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Localization and persistence of hepatitis A virus in artificially contaminated oysters

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ABSTRACT

Bivalve molluscan shellfish, such as oysters, clams, and cockles, are well-recognized as vectors that concentrate foodborne pathogens by filter feeding. The objective of this study was to investigate the distribution and persistence of hepatitis A virus (HAV) in experimentally contaminated oysters that were either fed or not fed with algae. Oysters were experimentally contaminated with HAV and maintained in depuration conditions. qRT-PCR, immunohistochemistry (IHC), and *in situ* hybridization (ISH) were performed on oyster samples collected at 0, 1, 3, 5, and 7 days post-inoculation. When HAV-contaminated oysters were depurated for 7 days, HAV was detected in 91.1–97.8% of the digestive glands and gills. While the high viral load in the digestive glands in oysters did not change significantly regardless of algae-feeding, the viral load of the gills gradually decreased in both groups during the depuration. HAV antigen and RNA were detected in the digestive diverticula and connective tissues by both IHC and ISH. HAV was detected in the stomach, intestine, and gills by only ISH. The distribution of HAV in various oyster tissues may explain the persistence of contamination in oysters during the depuration process.

1. Introduction

Bivalve shellfish including oysters, clams, and cockles, are important vectors for viral enteric diseases because raw or undercooked oysters are widely consumed (Gerba and Goyal, 1978; Kingsley and Richards, 2003; McLeod et al., 2009a). They can accumulate various foodborne pathogens efficiently by filtering as much as 40 l of seawater per hour as part of their feeding activities (Goyal et al., 1979; Provost et al., 2011). Hepatitis A virus (HAV) and human norovirus (HuNoV) commonly cause shellfish-borne illness, which is linked with the consumption of raw or undercooked shellfish in many countries (Koopmans et al., 2002; McLeod et al., 2009b). The contamination of hepatitis E virus, astrovirus, rotavirus, sapovirus, and aichivirus in shellfish also cause outbreaks. Although shellfish was grown from the “class A” harvesting area of Portugal, the contamination of HuNoV and HAV in them was reported (Mesquita et al., 2011). The viral contamination of shellfish can be a serious health risk to shell fish consumers and enormous economic loss to the seafood industry (Kingsley and Richards, 2003; Richards et al., 2010).

Relay and depuration processes are used in the seafood industry to reduce the enteric bacterial or viral contamination in oysters (Dore and Lees, 1995; Power and Collins, 1989). Generally, the commercial depuration process takes 2–3 days, where in the shellfish are transferred into clean seawater, under conditions which allow the shellfish to eliminate the ingested contaminants. During the depuration process, the fecal bacteria accumulated in shellfish are purged within 48 to 72 h. However, several studies showed that human enteric non-enveloped viruses are not eliminated effectively in oysters by the commercial depuration process (Kingsley and Richards, 2003; Maalouf et al., 2010; Provost et al., 2011). Although viruses do not replicate within the shellfish, clearance of human enteric viruses in oysters is very slow. Viral contamination in oysters persists for up to several weeks of depuration (Calci et al., 2005). Many natural factors including tidal cycle, seasonal variation, salinity, temperature, light, pH, and turbidity are associated with the physiologic state of shellfish including the metabolism and immune system. And the depuration process at a low temperature reduced the elimination rate of human norovirus in Eastern oysters (Choi and Kingsley, 2016; Kennedy et al., 1996).

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In the ISO TS 15216-1 standard protocol, the digestive tract of shellfish is used as an exclusive target tissue to detect human norovirus and hepatitis A virus. As shellfish entrap nutrients on their mucous membranes during filter feeding and eventually transfer them to the digestive tract, the persistent contamination of HuNoV in the digestive tissues was observed (McLeod et al., 2009a, 2009b). According to the protocol of the United States Food and Drug Administration, 10–15 g of whole oysters should be used for virus testing (Kingsley and Richards, 2003; Richards et al., 2010). The gills of oysters are the largest non-digestive organ, whose surfaces collect filtered material and organic matter dissolved in seawater, including viruses and associated solids, and which sort the food particles to the labial palps (Richards et al., 2010). Recently, gills were suggested as a better target tissue to detect and localize HuNoV than digestive tissues (Wang et al., 2008). Although the contamination of human enteric viruses has been examined by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) in previous studies (Choi and Kingsley, 2016; McLeod et al., 2009a, 2009b), the target tissues and cells of HAV in oysters have not been determined.

Since the unfiltered natural seawater of aquarium was supplied to mimic tidal change in previous studies, artificially-contaminated oysters were restricted to obtain enough nutrients in laboratory setting (Choi and Kingsley, 2016; McLeod et al., 2009a, 2009b). Because the restriction of feeding lower metabolic rate and phagocytosis of oysters (Kennedy et al., 1996), it is necessary to investigate the effect of feeding on the virus accumulation and persistence of HAV in oysters. Therefore, this study aimed to compare the viral load of the digestive tract and nondigestive tract in HAV-contaminated oysters depending on the depuration time and algae feeding and to identify the target cells and the distribution of HAV in the oyster tissues by immunohistochemistry (IHC) and *in situ* hybridization (ISH).

2. Materials and methods

2.1. Preparation of virus inoculum

Hepatitis A viral strain HM-175 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HAV was cultivated following the previous protocol (Lee et al., 2013). Briefly, it was propagated on confluent fetal rhesus monkey kidney cells (FRhK-4, ATCC CRL-1688) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The titer of HAV stock determined by plaque assay was 1.7×10^6 plaque forming unit (PFU)/ml.

2.2. Experimental design

Oyster samples used in this study were purchased from a retail market in Tongyeong-si, Gyeongsangnam-do, Republic of Korea. Prior to the experimental challenge with HAV, three oysters of each batch were randomly selected and shucked for confirming the lack of contamination with HuNoV GI, HuNoV GII, HAV, and hepatitis E virus. All oysters were acclimated for 1 week in 65-liter aquariums equipped with air stones, aerators, and a protein skimmer primarily used in saltwater aquariums in order to remove dissolved organic compounds and other harmful substances. Forty liters of artificial seawater was freshly prepared with sea salt (Red sea, TX, USA) according to the manufacturer's instructions, and the temperature was set at 15–18 °C. Composition of the artificial seawater was as followed: Salinity 31 ppt, pH 6.8–7.2, Mg 1090–1150 mg/l, Ca 365–385 mg/l, K 330–350 mg/l.

In order to examine the effect of feeding in oysters, 90 oysters were divided into two groups after the acclimation period. Forty-five oysters were fed daily with phytoplankton concentrates (KENT Marine, TN, USA) containing *Nannochloropsis*, *Tetraselmis*, and *Isochrysis* species over the acclimation period. The other 45 oysters were not fed. Then, individual oysters were challenged with 1.7×10^6 PFU/ml of HAV for 16 h in a 1-liter glass beaker with 500 ml artificial seawater. Thereafter, HAV-challenged oysters were transferred to a new aquarium with HAV-negative fresh artificial seawater. This study used the same setup for mimicking the depuration process reported in a previous study (Choi and Kingsley, 2016). Freshly made artificial seawater was changed every 24 h. Three HAV-challenged oysters per experimental group were harvested at 0, 1, 3, 5, and 7 dpi. They were shucked with a sterile shucking knife. Their digestive gland, gills, and adductor muscles were removed for further testing.

2.3. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The detection of HAV in oysters was performed by following the guidance of the ISO/TS 15216-1:2013 protocol. Murine norovirus (MNV-1) was used as a processing control. Briefly, viral RNA was extracted using BioMerioux NucliSens miniMAG (bioMérieux, Korea) according to the manufacturer's protocol. One-step qRT-PCR was performed on a total volume of 25 μl, containing 5 μl of 5× Ultrasense reaction mix (Invitrogen, Carlsbad, CA, USA), 1.25 μl of RNA Ultrasense enzyme mix (Invitrogen), 0.5 μl of 10 pmol/μl HAV primers, 0.5 μl of 10 pmol/μl HAV probes, 5 μl of viral RNA, and 12.25 μl of diethyl pyrocarbonate (DEPC)-treated water. The primer sets developed by Jothikumar et al. (2005) were as follows: forward (5′-GGT AGG CTA CGG GTG AAA C-3′), reverse (5′-AAC AAC TCA CCA ATA TCC GC-3′), and probe (5′-CTT AGG CTA ATA CTT CTA TGA AGA GAT GC-3′). qRT-PCR was performed on a Thermal Cycler Dice Real-time System (TaKaRa Bio Inc., Shiga, Japan) at 50 °C for 15 min and 95 °C for 2 min with 45 cycles of 10 s at 95 °C and 20 s at 55 °C for HAV. The synthetic HAV RNA was synthesized by MBIOTECH (Gyeonggido, South Korea) and was used as a quantification standard to determine the quantity of viral RNA.

2.4. Plaque assay

Plaque assays for HAV were performed as previously described (Lee et al., 2013). FRhk-4 cells were seeded on 12-well plates and incubated at 37 °C, 5% CO_2. The virus eluted from oyster samples was serially diluted in serum-free DMEM. Each well of confluent FRhK-4 cells was inoculated with 0.2 ml of diluted HAV, and plates were incubated at 1 h at 37 °C. Each well was overlaid with 1 ml of 2× type II agarose mixed with 2× DMEM at room temperature and incubated for 6–7 days at 37 °C. Each cell was fixed with 10% neutral formalin (Sigma-Aldrich, St. Louis, MO, USA) and stained with 0.1% crystal violet solution for 20 min at room temperature. The HAV titer was expressed as $\log_{10}$ PFU/ml.

2.5. Immunohistochemistry (IHC)

Oysters were fixed with 10% neutral formalin solution (Sigma) for 24 h. They were submitted to routine tissue processing and embedded in paraffin. Formalin-fixed paraffin-embedded tissue (FFPE) sections with 4-μm thickness were prepared on silane coating slides (Muto Pure Chemicals, Japan). The tissue sections were deparaffinized in xylene and then rehydrated in serially graded alcohols. For antigen retrieval, the slides were treated with 100 μg/ml RNase-free proteinase K (Invitrogen) for 5 min at 37 °C. Non-specific interactions were blocked using 10% normal horse serum (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. The slides were incubated with mouse anti-HAV monoclonal antibody (Boreda Biotech, Korea) overnight at 4 °C. Biotinylated horse anti-mouse IgG and universal ABC-AP (Vector Laboratories) were applied sequentially. The tissue sections were visualized with ImmPACT Vector Red (Vector Laboratories, SK-5105) for 5–20 min. They were counter-stained with hematoxylin for 30 s and cover-slipped for microscopic observation.

2.6. In situ hybridization (ISH)

A double-stranded DNA probe was prepared with slight modifications according to a previous publication (Seo et al., 2018; Tahk et al., 2012). Briefly, RT-PCR products in the VP1/P2A region of HAV were amplified with 5′-TTG TCT GTC ACA GAA CAA TCA G-3′ (BR5; nucleotide 2950 to 2972) and 5′-AGT CAC ACC TCT CCA GGA AAA CTT-3′ (BR9; nucleotide 3310 to 3286) (Bruisten et al., 2001). The 360 base pairs of the RT-PCR product were purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WA, USA) and were labeled with a digoxigenin-DNA labeling kit (Roche, Switzerland).

To detect HAV RNA in the oyster tissues, *in situ* hybridization was performed by following the previous protocol (Seo et al., 2018; Choi et al., 2004). Briefly, FFPE tissue sections were deparaffinized and rehydrated. They were treated with 0.2 N hydrochloric acid for 20 min at room temperature and digested with 100 μg/ml proteinase K (Invitrogen) for 15 min at 37 °C. The tissue sections were hybridized with digoxigenin-labeled dsDNA probe diluted in a hybridization buffer (Enzo Life Sciences, Farmingdale, NY, USA). After hybridization, the slides were washed with saline sodium citrate (50 mM NaCl and 15 mM sodium citrate, pH 7.0). Then, each slide was rinsed with a maleic acid buffer (100 mM maleic acid and 150 mM NaCl, pH 7.5) for 5 min followed by 1 × blocking solution (Roche) for 40 min. Alkaline phosphate (AP)-conjugated anti-digoxigenin antibody was applied for 1 h at 37 °C. The hybridized probe with HAV RNA was visualized as a dark purple color by nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). Counterstaining was performed with 3% methyl green (Sigma). Slides were covered with glass coverslips using vectamount (Vector Laboratories).

2.7. Statistical analysis

Each experimental group had three replicates per each experiment. All experiments were repeated three times independently. One-way analysis of variance (ANOVA) and Duncan's multiple range testing were performed to compare the viral load on digestive glands and gills of oysters using the Statistical Analysis System software (SAS 9.1 version, Cary, NC, USA). Statistical significance was determined by $P < 0.05$.

3. Results

3.1. Reduction of HAV titer during the depuration of oysters

qRT-PCR detected HAV in the oysters depurated for up to 7 days post-inoculation (dpi) (Table 1). Regardless of algae-feeding, all oysters were positive for HAV at 0 dpi. In algae-unfed oysters, 44/45 (97.8%) of digestive glands and 41/45 (91.1%) of the gills were positive for HAV. In algae-fed oysters, 42/45 (93.3%) of both digestive glands and gills were positive for HAV. Although several oysters were negative for HAV at 7 dpi, the detection rate of HAV was not significantly different between algae-fed and algae-unfed oysters.

Table 1
qPCR detection of hepatitis A virus in experimentally contaminated oysters during the depuration process (no. of HAV-positive samples/no. of total samples).

Days post-inoculation (dpi)	Digestive glands		Gills	
	Without algae-feeding	With algae-feeding	Without algae-feeding	With algae-feeding
0 dpi	9/9 (100%)	8/9 (88.9%)	9/9 (100%)	8/9 (88.9%)
1 dpi	9/9 (100%)	9/9 (100%)	9/9 (100%)	9/9 (100%)
3 dpi	9/9 (100%)	9/9 (100%)	9/9 (100%)	9/9 (100%)
5 dpi	9/9 (100%)	9/9 (100%)	9/9 (100%)	8/9 (88.9%)
7 dpi	8/9 (88.9%)	7/9 (77.8%)	5/9 (55.6%)	8/9 (88.9%)
Detection rate	44/45 (97.8%)	42/45 (93.3%)	41/45 (91.1%)	42/45 (93.3%)

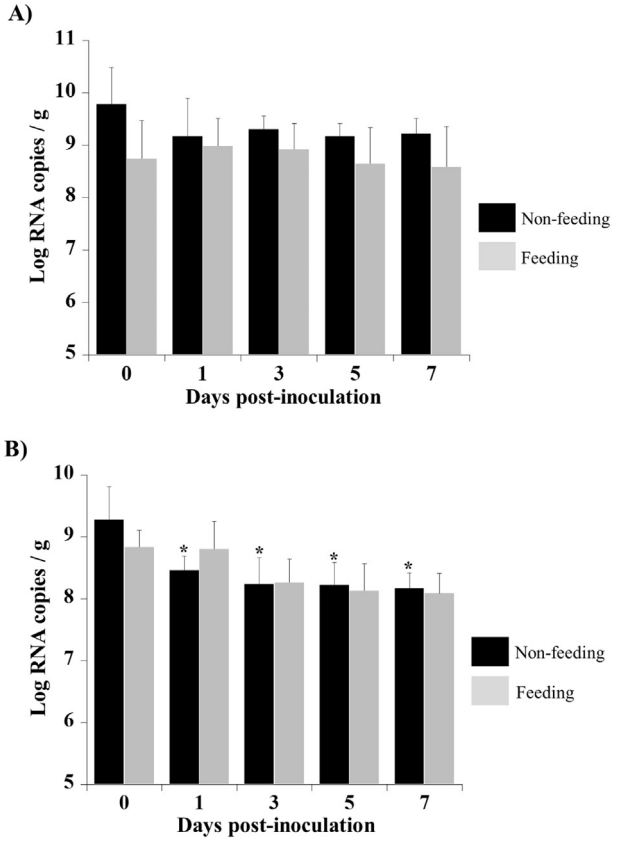

Fig. 1. Persistence of hepatitis A virus in the digestive gland and gill tissues of oysters during the depuration process. A) The digestive glands of algae-unfed oysters (black bars) and algae-fed oysters (gray bars), B) the gills of algae-unfed oysters (black bars) and algae-fed oysters (gray bars). Asterisk indicates statistical significance ($P < 0.05$).

The plaque assay was attempted to examine the viability of HAV remaining in the oyster tissue. However, oyster homogenates containing proteinase K and various tissue enzymes were highly cytotoxic to FRhK-4 cells because they were prepared following the ISO/TS 15216-1 protocol. Therefore, the HAV plaque assay was not successful in FRhK-4 cells (data not shown).

As an alternative to the HAV plaque assay, qRT-PCR determined the viral load of HAV-challenged oyster tissues during the depuration process (Fig. 1). In the digestive gland of the algae-fed oysters, the HAV titer decreased from 8.74 ± 0.72 $\log_{10}$ RNA copies/g at 0 dpi to 8.58 ± 0.77 $\log_{10}$ RNA copies/g at 7 dpi. In the digestive glands of algae-unfed oysters, the HAV titer decreased from 9.78 ± 0.70 $\log_{10}$ RNA copies/g at 0 dpi to 9.22 ± 0.29 $\log_{10}$ RNA copies/g at 7 dpi. Although depuration reduces HAV slightly in the digestive glands, the HAV reduction was not statistically significant in algae-fed and algae-unfed oysters.

In the gills of algae-unfed oysters, the HAV titer decreased from 9.27 ± 0.53 $\log_{10}$ RNA copies/g at 0 dpi to 8.17 ± 0.25 $\log_{10}$ RNA copies/g at 7 dpi. In the gills of algae-fed oysters, the HAV titer decreased from 8.84 ± 0.27 $\log_{10}$ RNA copies/g at 0 dpi to 8.09 ± 0.32 $\log_{10}$ RNA copies/g at 7 dpi. Depending on the depuration time, the HAV titer of gill tissues decreased significantly both in the algae-fed and algae-unfed oysters ($P < 0.05$). HAV reduction in the gills of algae-unfed oysters was significantly higher than in the gills of algae-fed oysters ($P < 0.05$).

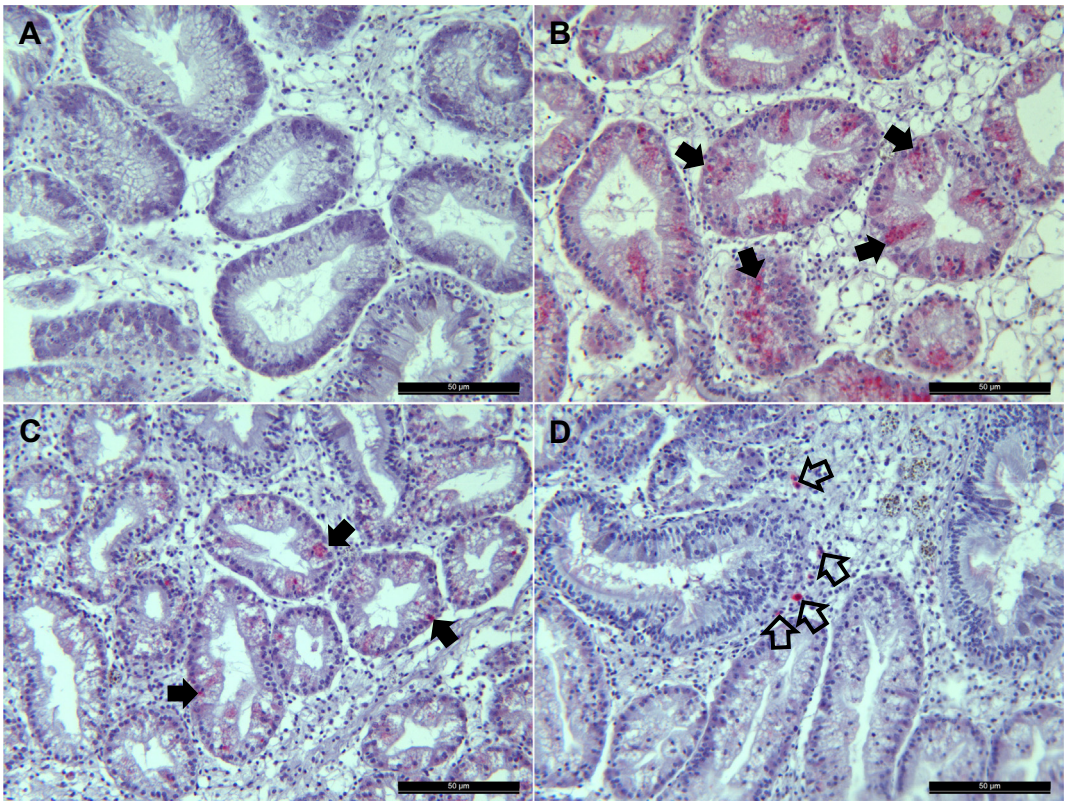

Fig. 2. Localization of hepatitis A virus in experimentally contaminated oysters by immunohistochemistry. A) Digestive diverticula of negative control oyster, B) digestive diverticula of HAV-contaminated oyster at 0 days post-inoculation (dpi), C) digestive diverticula of HAV-contaminated oyster at 3 dpi, D) digestive diverticula of HAV-contaminated oyster at 7 dpi, A–D magnification (×400). Positive signal was visualized as red color with ImmPACT Vector Red substrate. All slides were counterstained with hematoxylin. Black arrow indicated HAV capsid antigen in epithelium. Open arrow indicates HAV-positive hemocytes. Scale bar indicates 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Localization of HAV capsid in oysters by IHC

IHC was performed in the digestive tissues (digestive diverticula, intestine, and stomach) and nondigestive tract (connective tissues, gills, and adductor muscle) of the HAV-challenged oysters (Fig. 2 and Supplementary Fig. 1). IHC for HAV was completely negative in the negative control oysters. The IHC positive signal against the HAV capsid was observed predominantly in the epithelium and lumen of the digestive diverticula. The intensity of the IHC signal in the epithelium of the digestive diverticula decreased according to the depuration time (0, 3, and 7 dpi) (Fig. 2). Regardless of depuration time, individual hemocytes in the connective tissue surrounding the digestive diverticula were strongly positive for HAV by IHC. HAV-positive hemocytes in the lumen of the intestine were particularly observed near the typhlosole groove of the intestine. However, the intestinal epithelium, the stomach, the gills, and the adductor muscle were negative for HAV by IHC staining.

3.3. Localization of HAV RNA in oysters by ISH

ISH strongly detected viral RNA in the HAV-challenged oysters (Fig. 3 and Supplementary Fig. 2). A positive ISH signal was not detected in the negative control oysters. HAV was detected in various tissues including both the digestive tract (digestive diverticula, stomach, and intestine) and the nondigestive tract (connective tissues and gills) (Fig. 3). An ISH-positive signal was observed predominantly in the epithelium and lumen of the digestive diverticula. The signal intensity of ISH in the epithelium of the digestive diverticula decreased by the depuration time (0, 3, and 7 dpi) (Supplementary Fig. 2). Regardless of depuration time, individual hemocytes in the connective tissues and the lumen of the stomach and intestine showed strong positive signal for HAV by ISH. Unlike the IHC results, the exterior of the gill filament and the epithelium of the gills, stomach, and intestine were shown to be positive for HAV by ISH. ISH for HAV was negative in the adductor muscle.

4. Discussion

Depuration is an important processing strategy to ensure food safety in the shellfish industry (Richards et al., 2010). As shellfish can bioconcentrate not only nutrients but also various pathogenic microorganisms present in the production environment via filter feeding, sewage effluent in the marine environment caused the contamination of oysters (*Crassostrea gigas*) with *E. coli* (Corrêa et al., 2012; Dore and Lees, 1995). When shellfish are depurated in hygienic seawater, 98% or more oysters cleared the *E. coli* within 48 h in UV depuration conditions (Dore and Lees, 1995). Although depuration is used to eliminate pathogenic bacteria worldwide, virus contamination in depurated shellfish have caused several foodborne outbreaks (Grohmann et al., 1981; Hernroth and Allard, 2007; Rodriguez-Manzano et al., 2014). Therefore, the clearance of virus by depurated oysters has been examined in this study.

In this study, oysters were persistently contaminated with HAV under depuration conditions up to 7 dpi. While the HAV load in the gills of oysters gradually reduced after 7 days of depuration, it still remained high in the digestive glands. Similar to this study, contamination with adenovirus persisted in the gills and the digestive glands of oysters over 5 weeks under depuration conditions. Particularly, adenovirus was detected in the gills after over 10 weeks at 4 °C (Hernroth and Allard, 2007). HuNoV was also persistently detected in Eastern oysters after

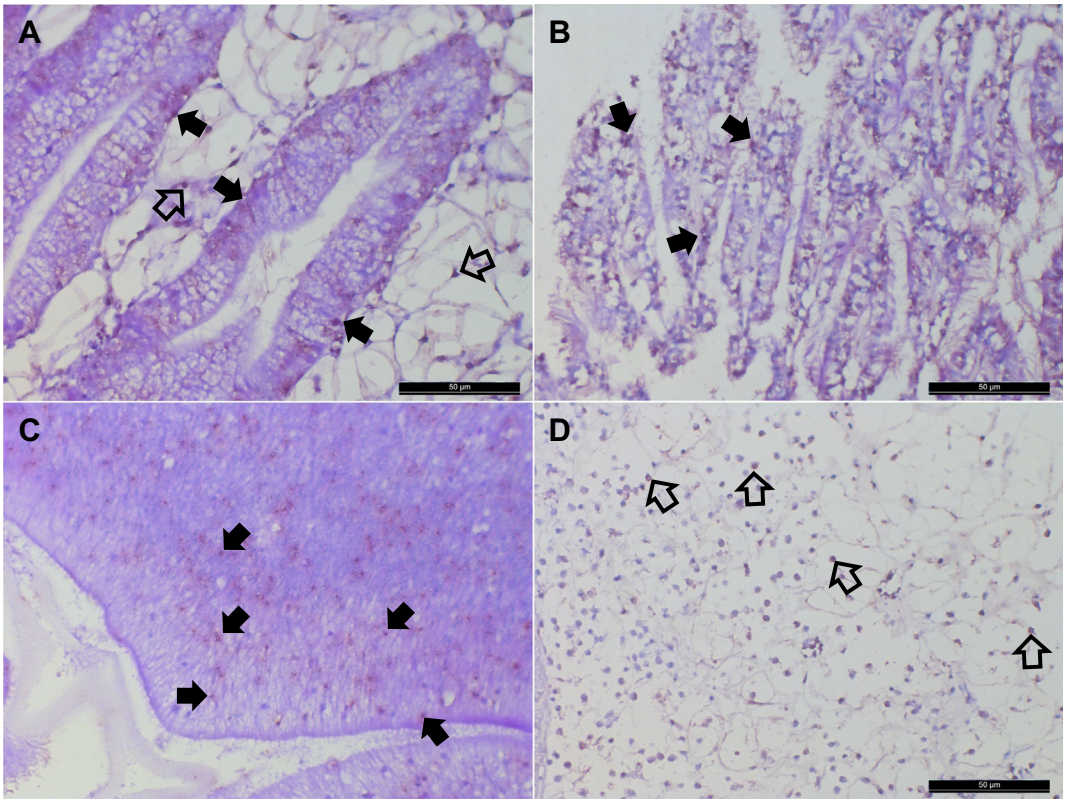

Fig. 3. Localization of hepatitis A virus in experimentally contaminated oysters by *in situ* hybridization. A) Digestive diverticula of HAV-contaminated oyster at 0 days post-inoculation (dpi), B) gills of HAV-contaminated oyster at 0 dpi, C) stomach of HAV-contaminated oyster at 7 dpi, D) connective tissue at 3 dpi, A–D magnification (×400). Positive signal was visualized as dark brown to black color with nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate. All slides were counterstained with 3% methyl green. Black arrow indicated HAV RNA in epithelium. Open arrow indicates HAV-positive hemocytes. Scale bar indicates 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

over 6 weeks of depuration-mimicking conditions (Choi and Kingsley, 2016). Although the mechanism of viral persistence has not been clearly elucidated so far, it is reported that the histo-blood group antigen (HBGA) expressed in oysters is associated with the accumulation of HuNoV (Tian et al., 2007). The association of HBGA and HAV in viral persistence in oysters needs to be examined in further study.

Since oyster feeding is affected by the water flow, tidal change, nutrients, and temperature, it is very hard to predict whether the viral contamination of oysters is affected by feeding in a laboratory setting (Kennedy et al., 1996). In this study, the initial HAV load in algae-fed oysters was approximately 1 $\log_{10}$ copy/g lower than that in algae-unfed oysters. On the contrary, a previous study reported that the initial viral load of norovirus, tulane virus, and mengovirus in the digestive tissue was higher in feeding conditions than in non-feeding condition (Drouaz et al., 2015). While the tulane virus was significantly reduced in the digestive tissue after 8 days of feeding, the reduction of norovirus and mengovirus was not significantly different in the digestive tissue regardless of the feeding conditions (Drouaz et al., 2015). Similarly, HAV reduction in the digestive glands was not significantly different between algae-fed and algae-unfed oysters during depuration. Taken together, algae-feeding does not seem to be a critical factor in determining the concentration and clearance of HAV in oysters.

Oysters have well-developed gills not only to exchange gas but also to capture food particles in seawater; they are classified as lamellibranch bivalves with ciliated rejection tracts. The food particles are highly processed in the ciliated gill surfaces and transferred to the digestive system of oysters. The retention of food particles in the gills of oysters is dependent on the particle size. Whereas > 6 μm particles are well-retained in the gills of Eastern oysters, < 1 μm particles are poorly retained (Kennedy et al., 1996). Interestingly, ISH detected HAV RNA in the gill epithelium and individual hemocytes whereas IHC could not detect the HAV capsid in them. Considering that the HAV virion has a 27-nm diameter, the retention of HAV in the gills might be low. This might explain why IHC could not detect HAV in the gills.

The epithelium of the stomach and digestive diverticula in HAV-contaminated oysters were strongly positive for HAV by IHC and ISH. Romalde et al. (1994) also demonstrated that *in situ* transcription (IST) with a radioactive cDNA probe primarily detected HAV RNA in the ciliated epithelium of the stomach, the epithelium of digestive diverticula, and hemocytes in the lumen and connective tissues. Similarly, previous studies demonstrated that IHC visualized the human viral antigens of poliovirus and HuNoV in the epithelium of digestive glands and gills (Le Guyader et al., 2006; Maalouf et al., 2010; McLeod et al., 2009b). As the HAV signal of IHC and ISH in the depurated oysters became weaker in a time-dependent manner, the epithelium of the digestive diverticula and stomach seemed to be the primary deposition site for the HAV in oysters.

It is also an important finding that both IHC and ISH were used to detect abundant HAV-positive hemocytes present in various tissues of depurated oysters. In general, hemocytes of oysters are of two types: granular hemocytes and agranular hemocytes. Among them, granular hemocytes are commonly found in the digestive tracts of adult oysters and oyster larvae. Because they have numerous digestive enzymes, their principal functions are associated with the digestion of food particles in the intestinal lumen and transport of the nutrients to tissues surrounding the gut (Kennedy et al., 1996). During the digestion process, hemocytes take up the foreign materials and microorganisms concentrated in the epithelium of the digestive tract and gills of oysters. As hemocytes circulate through all oyster tissues like immune cells of other mammals, they carry HAV particles from the epithelial cells of the

digestive glands and gills to other organs. Therefore, it is hard to eliminate the HAV present in circulating hemocytes. This may be the reason why depuration itself is not enough to eliminate the virus accumulated in oysters effectively.

Using both IHC and ISH, we could not detect HAV in abductor muscles. IST was utilized to also reveal that the adductor muscles were negative for HAV RNA (Romalde et al., 1994). In other viral contamination studies, the abductor muscles of HuNoV-challenged oysters were also negative, as determined by IHC (McLeod et al., 2009b; Wang et al., 2008). Interestingly, Provost et al. (2011) used the abductor muscle area to collect hemocytes. HuNoV, HAV, and other viruses were detected with oyster hemocytes but not with the adductor muscle (Provost et al., 2011). Taken together, the abductor muscle cells are not direct target cells for viral contamination in oysters.

For the first time, this study used the ISH technique to localize HAV RNA in various target tissues of virus-contaminated oysters. While both IHC and ISH detected HAV antigen and RNA in the hemocytes of connective tissues and the epithelium of the digestive diverticula, only ISH detected HAV RNA in the epithelium of gills and the stomach. When a previous study contaminated oysters with HuNoV and poliovirus, both were detected in the digestive tract and gills by qRT-PCR. However, IHC showed positive staining in the digestive tracts but negative in the gills (McLeod et al., 2009b). The difference in detection sensitivity and localization between IHC and ISH could be explained as follows. First, the sensitivity of IHC and ISH is dependent on both the antibody and probe because they target protein and DNA/RNA, respectively. Second, the stability of HAV RNA captured in the gills or hemocytes may be better than the HAV capsid antigen. Third, HAV capsid captured in the gills may be degraded or low abundant. Therefore, ISH could be a more useful technique to trace foodborne virus accumulation in oysters than IHC.

In this study, algae feeding was not a critical factor in determining the bioaccumulation and persistence of HAV. IHC and ISH were used to identify the HAV in the epithelium of digestive diverticula and hemocytes in gills, connective tissues, and digestive tracts. We found that HAV was persistent in oysters after over 7 days of depuration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.03.017.

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