

# Risk Assessment in Shellfish-Borne Outbreaks of Hepatitis A<sup>∇</sup>

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Received 21 May 2009/Accepted 30 September 2009

**In the present work, we aimed at determining the relationship between the hepatitis A virus (HAV) numbers in imported frozen coquina clams involved in two hepatitis outbreaks, as well as the risk for human health. Due to HAV unculturability, a standardized TaqMan real-time reverse transcription-PCR controlling the virus/nucleic acid extraction and enzyme efficiencies was employed to figure the exposure dose for clams responsible for hepatitis cases. HAV numbers were then employed to figure the risk of infection based on a dose-response model for echovirus 12. The estimated risk of infection after consumption of lightly cooked clams matched actual attack rates. Our data show that prospective monitoring of bivalve samples may fail to prevent the occurrence of outbreaks, since HAV was detected in 44% of samples directly associated with cases but was undetectable in samples that were randomly collected from the importers and belonged to the same batches. A correlation was nevertheless observed between the prevalence of hepatitis A cases in the harvesting areas and positive HAV isolation in clams, which points to the need to identify and prevent hazards rather than relying on random sampling of finished products to ensure safety. However, when evidence shows that a critical limit of viral contamination has been exceeded in the potential sources of contamination discharging into the shellfish-growing beds, quantitative virological analysis addressing quality assurance and quality control requirements should be performed with the bivalves. This work provides the first evidence of accurate HAV levels in shellfish involved in outbreaks that could be of use for risk assessment purposes.**

Epidemiological evidence suggests that human enteric viruses are the most common pathogens transmitted by bivalve shellfish (22, 23, 31) and that hepatitis A virus (HAV) infection is the most serious viral infection linked to shellfish consumption, causing a debilitating disease and, occasionally, death. The first documented shellfish-borne outbreak of “infectious hepatitis” occurred in Sweden in 1955, when 629 cases were associated with raw oyster consumption (33). However, the most significant outbreak of HAV infection occurred in Shanghai, China, in 1988, in which almost 300,000 cases were caused by consumption of clams harvested from a sewage-polluted area (17). In fact, this is so far the largest virus-associated outbreak of food poisoning ever reported. Smaller outbreaks have been reported worldwide (10, 13, 24, 27, 29, 35, 37). Several issues, such as the fact that fecal HAV excretion precedes the onset of symptoms, together with the difficulties in completely removing and/or inactivating viruses through sewage treatment (6, 7), make hepatitis shellfish-borne outbreaks hard to prevent if the virus is circulating among the population.

Risk analysis may provide a sensible approach to increase the safety of shellfish consumption. Quantitative microbial risk assessment (QMRA) is a powerful statistical tool for estimating the probability of an event, e.g., virus infection, taking place, based on exposure and dose-response models (15, 16, 18, 34). Exposure assessment requires the quantitative determination of viruses present in the transmission vehicle. Due to the lack of a reliable cell system for the recovery of wild-type HAV, standardized quantitative molecular approaches, including ap-

propriate quality assurance and quality control measures to assess the efficiency of critical steps in virus analysis, open the possibility of producing consistent and accurate exposure data (11). Additionally, the lack of an actual dose-response model for HAV makes it necessary to apply models developed for other enteric viruses (21, 25, 36, 39).

In the present work, we aimed at determining the relationship between the HAV numbers in imported coquina clams directly involved in two hepatitis outbreaks and the derived likelihood of acquiring hepatitis A infection from consumption of the contaminated product. To our knowledge, no quantitative analysis of the health risk of HAV-contaminated bivalve mollusks has previously been reported. The information generated in this work may be used to formulate cost-effective risk management measures in shellfish-borne outbreak situations.

## MATERIALS AND METHODS

**Cells and viruses.** The cytopathogenic pHM175 43c strain of HAV (courtesy of T. Cromeans, Centers for Disease Control and Prevention, Atlanta, GA) has been used throughout this study as a positive control of the detection technique. Virus replication in FRhK-4 cells was performed as previously described (4).

Infectious mengovirus strain MC<sub>0</sub> (ATCC VR-1957) was obtained after transfection of a cDNA clone, pMC<sub>0</sub> (kindly provided by Ann Palmenberg, University of Wisconsin), into HeLa cells as previously described (28). The MC<sub>0</sub> mutant strain, which lacks the poly(C) tract in comparison to the wild-type mengovirus, shows growth properties identical to those of the wild-type virus but with a completely avirulent phenotype (28). Mengovirus stocks were thereafter produced in the same cells and used as controls for procedures in each virus/nucleic acid extraction from shellfish as described elsewhere (11).

**Shellfish-borne hepatitis A outbreaks.** In a period of 10 years, two hepatitis A outbreaks occurred in Valencia (Spain) that were associated with the same source: frozen coquina clams imported from Peru. These molluscan bivalves complied with the European Union (EU) standards, based on the numbers of *Escherichia coli* in shellfish meat, which must be below 230 most probable numbers per 100 g of flesh to allow their market distribution (2). It is, however, well documented that bacterial microorganisms fail to give a reliable indication of the

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<sup>∇</sup> Published ahead of print on 9 October 2009.

TABLE 1. Parameters employed for the estimation of HAV exposure<sup>a</sup> from consumption of clams from contaminated batches

Parameter	Description	Reference(s)
<i>P</i>	Prevalence or percentage of units contaminated. In our case, the unit was the batch package, and in the 1999 outbreak, since all analyzed packages were associated with cases, $P = 1$ .	This study
<i>C</i>	Concn of viruses, in our case the no. of genome copies per g of clam meat as determined by real-time RT-PCR.	11
<i>R</i>	Percent recovery of the detection method. In our case, it is estimated from the percent of recovery of a known concn of added mengovirus (as an extraction process control) and a known concn of an added external RNA molecule (as an RT-PCR control).	11
<i>I</i>	Proportion of infectivity of the detected viruses. In our case, the ratio of infectious/physical particle has been determined to be 1/60.	12, 20
<i>PR</i>	Percent virus titer reduction associated with clam cooking. The final concn of infectious virus after cooking was estimated by applying a reduction of the initial infectious virus of 99.46% in lightly cooked shellfish (until valves are opened) and 99.86% in well-cooked shellfish (5 min after opening of the valves).	1, 8
<i>W</i>	Amount of food consumed per serving. In our case, having in mind the culinary habits for consumption of clams in the outbreak location, mostly as tapas or in paella, we chose a serving size of 60 g of meat.	34

<sup>a</sup> Dose =  $P \times C \times 1/R \times I \times PR \times W$  (18, 38).

virological quality of bivalve molluscan shellfish or their waters (1, 23, 32). The first outbreak (9) occurred in 1999, lasted 3 months, and affected 184 patients. The amount of clams involved in the outbreak was 189 tons. In this outbreak, the transmission vehicle was ascertained through the detection of the same HAV sequences in patient and coquina samples (35). The second outbreak occurred in 2008, lasted 7 months, and affected 100 patients. The amount of clams involved in this outbreak was 3,766 tons. Again the same sequences, closely related to those of the 1999 outbreak, were detected in clinical and bivalve samples (unpublished data).

Frozen samples of clams directly associated with the outbreaks were tested for the presence of HAV. In the 1999 outbreak, 20 samples from households with cases were analyzed by standard reverse transcription-PCR (RT-PCR) (35). In the 2008 outbreak, nine package samples from households, seven from retail stores, and seven from importers were assayed by real-time RT-PCR (11). Details of the procedures are provided below.

**Virus and nucleic acid extraction.** Processing of shellfish samples from the 1999 outbreak was performed essentially by the method described by Atmar and coworkers (5). Briefly, the stomachs and digestive diverticula were dissected from the clams and subjected to high-speed homogenization (Sorval OCI Omni mixer; Omni Intl., Waterbury, CT). Viruses were extracted from the homogenates (corresponding to 1.5 g of shellfish tissue) by sequential extractions with chloroform-butanol and Cat-Floc T (Calgon Corp., Elwood, PA), and concentrated by polyethylene glycol precipitation. Nucleic acids from these concentrates were extracted with the RNeasy plant mini kit (Qiagen) following the manufacturer's instructions.

Samples from the 2008 outbreak were processed as described by Lowther and colleagues (26) with minor modifications. Briefly, for each sample, 2.0 g of digestive glands was finely chopped using a razor blade. Homogenates were then prepared by treating the chopped digestive glands with 2 ml of a 100- $\mu$ g/ml proteinase K solution (30 U/mg; Promega). Samples were incubated at 37°C with shaking for 60 min, followed by an additional incubation at 60°C for 15 min. After a low-speed centrifugation at  $3,000 \times g$  for 5 min, supernatants were recovered and employed for RNA extraction using the NucliSens miniMAG magnetic system (BioMérieux), following the manufacturer's instructions.

The high variability inherent to the procedures for virus recovery from shellfish calls for the use of process controls. Mengovirus MC<sub>0</sub> (ATCC VR-1957) was used to control the efficiency of the extraction procedures described above. Ten microliters of a virus stock, containing  $10^7$  50% tissue culture infective doses per ml, was added to the homogenates prior to the extraction processes. The efficiency of the nucleic acid extraction was calculated by comparing the detected and added mengovirus genomes. Differences in the extraction efficiencies between the employed methods and also within repeated extractions using a given method were monitored through the use of the mengovirus. Appropriate corrective factors were subsequently applied to the raw figures obtained in the real-time assay. The final virus numbers for a given sample were of the same order of magnitude regardless of the extraction procedure employed.

**Primers, probes, and molecular assays.** HAV quantification was performed through a standardized real-time TaqMan RT-PCR with primers HAV240 and HAV68, probe HAV150(-) labeled at the 5' end with 6-carboxyfluorescein

(FAM) and modified at the 3' end with the addition of a minor groove binder (MGB), and assay conditions described elsewhere (11). This method includes the use of mengovirus as a process control for virus/nucleic acid extraction from shellfish tissues, as mentioned above. Quantification of mengovirus was carried out through a real-time procedure following the same conditions as for HAV with the reverse primer Mengo209 (5'-GAAGTAACATATAGACAGACGCA CAC-3'), the forward primer Mengo110 (5'-GCGGGTCCTGCCGAAAGT-3'), and the FAM-MGB probe Mengo147 (5'-ATCACATTACTGCGCCGAAGC-3'). The target region for the quantification of mengovirus was selected to be as similar as possible to that of HAV in terms of structure, length, and base composition (11). Additionally the efficiencies of the RT-PCR enzymes were monitored by spiking an RNA control, as specified elsewhere (11).

**Estimation of accurate virus genome copy numbers in bivalve tissue.** The accurate number of genome copies in bivalve shellfish may be estimated by applying the corrective factors derived from the virus/nucleic acid extraction efficiency on the one hand and the efficiency of the RT-PCR amplification on the other (11). However, extraction efficiencies in shellfish samples usually range from 0.1% to 10%, while RT-PCR efficiencies range from 50% to 100%. Only in those cases in which the extraction efficiencies are  $\geq 10\%$  are both corrective factors considered, since the corrective factors are in the same log magnitude. This is usually observed when analyzing clinical samples. When extraction efficiencies are  $< 10\%$  and RT-PCR efficiencies are  $> 50\%$ , as occurred in the present study, it is not necessary to sum up both corrective factors, since they are not in the same log magnitude, the actual bottleneck being the extraction procedure.

**Quantitative risk assessment.** The risk of infection is the probability of acquiring the infection from a given exposure dose. The two main factors affecting this probability after consumption of food are the level of exposure (i.e., the number of infectious virus units per gram of clams and the ingested amount of clams) and the interaction of HAV and the host (defined by the dose-response curve). Table 1 depicts the parameters employed for the estimation of the HAV exposure from consumption of clams from contaminated batches, employing an adaptation of a general model for estimation of exposure of viruses in food proposed by Havelaar and Rutjes (18) based on the formula developed by Teunis et al. for estimation of exposure to protozoa in drinking water (38):  $dose = P \times C \times 1/R \times I \times PR \times W$ , where dose is the number of infectious virus particles in a single meal,  $P$  is the prevalence (the percentage of units contaminated by one or more infectious particles) at a specified step in the food chain,  $C$  is the measured concentration in contaminated units at that step in the food chain,  $R$  is the percent recovery of the detection method,  $I$  is the proportion of infectious units in the total physical virus population,  $PR$  is the percent virus titer reduction of downstream processes including preparation for consumption, and  $W$  is the amount of food consumed (per serving). The HAV dose was employed to estimate the risk of infection based on dose-response models obtained in human volunteers with different enteric viruses reported to be isolated in shellfish, assuming a single serving of 60 g of meat tissue (34). The beta-Poisson formula,  $P_i = 1 - (1 + N/\beta)^{-\alpha}$ , was applied in this work, with  $P_i$  being the probability of infection,  $\alpha$  and  $\beta$  the dose-response parameters, and  $N$  the exposure expressed as the number of infectious units after cooking. Previously

TABLE 2. HAV numbers in clams from batches linked to the 1999 hepatitis A outbreak<sup>a</sup>

Sample batch	Genome copies/g <sup>b</sup>		Infectious units/g <sup>c</sup>		Infectious units/g after light cooking <sup>d</sup>	
	Without correction	With correction	Without correction	With correction	Without correction	With correction
20757	$8.0 \times 10^1$	$8.0 \times 10^4$	$1.3 \times 10^0$	$1.3 \times 10^3$	$7.0 \times 10^{-3}$	$7.0 \times 10^0$
49254	$1.4 \times 10^1$	$1.4 \times 10^4$	$2.3 \times 10^{-1}$	$2.3 \times 10^2$	$1.2 \times 10^{-3}$	$1.2 \times 10^0$
53323	$1.3 \times 10^1$	$1.3 \times 10^4$	$2.2 \times 10^{-1}$	$2.2 \times 10^2$	$1.2 \times 10^{-3}$	$1.2 \times 10^0$
1054	$1.1 \times 10^2$	$1.1 \times 10^5$	$1.8 \times 10^0$	$1.8 \times 10^3$	$9.7 \times 10^{-3}$	$9.7 \times 10^0$

<sup>a</sup> Estimations are figured with or without applying corrective factors derived by extraction and enzyme efficiencies.

<sup>b</sup> Genome copy numbers per g of tissue were estimated by a TaqMan real-time RT-PCR (11).

<sup>c</sup> The numbers of infectious viruses per g of tissue were figured by applying a 1:60 factor to the number of physical particles (12, 20).

<sup>d</sup> The final numbers of infectious virus per gram of tissue after cooking were estimated by applying a reduction of 99.46% of the initial infectious virus, which is the reduction experimentally observed after light cooking (1, 8).

reported values of  $\alpha$  and  $\beta$  for rotavirus (39), echovirus 12 (36), poliovirus 1 (25), and poliovirus 3 (21) were employed.

The probability of infection was figured with or without applying corrective factors derived by extraction and enzyme efficiencies ( $R$  in Table 1), and since the vast majority of cases in both outbreaks occurred in the adult population (correlating with the primary population consuming shellfish), we assumed that all infections were symptomatic (risk of illness = risk of infection).

The actual attack rate could be determined only for the 1999 outbreak and was figured from the number of cases and the number of consumers theoretically associated with a given batch package, assuming a single serving of 60 g of shellfish. The net weight of the analyzed packages was ~2 kg. The numbers of consumers who developed hepatitis A after consumption of clams from packages of batches 20757, 49254, 53323, and 1054 were 9, 5, 4, and 18, respectively.

## RESULTS AND DISCUSSION

QMRA requires an accurate estimation of the exposure dose (14, 16, 18, 34). For unculturable viruses, such as HAV, for which cell culture is not adequate for primary isolation, standardized quantitative molecular procedures, including adequate controls to ascertain the efficiency of critical steps in food matrices, such as virus/nucleic acid extraction and enzyme inhibition, must be used (30). In the present study, the numbers of HAV genome copies per gram of tissue in clams associated with two outbreaks of hepatitis were figured by a standardized TaqMan real-time RT-PCR (11). Raw numbers of genome copies were corrected, taking into consideration the virus/nucleic acid extraction efficiencies and enzyme efficiencies.

Table 2 depicts the HAV levels in package samples from four batches of clams linked with the 1999 outbreak. The

numbers of infectious viruses were figured by applying a 1:60 factor to the number of physical particles (12, 20). In addition, since clams were sold frozen, it was assumed that they were not consumed raw but lightly cooked, following the local culinary habits. In this scenario, the final numbers of infectious virus after cooking were estimated by applying a reduction of 99.46% of the initial infectious virus, which is the reduction experimentally observed for HAV after light cooking (1, 8). From the data depicted in Table 2, the need to apply the corrective coefficients is evident, since otherwise the exposure dose for clams responsible for hepatitis cases would be far below a single infectious unit, assuming a single serving of 60 g of clam meat. The HAV infectious dose is unknown but according to the U.S. Food and Drug Administration presumably is around 10 to 100 virus particles (<http://www.seafoodhaccp.com/SeafoodData/BadBugBook/chap31.html>).

The actual attack rate for these batches is shown in Table 3. The number of HAV genome copies in tissues of lightly cooked clams, with or without correction, was employed for the estimation of the risk of infection using the beta-Poisson parameters obtained for human volunteers after ingestion of rotavirus (39), echovirus 12 (36), poliovirus 1 (25), and poliovirus 3 (21). From these data we can conclude that overall the fittest model after applying the required corrective factors is the one corresponding to echovirus 12, as was also suggested by Rose and Sobsey (34). This model was employed thereafter for HAV QMRA. According to our data (Tables 2 and 3), an infectious dose of 72 infectious particles may be associated

TABLE 3. Actual attack rate of HAV present in different clam batches linked to the 1999 outbreak and estimated risk of illness<sup>a</sup>

Sample batch	Attack rate <sup>b</sup>	Risk of illness based on dose-response curves for enteric viruses <sup>c</sup>							
		Rotavirus		Echovirus 12		Poliovirus 1		Poliovirus 3	
		Without correction	With correction	Without correction	With correction	Without correction	With correction	Without correction	With correction
20757	0.27	0.17	0.83	0.0008	0.36	0.000030	0.026	0.16	0.92
49254	0.14	0.04	0.74	0.0001	0.11	0.000005	0.005	0.03	0.84
53323	0.12	0.04	0.74	0.0001	0.11	0.000005	0.005	0.03	0.84
1054	0.54	0.20	0.85	0.0012	0.41	0.000042	0.035	0.20	0.93

<sup>a</sup> The risk of illness after consumption of lightly cooked clams was figured with or without applying corrective factors derived by extraction and enzyme efficiencies (11).

<sup>b</sup> The actual attack rate was calculated for each batch with the number of cases and the number of consumers associated with a batch package, assuming a single serving of 60 g of shellfish. Analyzed packages contained around 2 kg (net weight) of clams.

<sup>c</sup> Estimation based on reported dose-response models for different enteric viruses (34) and determined for consumption of 60 g of shellfish. Beta-Poisson formula:  $P_i = 1 - (1 + N/\beta)^{-\alpha}$ ,  $P_i$  being the probability of infection,  $\alpha$  and  $\beta$  the dose-response parameters, and  $N$  the exposure expressed as the number of infectious units after light cooking (see Tables 1 and 2). Values of  $\alpha$  and  $\beta$ , are, respectively, 0.26 and 0.42 for rotavirus (40), 0.374 and 186.69 for echovirus 12 (36), 0.1097 and 1,524 for poliovirus 1 (25), and 0.409 and 0.788 for poliovirus 3 (21).

TABLE 4. Genome copy numbers of HAV present in different clam batches linked to the 2008 outbreak and estimated risk of illness

Harvesting date	Genome copies/g shellfish	Estimated attack rate <sup>a</sup>		
		Uncooked	Lightly cooked	Well cooked
March 2007	$1.5 \times 10^4$	0.81	0.13	0.04
September 2007	$5.1 \times 10^3$	0.71	0.05	0.01
December 2007	$8.8 \times 10^4$	0.90	0.38	0.17
	$5.6 \times 10^3$	0.72	0.05	0.02
May 2008	$1.3 \times 10^4$	0.80	0.11	0.03
	$3.2 \times 10^3$	0.66	0.03	0.01
	$1.2 \times 10^5$	0.91	0.43	0.21

<sup>a</sup> Estimation based on the echovirus 12 reported dose-response model (34, 36). Exposure dose was figured by applying the formula and parameters described in Table 1. Data were figured by applying corrective factors derived by extraction and enzyme efficiencies. The final concentration of infectious virus after cooking was estimated by applying a reduction of the initial infectious virus in cooked shellfish as specified in Table 1 (1, 8).

with an 11% probability of infection (batches 49254 and 53323), while a dose of 420 infectious units may be associated with a 36% likelihood of infection (batch 20757) and 582 infectious units may be associated with a 41% probability of infection (batch 1054). From these data, it may be suggested that the likelihood of infection of HAV may be proportional to the dose. However, further studies are required with other outbreaks once the tools for quantitative molecular detection of the virus are implemented.

The 2008 hepatitis outbreak was long lasting (7 months) due to the involvement of nearly 4,000 tons of clams harvested in the Piura and Sechura provinces, Peru. When the outbreak was declared, risk management measures implemented by the Spanish Ministry of Health were (i) activating the national system of epidemiological surveillance to prevent the distribution of any further coquina shipments and (ii) reporting to officials at border checkpoints and the European Community Rapid Alert System for Foodstuffs. All Peruvian shellfish imports were banned in the EU, and around 1,000 tons of shellfish were immobilized. Besides the sanitary impact, the commercial economical burden of this hepatitis outbreak was overwhelming; the loss due only to the immobilized product was US\$4 million, and the losses derived from the EU ban were of enough magnitude to bankrupt the Peruvian fishery sector.

The actual attack rates associated with different clam batches could be figured for the 1999 outbreak due to the high number of cases occurring in a relatively narrow time period (184 cases in 3 months). However, the wide period in which hepatitis cases were reported in the 2008 outbreak (100 cases in 7 months) made the same quality level of case control studies unattainable. These 100 hepatitis A cases were associated with around 3,000 tons (gross weight equivalent to 1,500 tons of meat) of clams that were already consumed when the alert measures were implemented. From these data, an overall attack rate of 0.000004, assuming a single serving of 60 g, could be roughly figured, which is far below the estimated attack rate for specific HAV-positive batches (Table 4), pointing to a

small proportion of contaminated clams within the huge amount of involved mollusks.

One major issue in the monitoring of food products for virus occurrence is the representativity of the assayed sample, particularly in situations of a low proportion of contaminated samples, as occurred in the 2008 outbreak. Coquina samples from households ( $n = 9$ ) as well as from retail stores ( $n = 7$ ) directly associated with cases in the 2008 outbreak were sampled, and HAV genome copies were determined in our laboratory. Additionally, samples belonging to the same batches but obtained from import companies ( $n = 7$ ) were also analyzed. While HAV was detected in five (56%) home and two (30%) retail store samples, no virus was detected in samples from import companies (0%). Overall, HAV was detected in 44% of samples directly associated with cases but was undetectable in samples belonging to the same batches but randomly collected from the importers. Additionally, 50 samples from immobilized batches with no associated cases were also analyzed with negative results, altogether showing the difficulty of preventing outbreaks through the prospective monitoring of samples prior to the occurrence of cases in this kind of scenario. Prospective virological analysis of food is envisaged to ensure the safety of the foodstuff before public consumption. However, it is such a highly complex and costly process that obviously it cannot be universally applicable to a huge production of bivalves. The need to analyze representative samples is even more critical when the screening procedures are based on molecular analysis of minute amounts of a sample whose virus contamination, if present, is very low. Obviously any error at this step is critically carried over and impossible to correct even when highly sophisticated detection procedures are subsequently applied.

The genome copy numbers of HAV per gram of clams in positive samples from the 2008 outbreak are given in Table 4. QMRA for consumption of raw, lightly cooked, or well-cooked coquinas is also shown in Table 4. As expected, the level of heat treatment applied to the bivalves greatly influences the estimated attack rate. This has raised the possibility of including on the product label the recommendation to consume the clams well cooked, an option which is under discussion among shellfish producers and importers.

The harvesting dates of the positive samples expanded over a 1-year period, suggesting that the harvesting area contamination was a frequent event, although it likely occurred at a very low level, as suggested by the low proportion of positive isolations in the imported product. Data from the Red Bajo Piura of the Peruvian Health Authority on the occurrence of hepatitis A in the Piura and Sechura provinces reflect a moderate endemicity of HAV infection, with some symptomatic cases among a majority of asymptomatic cases. The number of hepatitis A cases per 100,000 inhabitants in the area was figured for 6-month periods from January 2007 to June 2008 (Fig. 1). The number of hepatitis A cases increased from July 2007 on, correlating with an increase both in the distribution of positive clam samples and in the number of HAV genome copies. Of note, 50% of the positive samples were harvested during the January–June 2008 period (cases per 100,000 inhabitants: 13.3), 33% during July–December 2007 (cases per 100,000 inhabitants: 11.6), and the remaining 17% during January–June 2007 (cases per 100,000 inhabitants: 3.3). Addition-



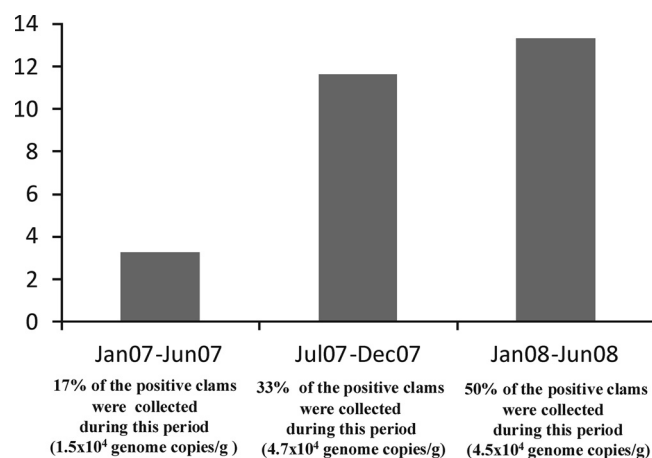


FIG. 1. Number of hepatitis A cases per 100,000 inhabitants in 6-month periods in the Piura and Sechura provinces, Peru (data from the Red Bajo Piura of the Peruvian Health Authority).

ally, the mean number of genome copies per gram of tissue of positive samples in the July–December 2007 and January–June 2008 periods also tended to be higher than that for January–June 2007 (Fig. 1), with some samples bearing a significantly higher virus load (Table 4).

The correlation between the prevalence of symptomatic hepatitis A cases among the population from the harvesting areas and positive HAV isolation in clams points to the need to identify and prevent hazards that could cause food-borne illnesses, rather than relying on random sampling of finished products to ensure safety. This constitutes the basis of the hazard analysis and critical control point system (19). A cost-effective hazard analysis and critical control point-based system applied to the viral safety of bivalves should rely on assessing hazard identification, critical limits, record keeping, and corrective actions in order to control the virological quality of harvesting areas through the continuous quantitative monitoring of such potential sources of contamination as treated or untreated wastewater or river water discharging into the shellfish-growing beds rather than the monitoring of shellfish for viruses (30). However, when evidence shows that a critical limit of viral contamination has been exceeded, quantitative virological analysis of bivalves should be performed before commercial distribution. These molecular assays for virus determination in harvesting areas and final product should be performed to address quality assurance and quality control requirements, like the one herein employed, in order to be internationally accepted in the future by regulatory agencies.

There is presently no internationally harmonized approach to shellfish testing, although bacterial indicators are almost universally employed to ensure the virological quality of bivalve mollusks. The United States and other countries require monitoring shellfish-harvesting waters, while the EU and other countries require testing the flesh of the animals (2, 3). In some particular situations, guidelines to ensure the specific virological safety of bivalve shellfish in the current situation of global food trade may be formulated and cost-effective risk management measures may be implemented. This work provides the first evidence of accurate HAV levels in shellfish involved in

outbreaks that could be of use for these risk assessment purposes.

## ACKNOWLEDGMENTS

This work was supported in part by EU projects SEAFOODplus, Food-CT-2004-506359, and Healthy-Water, Food-4B-2005-36306; Spanish Ministry of Science and Innovation projects BIO2005-05022, BIO2008-01312, and CSD2007-00016; and Generalitat de Catalunya projects 2005SGR00966 and Biotechnology Reference Network (XRB). M. I. Costafreda is a recipient of an FPI fellowship from the Ministry of Science and Innovation.

We thank the Epidemiology Section of the Directorate of Public Health of the Generalitat Valenciana for their cooperation in the present study.

## REFERENCES

- Abad, F. X., R. M. Pinto, R. Gajardo, and A. Bosch. 1997. Viruses in mussels: public health implications and depuration. *J. Food Prot.* **60**:677–681.
- Anonymous. 2004. Regulation (EC) no. 854/2004 of the European Parliament and 16 of the Council of 29 April 2004 laying down specific rules for the organisation of 17 official controls on products of animal origin intended for human consumption. *Official Journal of the European Union* L226 of 25 June 2004:83–127.
- Anonymous. 2007. Shellfish Federal Regulations. National Shellfish Sanitation Program. Guide for the Control of Molluscan Shellfish. U.S. Food and Drug Administration. <http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/FederalStatePrograms/NationalShellfishSanitationProgram/ucm046353.htm>.
- Aragones, L., A. Bosch, and R. M. Pinto. 2008. Hepatitis A virus mutant spectra under the selective pressure of monoclonal antibodies: codon usage constraints limit capsid variability. *J. Virol.* **82**:1688–1700.
- Atmar, R. L., F. H. Neill, J. L. Romalde, F. Le Guyader, C. M. Woodley, T. G. Metcalf, and M. K. Estes. 1995. Detection of Norwalk virus and hepatitis A virus in shellfish tissues with the PCR. *Appl. Environ. Microbiol.* **61**:3014–3018.
- Blatchley, E. R., III, W. L. Gong, J. E. Alleman, J. B. Rose, D. E. Huffman, M. Otaki, and J. T. Lisle. 2007. Effects of wastewater disinfection on waterborne bacteria and viruses. *Water Environ. Res.* **79**:81–92.
- Bosch, A. 2007. Human viruses in water. Elsevier, Amsterdam, The Netherlands.
- Bosch, A., F. X. Abad, R. Gajardo, and R. M. Pinto. 1994. Should shellfish be purified before public consumption? *Lancet* **344**:1024–1025.
- Bosch, A., G. Sanchez, F. Le Guyader, H. Vanaeloch, L. Haugarreau, and R. M. Pinto. 2001. Human enteric viruses in Coquina clams associated with a large hepatitis A outbreak. *Water Sci. Technol.* **43**:61–65.
- Conaty, S., P. Bird, G. Bell, E. Kraa, G. Grohmann, and J. M. McNulty. 2000. Hepatitis A in New South Wales, Australia from consumption of oysters: the first reported outbreak. *Epidemiol. Infect.* **124**:121–130.
- Costafreda, M. I., A. Bosch, and R. M. Pinto. 2006. Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. *Appl. Environ. Microbiol.* **72**:3846–3855.
- Deng, M. Y., S. P. Day, and D. O. Cliver. 1994. Detection of hepatitis A virus in environmental samples by antigen-capture PCR. *Appl. Environ. Microbiol.* **60**:1927–1933.
- Dismukes, W. E., A. L. Bisno, S. Katz, and R. F. Johnson. 1969. An outbreak of gastroenteritis and infectious hepatitis due to raw clams. *Am. J. Epidemiol.* **89**:555–561.
- Gerba, C. P., J. B. Rose, C. N. Haas, and K. D. Crabtree. 1996. Waterborne rotavirus: a risk assessment. *Water Res.* **30**:2929–2940.
- Haas, C. N. 1983. Estimation of risk due to low doses of microorganisms: a comparison of alternative methodologies. *Am. J. Epidemiol.* **118**:573–582.
- Haas, C. N., J. B. Rose, C. Gerba, and S. Regli. 1993. Risk assessment of virus in drinking water. *Risk Anal.* **13**:545–552.
- Halliday, M. L., L.-Y. Kang, T.-Z. Zhou, M.-D. Hu, Q.-C. Pan, T.-Y. Fu, Y. S. Huang, and S.-L. Hu. 1991. An epidemic of hepatitis A attributable to the ingestion of raw clams in Shanghai, China. *J. Infect. Dis.* **164**:852–859.
- Havelaar, A. H., and S. A. Rutjes. 2008. Risk assessment of viruses in food: opportunities and challenges, p. 221–236. *In* M. Koopmans, D. O. Cliver, and A. Bosch (ed.), *Food-borne viruses: progress and challenges*. ASM Press, Washington, DC.
- Jansen, J. 2008. Use of the Codex risk analysis framework to reduce risks associated with viruses in food, p. 209–220. *In* M. Koopmans, D. O. Cliver, and A. Bosch (ed.), *Food-borne viruses: progress and challenges*. ASM Press, Washington, DC.
- Jansen, R. W., J. E. Newbold, and S. M. Lemon. 1988. Complete nucleotide sequence of a cell culture-adapted variant of hepatitis A virus: comparison with wild-type virus with restricted capacity for in vitro replication. *Virology* **163**:299–307.

21. **Katz, M., and S. A. Plotkin.** 1967. Minimal infective dose of attenuated poliovirus for man. *Am. J. Public Health Nations Health* **57**:1837–1840.
22. **Lees, D.** 2000. Viruses and bivalve shellfish. *Int. J. Food Microbiol.* **59**:81–116.
23. **Le Guyader, F., L. Haugarreau, L. Miossec, E. Dubois, and M. Pommepuy.** 2000. Three-year study to assess human enteric viruses in shellfish. *Appl. Environ. Microbiol.* **66**:3241–3248.
24. **Leoni, E., C. Bevini, S. Degli Esposti, and A. Graziano.** 1998. An outbreak of intrafamilial hepatitis A associated with clam consumption: epidemic transmission to a school community. *Eur. J. Epidemiol.* **14**:187–192.
25. **Lepow, M. L., R. J. Warren, V. G. Ingram, S. C. Daugherty, and F. C. Robbins.** 1962. Sabin type I (LSc2ab) oral poliomyelitis vaccine. Effect of dose upon response of newborn infants. *Am. J. Dis. Child.* **104**:67–71.
26. **Lowther, J. A., K. Henshilwood, and D. N. Lees.** 2008. Determination of norovirus contamination in oysters from two commercial harvesting areas over an extended period, using semiquantitative real-time reverse transcription PCR. *J. Food Prot.* **71**:1427–1433.
27. **Mackowiak, P. A., C. T. Caraway, and B. L. Portnoy.** 1976. Oyster-associated hepatitis: lessons from the Louisiana experience. *Am. J. Epidemiol.* **103**:181–191.
28. **Martin, L. R., G. M. Duke, J. E. Osorio, D. J. Hall, and A. C. Palmenberg.** 1996. Mutational analysis of the mengovirus poly(C) tract and surrounding heteropolymeric sequences. *J. Virol.* **70**:2027–2031.
29. **Mele, A., M. G. Rastelli, O. N. Gill, D. di Bisceglie, F. Rosmini, G. Pardelli, C. Valtriani, and P. Patriarchi.** 1989. Recurrent epidemic hepatitis A associated with consumption of raw shellfish, probably controlled through public health measures. *Am. J. Epidemiol.* **130**:540–546.
30. **Pintó, R. M., and A. Bosch.** 2008. Rethinking virus detection in food, p. 171–188. *In* M. Koopmans, D. O. Cliver, and A. Bosch (ed.), *Food-borne viruses: progress and challenges*. ASM Press, Washington, DC.
31. **Potasman, I., A. Paz, and M. Odeh.** 2002. Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. *Clin. Infect. Dis.* **35**:921–928.
32. **Romalde, J. L., E. Area, G. Sanchez, C. Ribao, I. Torrado, X. Abad, R. M. Pinto, J. L. Barja, and A. Bosch.** 2002. Prevalence of enterovirus and hepatitis A virus in bivalve molluscs from Galicia (NW Spain): inadequacy of the EU standards of microbiological quality. *Int. J. Food Microbiol.* **74**:119–130.
33. **Roos, B.** 1956. [Hepatitis epidemic transmitted by oysters.] *Sven. Lakartidn.* **53**:989–1003. (In Swedish.)
34. **Rose, J. B., and M. D. Sobsey.** 1993. Quantitative risk assessment for viral contamination of shellfish and coastal waters. *J. Food Prot.* **56**:1043–1050.
35. **Sanchez, G., R. M. Pinto, H. Vanaclocha, and A. Bosch.** 2002. Molecular characterization of hepatitis A virus isolates from a transcontinental shellfish-borne outbreak. *J. Clin. Microbiol.* **40**:4148–4155.
36. **Schiff, G. M., G. M. Stefanovic, E. C. Young, D. S. Sander, J. K. Pennekamp, and R. L. Ward.** 1984. Studies of echovirus-12 in volunteers: determination of minimal infectious dose and the effect of previous infection on infectious dose. *J. Infect. Dis.* **150**:858–866.
37. **Stroffolini, T., W. Biagini, L. Lorenzoni, G. P. Palazzesi, M. Divizia, and R. Frongillo.** 1990. An outbreak of hepatitis A in young adults in central Italy. *Eur. J. Epidemiol.* **6**:156–159.
38. **Teunis, P. F. M., G. J. Medema, L. Kruidenier, and A. H. Havelaar.** 1997. Assessment of the risk of infection by *Cryptosporidium* or *Giardia* in drinking water from a surface water source. *Water Res.* **31**:1333–1346.
39. **Ward, R. L., D. I. Bernstein, E. C. Young, J. R. Sherwood, D. R. Knowlton, and G. M. Schiff.** 1986. Human rotavirus studies in volunteers: determination of infectious dose and serological response to infection. *J. Infect. Dis.* **154**:871–880.