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Detection of Human Adenoviruses and Enteroviruses in Korean Oysters Using Cell Culture, Integrated Cell Culture-PCR, and Direct PCR

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Oysters are known to be carriers of food-born diseases, but research on viruses in Korean oysters is scarce despite its importance for public health. We therefore tested oysters cultivated in Goheung, Seosan, Chungmu, and Tongyeong, for viral contamination using cell culture and integrated cell culture PCR (ICC-PCR) with Buffalo green monkey kidney (BGMK) and human lung epithelial (A549) cells. Additional screens via PCR, amplifying viral nucleic acids extracted from oysters supplemented our analysis. Our methods found 23.6%, 50.9%, and 89.1% of all oysters to be positive for adenoviruses when cell culture, ICC-PCR, and direct PCR, respectively, was used to conduct the screen. The same methodology identified enteroviruses in 5.45%, 30.9%, and 10.9% of all cases. Most of the detected enteroviruses (81.3%) were similar to poliovirus type 1; the remainder resembled coxsackievirus type A1. A homology search with the adenoviral sequences revealed similarities to adenovirus subgenera C (type 2, 5, and 6), D (type 44), and F (enteric type 40 and 41). Adenovirus-positive samples were more abundant in A549 cells (47.3%) than in BGMK cells (18.2%), while the reverse was true for enteroviruses (21.8% *vs.* 14.5%). Our data demonstrate that Korean oysters are heavily contaminated with enteric viruses, which is readily detectable via ICC-PCR using a combination of A549 and BGMK cells.

Keywords: adenovirus, enterovirus, oyster, cell-culture, ICC-PCR

Human enteric viruses are excreted in large numbers in feces and sewage and thereby find their way into the surface water, the groundwater, and the seawater. This constitutes a major public health threat as oysters, which filter small particles from the surrounding seawater, are a common part of our diet (Lees, 2000). The American Food and Drug Administration conducted a study in 1998 that revealed accumulation of large amounts of enteric viruses in shellfish which was deemed a public health risk and confirmed by various other studies (U.S. FDA 1998; Formiga-Cruz *et al.*, 2002; Beuret *et al.*, 2003; Nishida *et al.*, 2003; Shieh *et al.*, 2003; Myrmel *et al.*, 2004). It has been difficult, however, to isolate and identify viral particles in shellfish due to low-level contamination, inefficient analysis, and high concentrations of natural polymerase chain reaction (PCR) inhibitors in oyster tissues (Shieh *et al.*, 1999; Legeay *et al.*, 2000).

To address this problem, the Environmental Protection Agency enacted the Information Collection Rule (ICR), which introduced the total culturable virus assay (TCVA), a method for the detection of viruses in the environment based on the expression of viral cytopathic effects (CPE) in Buffalo green monkey kidney (BGMK) cells. Unfortunately, several enteric viruses (e.g. adenovirus types 40 and 41) do not produce CPE in the BGMK cell line, which renders this type of screening method prone to gross underestimation and incorrect negatives (Chapron *et al.*, 2000).

Adenoviral infections are seasonless and adenoviruses, in particular types 40 and 41, are considered second only to rotaviruses as the primary cause for gastroenteritis in children (Allard *et al.*, 2001). These epidemiologically important viruses are not detected by the TCVA, which has prompted several research groups to employ human lung epithelial A549 cells due to their susceptibility to a broad range of enteric adenoviruses and enteroviruses (Witt and Bousquet, 1988; Hashimoto *et al.*, 1991; Greening *et al.*, 2002).

Conventional cell culture methods have limited sensitivity regarding the detection of viruses and new

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procedures are currently being tested, including integrated cell culture-PCR (ICC-PCR), which utilizes the sensitivity of conventional PCR in combination with an infectivity assay (Pinto *et al.*, 1995). Several studies have established ICC-PCR as an effective (Greening *et al.*, 2002; Reynolds *et al.*, 2001) and, compared to traditional cell culture, more sensitive method for the detection of enteric viruses in water (Lee and Kim, 2002; Lee *et al.*, 2004; Lee *et al.*, 2005).

Previous analyses have shown that infectious viral particles, especially human adenoviruses and enteroviruses, contaminate the Korean surface water throughout the year (Cho *et al.*, 2000). To date, however, no data has been collected on the viral contamination of Korean marine oysters, which are direct recipients of upstream surface water. The aim of this study was i) to screen for human adenoviruses and enteroviruses in Korean oysters grown in Goheung, Seosan, Chungmu, and Tongyeong and ii) to find an appropriate detection method for adenoviral and enteroviral contamination of oysters. Various methods were tested, including cell culture, ICC-PCR with two types of cells (BGMK and A549), and direct PCR.

Materials and Methods

Oyster sample collection

Fifty-five oyster samples were collected between January 2002 and March 2003 (no collection in July 2002) from the Noryangjin fishery wholesale market, which supplies oysters from the four major harvesting areas - Goheung, Seosan, Chungmu and Tongyeong (Fig. 1). All oyster samples were put on ice, transported

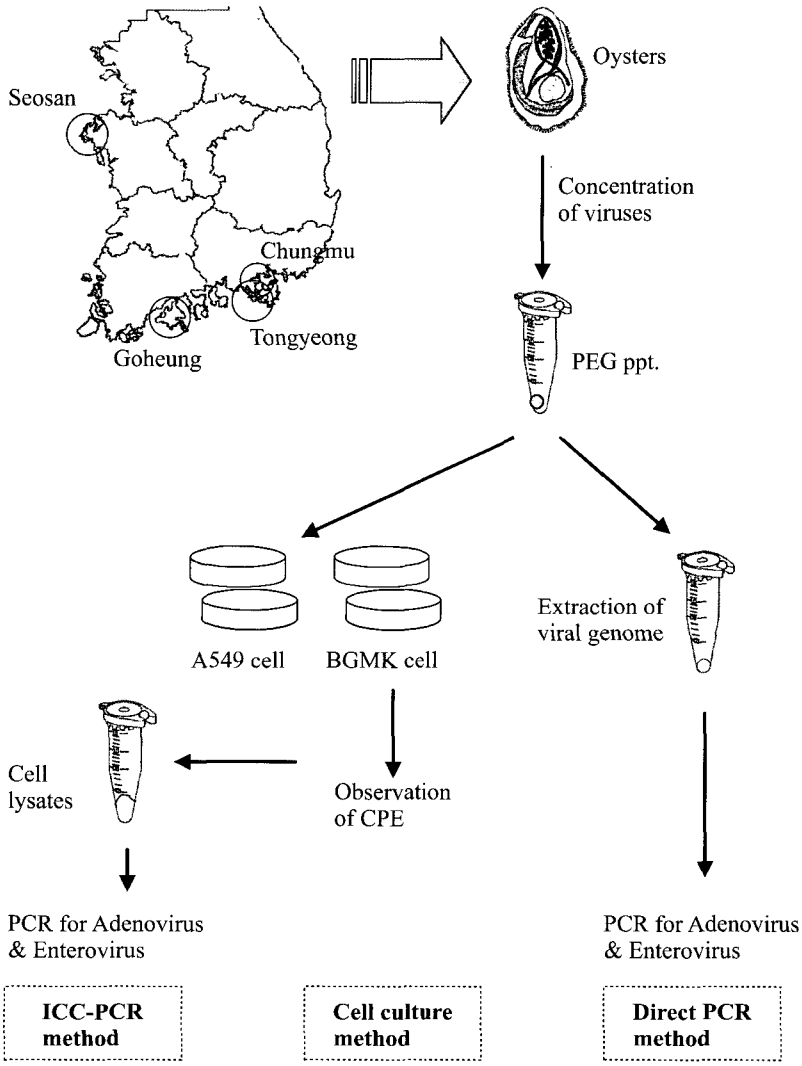

Fig. 1. Schematic diagram describing the strategy for the detection of adenoviruses and enteroviruses in oysters from four different harvesting areas

to the laboratory within 1 hour, washed twice externally with sterilized distilled water, divided into 25-g samples, and stored at −70°C until processing. The virus detection strategy is outlined in Fig. 1.

Virus concentration from oyster samples

All oyster samples were analyzed using a modified version of the protocol described by Mullendore *et al.* and Mendez *et al.*. Seven volumes of chilled, sterile, distilled water were added to 25 g of oyster tissue, which was homogenized twice for 60 s in an Omnimixer Waring blender (Omni International) at 25,000 rpm. The viruses were acid-adsorbed to the oyster solids by reducing the conductivity to < 2,000 S/cm via addition of distilled water, adjusting pH to 5 with 1 N HCl. After allowing for adsorption by 15 min of gentle stirring, the oyster solids with the adsorbed viruses were spun down for 20 min at 2,000 × g and eluted with seven volumes of chilled 0.05 M glycine/0.14 M NaCl (pH 7.5), with subsequent pH adjustment to 7.5. This was followed by 15 min of stirring, after which the oyster solids were pelleted by centrifugation (5,000 × g for 20 min at 4°C) and the supernatant was collected in a second tube to be stored at 4°C. The pellet was resuspended by vortexing for 60 s in 15 ml of 0.5 M threonine/0.14 M NaCl (pH 7.5). After an additional centrifugation as before, the supernatants were combined and the pellet discarded. Viruses in the supernatant were precipitated by polyethylene glycol 8,000 - NaCl solution (each final concentration is 8% [wt/vol] and 0.3 M, respectively) at 4°C for 2 h or overnight. The resulting floc was sedimented (6,700 × g for 30 min at 4°C) and resuspended in 15 ml of phosphate-buffered saline (PBS, pH 7.5). After addition of 15 ml of Vertrel XF (DuPont, USA) and 2 min of vortexing, the virus suspension was centrifuged at 2,000 × g for 30 min at 4°C. The supernatant was then removed and precipitated a second time by addition of PEG 8000/NaCl solution as above. This was followed by another centrifugation (14,000 × g for 15 min at 4°C), after which each of the PEG pellets was stored at −70°C until the cell culture assay and the extraction of nucleic acids for the direct PCR assay.

Virus detection via cell culture

For the cell culture assay, all oyster sample PEG pellets were resuspended in 10 ml PBS, divided equally, and inoculated in BGMK and A549 cells for 90 min at 37°C. The samples were removed and the cells washed twice with 10 mM PBS. Five milliliters of 2% (*v/v*) fetal bovine serum (FBS, HyClone, USA) supplemented minimal essential medium (MEM) or Dulbecco's modified Eagle medium (DMEM, Life Technologies, USA) were added to the BGMK and A559 cells, respectively. Adenovirus type 40 Dugan strain (VR-931, American Type Culture Collection, USA) was used as a positive control; PBS served as a negative control. When CPE was noted, the dish was frozen and thawed three times. All other dishes were similarly frozen and thawed on day 14 of the assay. One hundred microliters of cell lysate were then used for a second passage on a fresh cell monolayer and incubated at 37°C for 2 weeks to simulate the primary passage. CPEs were examined by microscope following the primary passage and a secondary incubation. Some cultures displayed presumptive CPE late in the second week of incubation.

Virus detection via ICC-PCR

After the second passage, all cell culture dishes were frozen and thawed three times. Two-hundred microliters of the supernatant were used to extract viral nucleic acids using the QIAamp Mini Elute Virus Spin Kit (Qiagen, Germany) to a final volume of 20 μl. Five microliters of the purified viral nucleic

Table 1. PCR primers

	Primer	Sequence (5' → 3')	Polarity	Reference
Adenovirus	Hex1deg	GCCSCARTGGKCWTACATGCACATC	Sense	Allard *et al.*, 2001
	Hex2deg	CAGCACSCCICGRATGTCAAA	Antisense	
	Hex3deg	GCCCGYGCMACIGAIACSTACTTC	Sense	
	Hex4deg	CCYACRGCCAGIGTRWAICGMRCYTTGTA		
Enterovirus	EV1	CAAGCACTTCTGTTTCCCCGG	Sense	Leparc *et al.*, 1994
	EV2	ATTGTCACCATAAGCAGCCA	Antisense	
	EV3	CTTGCGCGTTACGAC	Antisense	

acids were used as template in PCR amplifications according to Leparc *et al.* (1994) and Allard *et al.* (2001) (Table 1).

Virus detection via direct PCR

The PEG pellets of the oyster samples were resuspended thoroughly in 1 ml of 6 M guanidinium isothiocyanate and incubated at room temperature for 10 min. This was followed by a 10-min centrifugation at 12,000 × g. Half of the supernatant was used for RNA extraction with the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's protocol. Five-milliliter aliquots of the resulting viral nucleic acid solution were used for PCR and nested PCR assays for adenoviral detection using the conditions described above. All samples were run in triplicate and the results were added.

Cloning and sequencing

To avoid false-positives and to identify the recovered viral genomes, all nested PCR products were sequenced. Briefly, purified nested PCR products were inserted into the pGEM-T vector (Promega, USA), transformed into *Escherichia coli* DH5α competent cells, and re-isolated with Wizard Mini-Preps (Promega). Sequencing was done on an ABI 3730 automated sequencer (Applied Biosystems, USA). The resulting sequences were compared to those available in the EMBL/GenBank databases using the PubMed NCBI BLAST program and the genotype confirmed based on homology with known adenovirus sequences.

Results

Virus detection via cell culture

A total of 55 oyster samples from four different oyster-growing areas were analyzed by the cell culture method using BGMK or A549 cells (Table 2). For oyster samples from Goheung, two oyster samples exhibited CPE in BGMK and two another oyster samples exhibited CPE in A549 cells. Seosan oyster samples also tested positive for CPE (BGMK, N=1; A549, N=2). Oyster samples from Chungmu displayed CPE in both, BGMK and A549 cells (N=1 each), while 3 more samples exhibited CPE only in A549 cells. Two samples from Tongyeong showed CPE in BGMK and A549 cells (Table 2). Overall, of the 55 oyster samples analyzed, 6 (10.9%) exhibited CPE in BGMK cells, while 10 samples (18.2%) showed CPE in A549 cells. Taken together, infectious viruses were detected in 13 out of 55 oyster samples (23.6%).

Virus detection via ICC-PCR assay

After the second passage, reverse-transcription (RT)-nested PCR reconfirmed the presence of enteroviral RNA and adenoviral DNA in the cell lysates (Table 2). Some lysates with no CPE were also positive for PCR results. Similar to the cell culture assay, combination of the ICC-PCR results for both cell lines increased the detection sensitivity for infectious viruses. For oyster samples from Goheung, 3 samples originally deemed negative in the cell culture assay tested positive for either enteroviruses or adenoviruses in BGMK cell lysates. Lysates from A549 cells similarly produced 4 additional positive samples. Three samples tested positive in both cell lines. Thus, using ICC-PCR, 8 out of 14 Goheung oyster samples (57.1%) were contaminated with either adenoviruses or enteroviruses when the results from both cells lines were combined. For Seosan oyster samples, 2 and 5 additional oyster samples proved positive in BGMK and A549 cells, respectively, with 3 samples being virus positive in both cell lines. Hence, ICC-PCR showed that half of the Seosan oyster samples were positive for either adenoviruses or enteroviruses when the two cell line results were added. Using the same method, Chungmu oysters produced 5 and 3 positive

Table 2. Enteric virus detection frequency in cell culture and ICC-PCR assays with BGMK and A549 cells

Cell line	Method	No. of positive samples / No. of total samples				Total (%)
		Oyster-harvesting area				
		Goheung	Seosan	Chungmu	Tongyeong	
BGMK	Cell culture	2/14	1/14	1/13	2/14	6/55 (10.9)
	ICC-PCR	5/14	3/14	6/13	4/14	18/55 (32.7)
A549	Cell culture	2/14	2/14	4/13	2/14	10/55 (18.2)
	ICC-PCR	6/14	7/14	7/13	9/14	29/55 (52.7)
Total	Cell culture	4/14	3/14	4/13	2/14	13/55 (23.6)
	ICC-PCR	8/14	7/14	11/13	10/14	36/55 (65.5)

samples in BGMK and A549 cells, respectively, two samples being virus positive in both cell lines. In total, 11 out of 13 Chungmu oyster samples (84.6%) were positive for either adenoviruses or enteroviruses. The results for Tongyeong oysters were comparable, as 2 and 7 additional oyster samples were virus positive in BGMK and A549 cells, respectively, with 3 samples showing contamination in both cell lines, producing in total 10 contaminated samples out of the 14 collected (71.4%).

Overall, 18 (32.7%) and 29 (52.7%) out of 55 oyster samples were positive for either adenoviruses or enteroviruses in BGMK and A549 cells, respectively. Eleven samples (20.0%) were positive in both cell lines. Combination of the ICC-PCR test results from BGMK and A549 cells produced 36 positive samples in total (65.5%).

We next determined the virus type from the ICC-PCR results (Table 3). Adenoviruses were identified in 1 (BGMK) and 3 (A549) additional Goheung oyster samples, with a total of 3 additional samples in both cell lines. Seosan oysters had adenoviral contamination in 1 (BGMK) and 5 (A549) additional cases, with a total of 5 additional samples being positive in both cell lines. Chungmu oysters were adenovirally contaminated in 2 (BGMK) and 2 (A549) additional cases, with a total of 3 additional samples in both cell lines. Finally, Tongyeong oysters came up with no additional adenovirus positive samples in BGMK cells, while 6 additional samples were deemed positive in A549 cells. In summary, 10 (18.2%) and 26 (47.3%) oyster samples were found adenovirus-positive in BGMK and A549 cells, respectively, when tested via ICC-PCR, with 8 samples (20.0%) being positive in BGMK as well as in A549 cells. This method thus generated 28 adenovirus positive tests for a total of 55 oyster samples (50.9%, combined results).

Enteroviral contamination faired similarly, when examined via ICC-PCR. Goheung (for additional samples being positive in BGMK, A549, and both, respectively) 2, 2, and 3; Seosan: 1, 2, and 1; Chungmu: 3, 1, and 4; Tongyeong: 3, 3, and 6; overall: 12 (21.8%), 8 (14.5%), and 3 (5.5%). Thus, by combining the results from both cell lines, ICC-PCR produced 17 enterovirus positive samples from a total of 55 oysters (50.9%).

These data demonstrate that A549 cells performed

Table 3. Detection frequency of adenovirus and enterovirus in cell culture and ICC-PCR assays with BGMK and A549 cells

	Cell line	Method	No. of positive samples/ No. of total samples					
			G	S	C	T	Total (%)	
Adenovirus	BGMK	CC[a]	2/14	1/14	1/13	2/14	6/55 (10.9)	10/55 (18.2)
		PCR[b]	1/14	1/14	2/13	0/14	4/55 (7.27)	
	A549	CC	2/14	2/14	4/13	2/14	10/55 (18.2)	26/55 (47.3)
		PCR	3/14	5/14	2/13	6/14	16/55 (29.1)	
	subtotal[c]	CC	4/14	3/14	4/13	2/14	13/55 (23.6)	
		PCR	3/14	5/14	3/13	6/14	17/55 (30.9)	
	Total[d]		6/14	7/14	7/13	8/14	28/55 (50.9)	
Enterovirus	BGMK	CC	1/14	1/14	0/13	1/14	3/55 (5.45)	12/55 (21.8)
		PCR	2/14	1/14	3/13	3/14	9/55 (16.4)	
	A549	CC	0/14	0/14	0/13	0/14	0/55 (0.0)	8/55 (14.5)
		PCR	2/14	2/14	1/13	3/14	8/55 (14.5)	
	subtotal[c]	CC	1/14	1/14	0/13	1/14	3/55 (5.45)	
		PCR	3/14	1/14	4/13	6/14	14/55 (25.5)	
	Total [e]		4/14	2/14	4/13	7/14	17/55 (30.9)	

G: Goheung , S: Seosan, C: Chungmu, T: Tongyeong,
CC[a]: CPE positive and ICC-PCR positive,
PCR[b]: CPE negative and ICC-PCR positive,
subtotal[c]: sum of the CC[a] results or ICC-PCR[b] results from both, BGMK and A549 cells
Total[d]: adenoviruses detected with ICC-PCR, combined results from BGMK and A549 cells
Total[e]: enteroviruses detected with ICC-PCR, combined results from BGMK and A549 cells

better for adenoviral detection, while BGMK cells were better suited for enteroviral screening.

Detection of Adenoviruses and Enteroviruses via direct PCR

We additionally ran assays based on direct RT-nested PCR. Adenoviruses were detected in 49 out of 55 oyster samples (89.1%), while enteroviruses proved to present in 6 out of 55 oyster samples (10.9%). Compared to the ICC-PCR approach, the number of adenovirus positives increased from 28 to 49, whereas enteroviruses positives declined from 17 to 6.

Sequence analyses of the PCR products

Sequence analysis for adenoviral nucleic acids showed that both enteric and non-enteric adenoviruses were present in the oysters (Table 4). Among the identified types were human adenovirus types 40 and 41 (subgroup F, enteric adenoviruses), human adenovirus types 2, 5, and 6 (subgroup C, non-enteric adenoviruses), and type 44 (subgroup D, non-enteric adenoviruses). The ICC-PCR approach detected enteric adenoviruses and non-enteric adenoviruses in 73.3 and 26.7% of all samples, respectively. Direct PCR, on the other hand, produced positives for enteric and non-enteric adenoviruses in 57.1 and 42.9% of all cases, respectively.

Sequence analysis for enteroviral nucleic acids showed that almost all enteroviruses detected were similar to poliovirus type 1. With ICC-PCR, polioviruses were detected in 13 out of 16 samples (81.3%), with the remaining 3 positive samples (18.8%) resembling coxsackievirus type A1. Direct PCR only detected polioviruses.

Discussion

Cases of human enteric viruses as well as pathogenic bacteria causing gastroenteritis are epidemiologically linked to the consumption of shellfish, especially oysters (Rippey, 1994; Lees, 2000). Illegal overboard sewage discharge into areas where shellfish are cultivated is the most probable cause for recent U.S. outbreaks (Veazey *et al.*, 1998).

Many investigators have studied enteric pathogens: their incidence, ecology and pathogeny (Sayler, 1976; Kaper, 1979; Murphree, 1995; Kim, 2004; Jones, 2005), because they predominate as a disease vector in clinical statistics all over the world. The link to incidents of infectious diseases is a reflection of a traditional consumption style – raw or only lightly cooked, eaten in whole including the viscera. In

Table 4. Types of adenoviruses and enteroviruses detected in oysters harvested in Goheung, Seosan, Chungmu, and Tongyeong

Date		ICC-PCR				Direct PCR			
		G	S	C	T	G	S	C	T
2002	Jan	C1		A2	P1	P1, A2	P1	A41	
	Feb			P1		A40	A2	A5	A6
	Mar	A41		A4	C1	A41			P1, A40
	Apr	A41	A2	C1		A41	A41	A41	A41
	May			A40	P1, A40	A44	A2	A5	A40
	Jun	A40	A40	A40	P1, A40		A40	A40	A6
	Aug	P1, A5	P1, A5	NA	A44	P1, A5	A5	NA	A44
	Sep		P1, A41		P1, A5	A41	A41	A41	A41
	Oct		A41	A41	A41	P1, A41	A41	A41	A41
	Nov	P1		P1	P1, A41	A2	A2	A41	A41
	Dec	A41			A41	A6	A5	A41	A2
2003	Jan					A2		A2	A41
	Feb	P1, A40	A41	A40	P1, A2	A40	A41	A40	A2
	Mar	A41	A2	A41	A41	A2	A41	A6	A41

G: Goheung, S: Seosan, C: Chungmu, T: Tongyeong, NA: Not assayed,
P1: poliovirus type 1, C1: coxsackievirus A type 1, A2: human adenovirus type 2, A5: human adenovirus type 5, A6: human adenovirus type 6, A40: human adenovirus type 40, A41: human adenovirus type 41, A44: human adenovirus type 44

Korea, raw shellfish is a common part of the daily diet. New recipes regarding the preparation of raw shellfish are continuously developed due to a prevailing public ignorance about the dangers of this food source. To get a better understanding of the type, frequency, and level of the viral contamination, we screened oysters for human adeno- and enteroviruses using various detection methods including cell culture, ICC-PCR, and direct PCR.

The results of our study show that ICC-PCR is most sensitive for the detection of infectious viruses in oysters, which is in agreement with a previous water environment survey (Chaperon *et al.*, 2000; Lee and Kim, 2002). While ICC-PCR detected many infectious enteric viruses, 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.

In particular, enteric adenovirus type 40 and 41 are cannot be propagated efficiently in BGMK cells, which are most commonly used in environmental enteric virus sampling. To reduce false-negatives, several investigators have turned to combine appropriate cell lines for an expanded range of virus detection. Grabow and co-workers applied to polioviruses. Earlier cell culture work in our own laboratory has shown that combination of A549 and BGMK cells is a useful tool for the detection of adenoviruses and enteroviruses in water environments (Lee *et al.*, 2004). We therefore used these two cell lines to examine virus infections in oysters. The results of this study show that, for the detection of adenoviruses and enteroviruses, combined-cell ICC-PCR is superior to conventional cell culture and ICC-PCR using only a single cell line.

When the ICC-PCR results for the two cell lines are compared, sensitivity for adenoviruses was higher in A549 cells (47.5%) than in BGMK cells (18.2%). The reverse was true for enteroviruses (14.5 *vs.* 21.8%). It has been reported in the literature that it is difficult to induce adenoviral CPE in BGMK cells, as these cells seem to select for enteroviruses which replicate more rapidly than adenoviruses (Lee and Kim, 2002). Our data do not confirm such notion. In four samples with CPE, two were positive for adenoviruses and another two were positive for both, adenoviruses and enteroviruses. It may be that the enterovirus concentration was too low to interfere with adenoviral amplification. The higher level of adenovirus detection by ICC-PCR is probably the result of infectious adenoviruses being too scarce in oysters to generate significant CPE.

The results of the sequence analysis showed that most of the detected enteroviruses were similar to polioviruses. Oral poliovirus vaccines may be the source of poliovirus dissemination (Bellmunt *et al.*, 1999), which, once discharged into the water environment, ultimately accumulated in oysters.

In many previous studies, enteric viruses were detected by PCR-based methods (Le guyader *et al.*, 2000; Formiga-Cruz *et al.*, 2002), because cell culture methods are time-consuming and expensive, and in many cases difficult to adapt to enteric virus screens. In this study, direct PCR results showed that almost all oyster samples were contaminated with adenoviruses. When the ICC-PCR results were taken into account, the detected viruses only with direct PCR assay proved to be noninfectious as in previously published reports. However, it was possible to underestimate adenovirus level due to cell susceptibility.

Our sequencing for adenoviral presence differed depending on the type of PCR employed. Nonenteric adenoviruses were found in 21.4% via ICC-PCR, but in 40.8% if direct PCR was used. Viral accumulation in oysters is nonspecific, and the fact that enteric adenoviruses appeared to be more concentrated may be a reflection of prolonged infectivity in enteric over nonenteric adenoviruses. Adenovirus serotype C (adenovirus type 2, 5, and 6) was detected by both, ICC-PCR and direct PCR, while adenovirus serotype D (adenovirus type 44) was detected only by direct PCR. To date, there are no reports that adenovirus serotype C and D are related to gastroenteritis or transmitted via the fecal/oral route. It is important to note, however, that adenovirus serotype C in oysters may be highly infectious and thus a potential public health hazard.

Detection of enteroviruses was less sensitive with direct PCR compared to ICC-PCR. The amount of oyster tissue analyzed by cell culture/ICC-PCR for one trial was ~50 g, while direct PCR assays required only 3.12 g for one PCR analysis. This implies that direct PCR can seriously underestimate viral concentrations compared to ICC-PCR, simply because the amount of enterovirus is below the detection limit of the PCR reaction.

Earlier work by Muniain-Mujika *et al.* (2003) found that the detection probability for adenoviruses, enteroviruses, and HAV in shellfish decreased as the temperature of the surrounding water increased. Our data, conversely, showed improved detection with elevated water temperature. This difference may originate from different harvesting areas, where the water quality is influenced by unequal upstream surface waters. It is hence crucial to employ appropriate screening methods to produce an accurate estimate of viruses in Korean oysters.

ICC-PCR as well as direct PCR detected adenoviral contamination in almost all oyster samples. Potentially, human adenovirus levels in oysters could be used as

an index for the presence of the same in humans. This is in line with data by Pina *et al.*, (1998) who suggested that human adenoviral PCR detection could be used as an index for the presence of human viruses in the environment based on their stability and their mostly human origin. Our current study shows that infectious adenoviruses can be recovered via ICC-PCR from A549 and BGMK cells.

In conclusion, we found infectious viral particles in Korean oysters throughout the year. Contamination control during the processing of shellfish tends to be little effective, but it may pose an alternative for countries with heavily contaminated waters. Two different approaches are used to decontaminate shellfish, namely heat treatment (cooking) and extending natural filtering in clean seawater to purge microbial contaminants. The latter can be performed in tanks (depuration) or in the natural environment (relaying). However, oysters are mostly consumed raw and rarely cooked. Additionally, the depuration process is complex, because the viral elimination efficiency is lower than bacterial elimination and influenced by the temperature and other physiochemically important parameters such as food availability, salinity, oxygen levels, and the overall condition of the shellfish (Jaykus *et al.*, 1994). Successful elimination is also critically dependent on the initial contamination level. It is therefore generally accepted that the most effective and reliable approach to controlling the contamination of shellfish is to harvest from areas with good water quality. To ensure protection from contaminated oysters, wastewater treatment plants must work effectively, and a continuous survey of viral pollution of the oysters' water environment must be carried out nationwide.

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