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Effectiveness of depuration for hepatitis A virus removal from mussels (*Mytilus galloprovincialis*)

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ABSTRACT

The efficacy and kinetic of depuration of hepatitis A virus (HAV) were evaluated under experimental conditions with Mediterranean mussels (*Mytilus galloprovincialis*) subjected previously to bioaccumulation processes. Seven independent trials (70 kg of mussels each) were performed in a closed experimental system using two different water temperatures (13 and 17 °C) during 7 days. The real time RT-PCR technique with TaqMan probes was used for viral quantification. Qualitative infectivity assays were conducted to test the presence of infectious viral particles at the end of the depuration period. The depuration trials showed an average reduction of HAV levels of aproximately 1.1 Log units (>90%). However, the average final viral loads in shellfish samples remain at relatively high levels (6.5×10^3 RNA copies/g digestive tissue) and still infectious. A positive correlation between the initial and the final numbers of the viral RNA copies was observed. The reduction of HAV showed a two-phase removal kinetic, an initial logarithmic trendline, with a rapid reduction of viruses during the first 24–48 h of depuration, and a subsequent stabilization with a slower depuration rate until the end of the process.

1. Introduction

Bivalve mollusks are one of the main vehicles for transmission and dissemination of a wide variety of food-borne enteric viruses (Lees, 2000; Polo et al., 2010). As a part of their filter-feeding activity, bivalves act as natural concentrators of different bacterial and viral pathogens present in fecal human pollution when discharged on harvesting areas (Butt et al., 2004; Lees, 2000). Furthermore, bivalves are traditionally consumed raw or lightly cooked, and whole including the viscera (where viruses are principally concentrated)(Romalde et al., 1994), which increases the risk of infection by these pathogens. Most shellfish-borne viral outbreaks are associated with norovirus (NoV), the leading agent of viral gastroenteritis, and hepatitis A virus (HAV), one of the most serious viral infections linked to shellfish consumption (Butt et al., 2004).

Shellfish depuration is a post-harvest commercial processing strategy and an extended statutory requirement for shellfish in many countries. The process, intended to reduce the likelihood of transmitting infectious agents to consumers, consists of placing shellfish in tanks with clean seawater and use their pumping activity to purge the contaminants. Depuration has demonstrated to significantly reduce the fecal bacteria levels in a rapid and efficient manner (Lee et al., 2008; Lees et al., 2010; Richards et al., 2010). This fact has guided the development of depuration systems and practices since its adoption one century ago. Due to this, current standards that establish which mollusks must be depurated, as well as the effective controls of the process are based on bacterial indicators (Anon, 2004).

The compliance with the end product standards is frequently seen as an evidence of satisfactory design and operation of purification plants. However, virus removal is known to be less effective than bacterial removal, and those standards cannot guarantee viral absence (Loisy et al., 2005; Romalde et al., 2002; Schwab et al., 1998). Epidemiological data demonstrate the periodic occurrence of HAV and NoV outbreaks linked to the consumption of depurated shellfish in conformity with legal standards (Chalmers and Mcmillan, 1995; Heller et al., 1986; Le Guyader et al., 2003, 2006; Lopman et al., 2004). The strong impact of these diseases in human populations has brought awareness by European authorities. In fact, a standard method (ISO/TS 15216-1) for virus detection in foodstuffs including shellfish has been recently developed (ISO, 2013), and is expected to be incorporated into EU legislation in the future.

The slow removal rates of enteric viruses from bivalve shellfish indicate that depuration is not a passive process (Richards et al., 2010). Recent studies suggest the involvement of different processes, including specific viral ligands present in the shellfish digestive tissues or the accumulation within hemocytes, that may influence the bioaccumulation and persistence of certain viruses or viral strains (Le Guyader et al., 2006; Maalouf et al., 2011; Provost et al., 2011; Tian et al., 2007). The different behavior would result in diverse removal rates depending on the

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virus and the bivalve species. The efficacy of viral depuration in certain conditions, the time required for a significant viral reduction, the specific kinetics under depuration processes, and the differences in removal rates depending on virus and bivalve species are crucial issues for developing new shellfish standards and purification processes.

This study evaluates the effectiveness of depuration and removal kinetics of HAV in Mediterranean mussels (*Mytilus galloprovincialis*) in an experimental depuration system during 7 days under two different water temperatures, 13 and 17 °C.

2. Materials and methods

2.1. Depuration system

A closed experimental system (isothermal ASE M BINS system) (Adriatic Sea Aquarium and Equipment SRL, San Clemente, Italy) with mechanical, biological and chemical static filter systems, thermal control and water sterilization by ozone and UV-C radiation was employed for the purification of mussels. The total system volume was approximately 1750 l with a complete water renewal time in the tanks of 30 min and with a depuration capacity of 500 kg of mollusks.

2.2. Cell culture and viral stocks

HAV HM-175/18f was obtained from the ATCC as a cell culture-adapted cytopathic clone of strain HM-175. A mutant non-virulent infective strain of Mengovirus (vMC_0), kindly provided by Dr. Albert Bosch (University of Barcelona, Spain) was employed as an extraction process control as it was previously described (Costafreda et al., 2006). Stocks of each viral strain were generated by inoculation onto confluent monolayers of appropriate cell lines: HeLa for Mengovirus vMC_0 and FRhK-4 for HAV HM-175/18f. Final viral concentrations in the stocks were 1×10^5 plaque-forming units (pfu)/ml (6×10^6 RNA copies/ml) for Mengovirus and 1×10^6 pfu/ml (7×10^6 RNA copies/ml) for HAV.

2.3. Bioaccumulation process

Mussels (70 kg/batch) were acclimated for 24 h in tanks of 115 × 72 × 50 cm with natural seawater and continuous aeration. All batches were checked to determine any initial viral contamination. The bioaccumulation process was performed in the same tanks. Mussels were laid on a monolayer disposal with 100 l of natural seawater and HAV (final concentration of 10^2 pfu/ml) were added to the tanks mixed with a 500 ml mix of two species of phytoplankton (1:1 *v/v*; *Chaetoceros* sp.: *Nannocloropsis* sp.). The presence of phytoplankton in the bioaccumulation process simulates the natural conditions, inducing the mussels to filter and facilitating the viral uptake and accumulation in shellfish digestive tissues (Gentry et al., 2009). The exposure-time of mollusks to the viral-plankton suspension was 24 h. Then, mussels were relocated to the experimental depuration system and the initial viral load (t0) was quantified.

2.4. Experimental design

Seven depuration trials (E1–E7) were performed. Mussels were maintained into the experimental depuration system for 7 days under an exhaustive control of water parameters, including dissolved O_2, pH, T, NH_3/NH_4, NO_2, NO_3, conductivity and salinity. These parameters were checked daily along the 7 days of depuration. Dissolved O_2, pH, T, conductivity and salinity were checked with the Multiprobe system YSI 556 MPS (YSI, UK). NH_3/NH_4, NO_2 and NO_3 were checked by a colorimetric method (Nutrafin® Test, Hagen, Spain). The water temperature of the system was maintained at 13 °C in five trials and at 17 °C in two trials to evaluate the effect of the temperature increase in the depuration dynamics. Moreover, the temperatures employed were chosen on the basis of the average marine water temperatures in our area in winter and summer respectively. Samples (10 mussels) were obtained during the purification process, every 24 h for 7 days (t1–t7). The presence of mortality was checked daily and the dead animals, if any, were removed.

2.5. Viral recovery and RNA extraction

Viral recovery from shellfish was carried out according to the ISO/TS 15216-1 method with minor modifications. Briefly, mussels were shucked and the digestive tissues (DT) were removed by dissection and pooled to get a final weight between 2 and 3 g. Known amounts of mengovirus clone (vMC_0) (10 μl of mengovirus stock) were spiked to each sample homogenate as an independent nucleic acid extraction efficiency control (Costafreda et al., 2006). Tissues were homogenized, chopping the digestive glands with a razor blade to a paste-like consistency, in one volume (1:1 *w/v*) in peptone water (0.1%; pH 7.4). Then, homogenates were strongly shaken for 1 h and centrifuged at 1000 ×*g* for 5 min, recovering the supernatant. Viral RNA was extracted in duplicate from each homogenate using *Nucleospin RNA Virus Kit* (Macherey-Nagel; Dürem, Germany), from a sample volume of 150 μl according to the manufacturer's protocol. The RNA was eluted in RNAse-free sterile water and stored at −80 °C.

2.6. Real time RT-PCR (RT-qPCR)

The RT-qPCR for Mengovirus and HAV was performed on an Mx3005p QPCR System (Stratagene, USA) thermocycler. Platinum Quantitative RT-PCR Thermoscript One-step System kit (Invitrogen, Saint Aubin, France) was used with a 25 μl total volume, using 5 μl of extracted RNA. Quantification was also carried out following the principles outlined in the CEN/ISO technical specification (ISO/TS 15216).

The extraction efficiency was determined by comparing the Cycle threshold (C_t) value of the Mengovirus-positive control with the C_t value of each sample for Mengovirus (Costafreda et al., 2006), and was classified as valid (>5%) or invalid (<5%). The presence of RT-PCR inhibitors and the determination of the RT-qPCR efficiency were tested by means of the external controls (EC). Briefly, 2.5 μl of EC, containing 10^3 genome copies of HAV, was mixed with 2.5 μl of each sample extracted RNA and the C_t values of these reactions were compared with the C_t value of the EC mixed only with RNA-free sterile water. The efficiency was classified as valid (>25%) or invalid (<25%). Following the CEN/ISO method, samples with a <5% extraction efficiency or a <25% RT-qPCR efficiency were re-extracted and tested again.

Primer set and probe used were: 0.9 μM of reverse primer HAV240 (5′-GGAGAGCCCTGGAAGAAAG-3′), 0.5 μM of forward primer HAV68 (5′-TCACCGCCGTTTGCCTAG-3′) and 0.45 μM of probe HAV150 (6-FAM-CCTGAACCTGCAGGAATTAA-MGB) (Costafreda et al., 2006). Negative controls containing no nucleic acid as well as positive controls were introduced in each run. A sample displaying a $C_t \leq 41$, with no evidence of amplification in the negative controls, was considered as positive. Quantification was estimated by standard curves constructed with serial dilutions of HAV RNA, plotting the number of genome copies against the C_t. This quantification was corrected with the extraction efficiencies and expressed as the number of RNA viral genome copies per gram of digestive tissue (RNAc/g DT). The limit of detection was 1×10^2 RNAc/g DT.

2.7. Infectivity assays

The infectivity of HAV remaining after the experimental depuration process was evaluated. Confluent FRhK-4 cells in 48-well cell culture plates were inoculated with diluted DT homogenates (40 μl/well) of the samples. Once inoculated, plates were incubated 1 h at 37 °C with slow agitation to promote virus attachment and internalization. After this period, the cells were washed with PBS (pH 7.4) and a maintenance medium was added to each well. The maintenance medium consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% fetal bovine serum, 1% L-glutamine, 1% non-essential amino-acids