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Depuration kinetics of hepatitis A virus in clams

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ABSTRACT

The efficacy and dynamic of depuration for the removal of hepatitis A virus (HAV) contamination were evaluated under experimental conditions using Manila clams previously subjected to bioaccumulation with this virus. Five independent trials were assayed in a closed experimental system with a total volume of approximately 1750 l, using clam batches of 60 Kg. The reverse transcriptase-real time PCR (RT-qPCR) technique was utilized for viral quantification. Infectivity assays were conducted at the end of depuration. Although the final viral loads in shellfish after 7 days remained relatively high and still infectious, an average reduction in HAV levels of 1.44 log units (approx. 93.1%) was observed. This reduction showed a two-phase removal kinetic, with an initial rapid reduction of viruses during the first 72 h of depuration, with a 0.6 log units (69%) of average decrease in HAV RNA copies/g digestive tissue, and a subsequent stabilization with a slower depuration rate in the remaining days.

1. Introduction

Bivalve mollusks are one of the most common foods implicated in the transmission and dissemination of a wide variety of human enteric viruses around the world (Lees, 2000; Polo et al., 2010). In addition, the traditional way of consuming shellfish, raw or slightly cooked, and whole, including digestive tissues (where viruses are mainly concentrated), makes the bivalve mollusks a high-risk food group (Romalde et al., 1994). Among the viral infections linked to shellfish consumption, acute hepatitis A is one of the most serious diseases, even causing occasionally death (Butt et al., 2004; Hollinger and Emerson, 2001; Koopmans and Duizer, 2004). Fecal excretion of hepatitis A virus (HAV) precedes the onset of symptoms, and the inability of sewage treatments to completely remove or inactivate viruses is well known (Bosch, 2007; Da Silva et al., 2007). Viruses discharged from sewage outfalls into shellfish growing waters can be easily uptaken and bio-concentrated by shellfish due to their filter-feeding nature (Lees, 2000; Vilariño et al., 2009), leading to shellfish-borne outbreaks of hepatitis A.

Historically, the study of food-borne diseases associated with contaminated shellfish has mainly focused on bacterial pathogens. As a result, the development of shellfish sanitary controls and depuration practices have been strongly influenced and guided by these studies. However, it is well known that enteric viruses significantly differ from traditional bacteria in terms of transmission, resistance to sewage treatment, persistence in the environment and resistance to shellfish purification processes (Da Silva et al., 2007; Maalouf et al., 2010; Ueki et al., 2007). Therefore, bacterial indicators of fecal contamination, on which sanitary controls are based (Anonymous, 2004), are not reliable tools to assess sanitary safety of single shellfish samples from a viral standpoint (Romalde et al., 2002), a fact evidenced by the occurrence of periodic outbreaks of gastroenteritis associated to depurated shellfish in compliance with the legal standards (Chalmers and McMillan, 1995; Heller et al., 1986; Le Guyader et al., 2003, 2006) and puntual hepatitis A outbreaks linked to imported shellfish (Pintó et al., 2009; Romalde et al., 2001).

The health risk associated with the consumption of faecally contaminated shellfish requires the development of new methods to prevent, detect, quantify and eliminate these pathogens. The depuration process allows the purging of bivalve mollusks in order to reduce the likelihood of transmitting infectious pathogens. The efficacy of viral depuration under certain conditions and the time required for a significant reduction of the viral load are critical issues for the development of new and improved shellfish sanitary controls, purification processes, and the enactment of viral legislation for this product.

The removal kinetics of enteric viruses from bivalve shellfish is not a passive process, a fact evidenced by their slower removal rates (Richards et al., 2010). Recent studies suggested the involvement of

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specific viral ligands to shellfish tissues in the bioaccumulation processes of certain viruses or viral strains (Le Guyader et al., 2006; Maalouf et al., 2011; Tian et al., 2006, 2007), which would result in different removal rates depending on the virus and the bivalve species. The development of new standard sanitary controls and depuration processes focused on these pathogens should take into account this virus-bivalve specific behavior and their depuration time frame, much longer than those currently used. In this sense, experimental depuration systems provide a useful tool for the study of viral removal in mollusks under conditions more similar to those expected in a commercial plant and for a much longer period of time.

This study evaluates the efficiency of depuration and removal kinetics of HAV in an experimental depuration system for 7 days.

2. Materials and methods

2.1. Depuration system

The depuration system was 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 (Fig. 1). The total system volume was approximately 1750 l with a complete water renewal time in the tanks of 30 min and with 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: FRhK-4 for HAV HM-175/18f and HeLa for Mengovirus vMC_0. 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 of virus by clams

Manila clams (*Venerupis philippinarum*) were checked to determine any initial HAV contamination and were acclimated for 24 h in tanks of 115 × 72 × 50 cm with natural seawater at 13 °C and continuous aeration. Five bioaccumulation trials were performed. Clams were laid in the same tanks on a monolayer disposal with 100 l of natural seawater. Then, HAV was added (10^2 pfu/ml final concentration) to the tanks together 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 simulate the natural conditions, inducing the clams to filter and facilitating the viral uptake by the molluscs and their accumulation in shellfish digestive tissues (Gentry et al., 2009). The exposure-time of clams to the plankton-viral solution was 24 h. Once contaminated, the clams were relocated to the experimental depuration system.

2.4. Experimental design

Five depuration trials, each with 60 kg (approx. 2400 individuals) of Manila clams previously subjected to bioaccumulation with HAV, were performed in the experimental depuration system. After bioaccumulation, the clams were relocated in the experimental depuration system under an exhaustive control of depuration parameters (O_2, pH, T[a], NH_3/NH_4, NO_2, NO_3, conductivity and salinity) for 7 days. Presence of mortality was checked daily and the dead animals, if any, were removed.

In all the experiments clams were randomly sampled before (t0) and during the purification process, every 24 h for 7 days (t1–t7). Each sample consisted of 20 clams and was processed within 4 h after sampling.

2.5. Viral recovery and RNA extraction

Clams were shucked and the digestive tissues (DT) (stomach and digestive diverticula) were removed by dissection, pooled to get a final weight between 2 and 3 g and homogenized with one volume (1:1 w/v) in peptone water (0.1%; pH 7.4). Known amounts of mengovirus clone vMC_0 were spiked in sample homogenates (10 μl of Mengovirus stock) to be employed as a control for nucleic acid

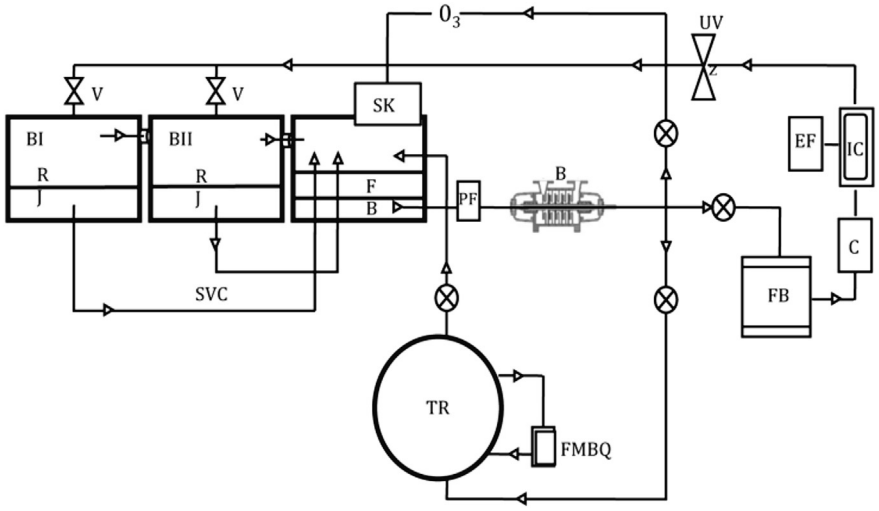

Fig. 1. Experimental depuration system. BI. Bin 1; BII: Bin 2; RJ: Grid on which the shellfish are placed; SVC: System of Communicating Vessels; FB: Biological Filter (Maëhl sand); SK: Skimmer; PF: Prefilter; B: Water pump (3500 l/h). T ° for renewal of total system volume = 1/2 h; TR: Reserve Tank; FMBQ: Mechanical. Biological and Chemical filter; C: Heater; EF: Cooler; IC: Heat Exchanger; SF: Cooling systems; UV: Ultraviolet Disinfection System; O3: Ozonator; V: Venturi Device;×: Water flow regulator.