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RESEARCH ARTICLE

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Global market: shellfish imports as a source of reemerging food-borne hepatitis A virus infections in Spain

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Abstract A total of 16 mollusk imports from South America to Spain, including clam and scallop species, were analyzed for hepatitis A virus (HAV), due to the great concern about this type of food after an important hepatitis A outbreak in eastern Spain in September 1999. In addition, clams from the stock that had caused the outbreak were also tested. Of the 17 stocks, four were positive for the presence of HAV RNA as demonstrated by RT-PCR and Southern hybridization. Contradictory analyses confirmed the results of the primary tests in all cases. The findings obtained in this work strongly support the role of mollusk imports from endemic areas of HAV as an important vehicle of hepatitis A, and demonstrate the imperative need for sanitary control measures to prevent future outbreaks of this disease.

Keywords Hepatitis A virus · Shellfish · Foodborne infection · International commerce

Introduction

Outbreaks of viral enteric diseases, mainly hepatitis A and gastroenteritis, associated with shellfish consumption constitute a major health problem worldwide [9]. Current sanitary control methods are based on bacterial indicators [4, 10], however, these may fail to detect viral contamination [12].

Hepatitis A is one of the most serious viral infections linked to shellfish consumption, causing a serious debilitating disease and even death. Although hepatitis A is a common endemic infection in developing areas, its prevalence in developed countries has declined due to improved sanitary conditions [1, 6, 7]. A consequence of this decline is that adult human populations are more susceptible to potential food- or water-borne hepatitis A epidemics or to acquiring the infection when traveling to endemic areas [1, 9, 11]. The increase in international trade between endemic and non-endemic areas can also account for the reemergence of hepatitis A infections. In fact, in recent years, countries historically considered to be important seafood consumers, such as Spain and France, have become the main importers of mollusks from diverse developing countries, where these natural resources have become essential for their economies.

In September 1999, an important outbreak of hepatitis A, affecting 188 people, was detected in Valencia (eastern Spain), a non-endemic area for this disease. Further studies [3] demonstrated its association with the consumption of wedge clams (*Donax* sp.) imported from Peru. Although not required by the regulations controlling imports, the Spanish sanitary authorities have since adopted, as a preventive measure, the systematic analysis of imported shellfish samples for the presence of hepatitis A virus (HAV). This work presents further evidence that shellfish imported from countries where hepatitis A is endemic can be a significant source of reemerging infection in developed countries.

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Materials and methods

Samples

From November 1999 to May 2001, 16 samples from South American imports of different types of bivalve mollusks, which had been distributed in several cities in Spain, were analyzed for the presence of HAV. Frozen mollusks were shipped under appropriate isothermal conditions. In addition, clams retained from the stock associated with the HAV outbreak in Valencia were also analyzed. Therefore, the study included six stocks of wedge clam (*Donax* sp.), six of carpet clam (*Tapes* sp.), and five of scallop (*Argopecten* sp.), which, taken together, represented an import volume of approximately 300 tons. The stocks were sampled following standard procedures [10].

Virus concentration

The shellfish were shucked, and the stomachs and digestive diverticula were removed and randomly pooled in 50-g aliquots. Viral particles were concentrated from the aliquots using a conventional adsorption-elution method [14]. Each 50-g aliquot was homogenated and then diluted (1:7 w/v) in sterile distilled water to achieve a salinity lower than 2,000 ppm NaCl/l, adjusting the pH to 5. After centrifugation at 1,850 *g* for 15 min, supernatants were discarded, and the pellets were resuspended in seven volumes of glycine saline buffer (0.37% glycine, 0.85% NaCl). The pH was then adjusted to 7.5 and the mixtures were gently shaken for 10 min. The supernatants obtained after centrifugation at 1,850 *g* for 15 min were adjusted to pH 4.5 and shaken for 20 min. Samples were then centrifuged as before and the pellets were resuspended in 2 ml of 1 N Na_2PO_4H (pH 7.4). The concentrates were filtered through Sephadex columns to eliminate possible PCR inhibitors (E. Area, M.Sc. Thesis, Universidad de Santiago de Compostela, 1998).

Nucleic acid amplification and detection

Viral RNA was extracted and subjected to RT-PCR amplification employing primers HAVp3 (5′-GGA AAT GTC TCA GGT ACT TTC TTT G-3′) and HAVp4 (5′-GGT TTG CTC CTC TTT ATC ATG CTA TG-3′) as previously described [2]. After a RT step at 42 °C for 1 h, PCR amplification was carried out in a Perkin Elmer 2400 thermocycler, employing the following cycling conditions: initial heat denaturation at 94 °C for 4 min; 40 cycles of template denaturation at 94 °C for 1 min; primer annealing at 55 °C for 1 min; primer extension at 72 °C for 90 s; and a final extension at 72 °C for 10 min. RNA obtained from FRhK-4 (fetal rhesus monkey kidney) cells infected with HAV strain HM-175 was employed as positive control in all RT-PCRs. The negative control consisted of a RT-PCR reaction tube without RNA.

To confirm the RT-PCR results, Southern hybridizations were carried out using a digoxigenin-labeled probe complementary to an internal fragment of the amplification product [2]. Prehybridization (3–4 h) and hybridization (overnight) were performed at 40 °C for HAV in hybridization solution containing 5×SSC, blocking reagent (1% w/v; Boehringer Mannheim), 0.1% (w/v) sarcosyl, 0.02% (w/v) SDS, and a final concentration of 8.5 pmol/µl cDNA probe. The hybridized probe was detected using the Dig DNA Labeling and Detection Kit (Boehringer Mannheim) according to the manufacturer's protocol.

Results

Table 1 shows the results of the RT-PCR detection studies carried out with mollusks obtained from different stocks imported into Spain between November 1999 and May 2001. HAV RNA was detected in four of the stocks analyzed (23.5%) by RT-PCR and Southern hybridization. HAV was detected in one out of six (16.6%) imports of wedge clams, two out of six (33.3%) imports of carpet clams and in one out of five (20%) imports of scallops. Figure 1 shows the results obtained from several aliquots of different clam and scallop imports. The appearance of an amplicon of the appropriate size (248 bp) indicated a positive result for the presence of HAV RNA in mollusk tissues (Fig. 1A).

In some experiments, additional bands were seen following ethidium bromide staining. The sizes of these bands were similar to those expected from specific amplification of HAV nucleic acid (data not shown). Southern hybridization was employed to discriminate between HAV-specific bands and non-specific ones (Fig. 1B). Note that 11 out of the 22 (50%) aliquots from the stock associated with the hepatitis A outbreak were positive in the RT-PCR analysis (Table 1). For imports positive for the presence of HAV, confirmatory analyses were performed in duplicate samples of the same stocks. Detection of the virus was confirmed in all cases (data not shown).

Discussion

Shellfish-associated HAV infections are currently uncommon in developed countries [1, 7]. However, continuing vigilance is necessary, since, on many occasions, bivalve mollusks have been demonstrated to be potential

Table 1 Detection of HAV in mollusks from imported into Spain

City of arrival	Date of analysis	No. of pooled aliquots	Mollusk species	No. of HAV-positive aliquots
Vigo	17.11.99	5	Wedge clam	0
Bilbao	24.11.99	6	Scallop	0
Bilbao	24.11.99	6	Wedge clam	0
Valencia	01.12.99	12	Wedge clam	0
Madrid	13.12.99	6	Wedge clam	0
Bilbao	14.01.00	8	Scallop	0
Vigo	24.03.00	2	Scallop	0
Valencia	07.04.00	3	Carpet clam	0
Valencia[a]	11.04.00	22	Wedge clam	11
Valencia	12.04.00	8	Carpet clam	2
Valencia	04.05.00	12	Carpet clam	0
Soria	09.05.00	6	Scallop	0
Bilbao	23.06.00	4	Scallop	3
Bilbao	23.06.00	8	Wedge clam	0
Valencia	12.07.00	6	Carpet clam	1
Bilbao	02.03.01	4	Carpet clam	0
Bilbao	11.05.01	6	Carpet clam	0

[a]This sample arrived in the laboratory in April 2000 and was derived clams associated with the hepatitis A outbreak of September 1999; the clams had been confiscated by the sanitary authorities

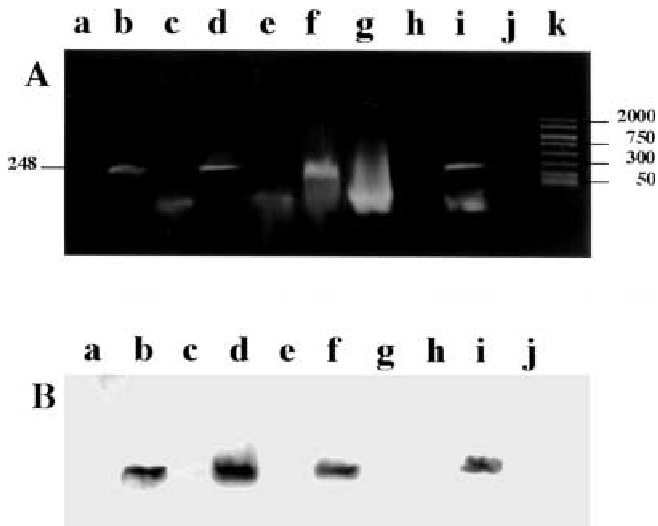

Fig. 1A, B Hepatitis A virus (HAV) detection in mollusks from imported stocks. **A** Detection of RT-PCR products on an ethidium bromide-stained agarose gel. *Lanes a–d* Carpet clam tissue: aliquots 1–4 of stock associated with the hepatitis A outbreak, *lane e* wedge clam tissue: aliquot 1 of stock from 12.04.00, *lanes f, g* scallop tissue: aliquots 1 and 2 of stock from 23.06.00, *lane i*, HAV RNA (positive control), *lane j* negative control, *lane k* molecular size marker (PCR marker 50–2,000 bp, Sigma). Numbers on the *left* (specific amplicon) and *right* (size markers) are expressed in bp. **B** Southern blot of gel from **A** with a digoxigenin-labeled, HAV-specific probe. Lanes are the same as in **A**

vectors for HAV epidemics. Moreover, human adult populations are now highly susceptible [9].

The long incubation period (average 4 weeks) of HAV makes it very difficult to associate the disease with a particular food vehicle, including mollusks, in individual or sporadic cases. A diligent epidemiological investigation is usually needed since the suspected food is rarely available for testing, and consumption histories are inconclusive [13]. In the case of large outbreaks, the existence of a common vehicle of infection is more obvious, as in the Spanish outbreak of September 1999, in which 188 people were infected [3].

Bivalve mollusks constitute a special case amongst the possible food vehicles for HAV transmission. In the process of filter-feeding, these shellfish can concentrate and retain human pathogens derived from sewage contamination of sea water. The bioaccumulation of potentially harmful microorganisms by shellfish is hazardous because shellfish are traditionally eaten whole (including the viscera) and raw or only mildly cooked. In addition, current control measures, based on bacterial indicators, are not effective to predict viral contamination [12]. In fact, although all imports subjected to HAV testing in this work fulfilled the health requirements of current European laws on microbiological safety of bivalve mollusks, HAV RNA was detected in four of them. Sequencing studies are in progress to evaluate the genetic variability among the viruses detected in order to find possible epidemiological markers associated with either the season of isolation or the bivalve mollusk host.

Biotechnological advances have led to the development of new PCR-based methods for the direct detection of HAV and other enteric viruses in shellfish tissues and cultivation waters [2, 8,9]. Despite their limitations, including the lack of standardization and the inability to quantify or assess viral infectivity, these procedures currently constitute the most suitable tool to assess viral contamination.

These facts, along with the results of the present study, show that there is a clear need to screen all mollusk stocks targeted for human consumption, especially those from endemic areas, for viral contamination. We recommend that the European Council considers legislation leading to the standardization of new measures to test viral contaminants in bivalve shellfish [5]. Such measures would improve the sanitary quality of shellfish and, consequently, public health.

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