas into a sterile tube. The collected products were mechanically homogenized, divided into 1.5g portions and stored at − 80 °C for virological analysis.

Viruses and cells

Sabin strain of Poliovirus type 1 (PS1) was used for experimental inoculation of shellfish. The virus strain was cultivated in Vero and HEp-2c cells. Cells were grown in Eagle's Minimum Essential Medium (MEM) (Gibco) supplemented with 2% fetal bovine serum (Gibco), 2% Penicillin 10.000 UI/mL and Streptomycin 10.000 µg/mL (Eurobio) and 2% L-Glutamine 200 mM (Eurobio). PS1 virus titration was carried out on Vero and HEp-2 cells in micro-titration plates and expressed by 50% tissue culture infection dose (TCID50) per volume unit, with 1 TCID50/mL. Consequently, the titer of Sabin poliovirus stock was 106 TCID50/mL.

Shellfish contamination

The bioaccumulation of PS1 strain was identified in aquarium, as described previously (**Legeay *et al*., 2000**), containing 5 *Acanthocardia tuberculatum* and 5 *Callista chione* in 5 liters of aerated sea water, spiked with 10^3 $TCID_{50}$/mL of PS1. Shellfish were observed for viability prior to inoculation and kept in the pool for 24 hours. They were then rinsed, opened and the hepatopancreas dissected and processed as described below.

Virus concentration

Virus concentration was performed using the protocol described previously **(Beuret *et al*., 2003; Mullendore *et al*., 2001**). This experimental protocol consists in virus elution with high-pH glycine buffer, followed by a virus concentration with polyethylene glycol.
Briefly, fifteen milliliters of Glycine-NaCl buffer (0.05 mol/L - 0.3 mol/L) were added to 1.5g of the hepatopancreas, and homogenized with a mixer mill (Retsch MM 301) and then centrifuged at 6000 ×g for 20 min at 4 °C; the supernatant was collected into a sterile tube. The virus was precipitated and mixed with an equal volume of PEG 8000- NaCl (12%- 0.3 mol/L) for two hours. The obtained mixture was then centrifuged at 6000 ×g for 15 min at 4 °C. The pellet was suspended in 1 mL of Na_2HPO_4 pH 9.5 as described previously (**Romalde *et al*., 2002**). After centrifugation at 2500×g for 10 min at 4 °C, the supernatant was filtered with a 0.22 µm-pore-size membrane filter (Millipore), and stored at -80°C.

Virus detection

Cell Culture: One milliliter of each viral sample concentrate was stored at -80°C and inoculated into two different confluent monolayer cell lines (Vero and Hep2). Subcultures of seven days were used to observe and detect cythopatic effects (CPE).
Viral nucleic acid extraction: For each sample, 200 µl of cell culture supernatant was used for a single RNA/DNA extraction method based on a guanidinium thiocyanate acid buffer (**Casas *et al*., 1995**).
Reverse transcription (RT): The EV reverse transcription was performed with 5 µL of the extracted RNA added to a "mix" containing: 0.5 mmol/L of dNTP mix, 10 pmol of Random Hexamer, 5×RT Buffer, and 2 units of AMV enzyme (Promega) in a final volume of 24 µL. The mixture was incubated for 1 h at 42°C, followed by incubation for 5 min at 95°C to inactivate the reverse transcriptase.
PCR: The highly conserved 5'-end untranslated region of enteroviruses was used as target for the synthesis of a 439 bp cDNA (**Hot *et al*., 2003**). For AdVs (Hexon gene) amplification, the used primers were Ad1 and Ad2 (**Hierholzer *et al*., 1993**) (Table 1).

Table 1 Primers sequences used for adenovirus and enterovirus PCR

	PRIMER	REGION	SEQUENCE (5'- 3')	REFERENCE
ENTEROVIRUS	P2	5'NC	CAAGCACTTCTGTTTCCCCGG	**Hot *et al*., 2003**
	P3	5'NC	ATTGTCACCATAAGCAGCCA	
ADENOVIRUS	Ad1	Hexon	TACGCCAACTCCGCCCACGCGCT	**Hierholzer *et al*., 1993**
	Ad2	Hexon	GCCGAGAAGGGCGTGCGCAGGTA	

The amplification of DNA and cDNA was performed separately in a final volume of 50 µl containing 5 µL cDNA or DNA extract, 5 mmol/L of PCR buffer, 1.5 mmol/L $MgCl_2$, 0.25 mmol/L of dNTPs, 10 pmol of each primer and 2U of Taq DNA polymerase (Promega).
PCR was carried out for 40 cycles in a thermal cycler (DNA Engine Dyad, Biorad). Each cycle consisted of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec (EV) - 60 °C for 30 (AdV), and primer extension at 72°C for 30 sec. After the last cycle, the extension was continued at 72°C for 15 min. In each serial, a control negative without DNA or RNA was included. Amplification products were analysed by electrophoresis method on a 2% Agarose gel and visualisation at the trans-lighting (Gel Doc system, Biorad).

RESULTS AND DISCUSSION

The results of experimental bioaccumulation of Poliovirus Sabin strain 1 in *Callista Chione* and *Acanthocardia tuberculatum* showed a titre of $10^{5.7}$ $TCID_{50}$/mL and $10^{3.9}$ $TCID_{50}$/mL respectively after titration on Hep-2 cell.

Virus detection via cell culture

A total of 44 shellfish samples were analysed for enteric viruses by cell culture procedure, 16 (36.4%) exhibited CPE in Vero cells, while 14 (31.8%) showed CPE in Hep2 cells. Taken together, infectious viruses were detected in 18 out of 44 samples (40.9%).

Virus detection via ICC-PCR assay

After cell culture, PCR reconfirmed the presence of enteroviral RNA and adenoviral DNA in the cell lysates, and some lysates with no CPE were positive for PCR. By using ICC–PCR method. Overall, 29 samples (65.9 %) was contaminated by at least one of the two studied viruses, Adenoviruses was detected in 23 (52%) of the 44 samples, and enteroviruses in 16 (36%). Simultaneous presence of enteroviruses and adenoviruses was observed in 10 samples (23%) (Table 2).

Table 2 Virus detection in cell culture and ICC-PCR with Hep-2 and Vero Cells

Cell line	Method	Number of positive samples / No of total samples		
		Collection Site		
		Zone 1	Zone 2	Total (%)
Hep 2	Cell culture	2/18 (11.1%)	12/26 (46.5%)	14/44 (31.8%)
	ICC-PCR	4/18 (22.2%)	15/26 (57.7%)	19/44 (43.2%)
Vero	Cell culture	3/18 (16.7%)	13/26 (50%)	16/44 (36.4%)
	ICC-PCR	8/18 (44.4%)	16/26 (61.5%)	24/44 (54.5)
Total	Cell culture	5/18 (27.8%)	13/26 (50%)	18/44 (40.9%)
	ICC-PCR	12/18 (66.7%)	17/26 (65.4%)	29/44 (65.9%)

Contamination with AdV and EV was detected in the two shellfish species and in all collection areas (12 in zone 1 and 17 in zone 2). 23 samples of *Callista chione* were positives for AdV and EV, 6 samples of *Acanthocardia tuberculatum* were positives only for AdV (Table 3).

Table 3 Virus detection in collections area and shellfish species

Collection site	Species	Number of positive samples / No of total samples	
		Adenovirus	Enterovirus
Zone 1	*Callista chione*	7/12 (58.33%)	4/12 (33.33%)
	Acanthocardia tuberculatum	1/6 (16.67%)	0
Zone 2	*Callista chione*	10/16 (62.5%)	12/16 (75%)
	Acanthocardia tuberculatum	5/10 (50%)	0

DISCUSSION

Viral contamination of shellfish might cause significant public health problems linked to the consumption of shellfish. To get a better understanding of the viral contamination in northern Morocco, we tested two species of shellfish for human adenovirus and enterovirus using cell culture and ICC-PCR.

The results of this study show that ICC-PCR is most sensitive for the detection of viruses, which is in agreement with previous studies (**Chaperon *et al*., 2000; Lee and Kim, 2002**). Our results showed that 65.9% samples were positive for viruses by ICC–PCR method compared with 40.9% samples positive by cell culture method. However it is still possible that the viral contamination level was underestimated because individual cells reproduce only certain types of the enteric viruses present in the samples.

The shellfish studied were growing in Tetouan region which was exposed to sewage and industrial waste water during the period study. The contamination of coastal waters by sewage is often cited to be the cause of shellfish contamination by enteric viruses (**Allard *et al*., 1992; Griffin et *al*., 2003; Hernroth *et al*., 2002**).

Detection and identification of viruses in shellfish is problematic because of the low density of contamination, inefficient recovery of viruses during the concentration process and the presence of natural inhibitors for the detection by PCR particularly in heavily contaminated waters. Viral pathogens include cultivable and non cultivable viruses whose detection methods are complex, laborious, time-consuming and expensive.

Different studies have demonstrated the successful application of PCR for the detection of the viruses in shellfish artificially contaminated in the laboratory and in naturally polluted shellfish (**Le Guyader *et al*. 2000; Chironna *et al*. 2002; Carducci *et al*. 2004**). Despite the success of PCR in detecting minimal starting quantities of nucleic acid, it has been pointed out that this method does not differentiate between infectious and non-infectious viruses because amplified nucleic acid could originate either from viable virus or from damaged non-infectious virus (**Lees 2000; Koopmans and Duizer 2004; Rompré *et al*., 2002**). In addition, molecular approaches can only be performed with qualified staff in specialized laboratories.

CONCLUSION

In our study, we used a simplified method to concentrate adenovirus and enterovirus in shellfish. We showed that the analysis of enteric viruses by the cell culture method alone might underestimate the concentrations of viruses in shellfish and that more viral particles could be detected by ICC–PCR method.

Our results confirm that shellfish could represent a potential health risk for consumers and require the development of an appropriate monitoring system in producing sites to improve shellfish safety.

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