Molecular Confirmation of Oysters as the Vector for Hepatitis A in a 2005 Multistate Outbreak

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ABSTRACT

Numerous hepatitis A outbreaks were linked to the consumption of raw molluscan shellfish in the United States between 1960 and 1989. However, there had been no major molluscan shellfish–associated hepatitis A outbreaks reported in the United States for more than a decade (1989 to 2004). Beginning in late August 2005, at least 10 clusters of hepatitis A illnesses, totaling 39 persons, occurred in four states among restaurant patrons who ate oysters. Epidemiologic data indicated that oysters were the source of the outbreak. Traceback information showed that the implicated oysters were harvested from specific Gulf Coast areas. A voluntary recall of oysters was initiated in September. Hepatitis A virus (HAV) was detected in multiple 25-g portions in one of two recalled samples, indicating that as many as 1 of every 15 oysters from this source was contaminated. Comparing 315 nucleotides within the HAV VP1-2B region, 100% homology was found among four amplicons recovered from a total of six independent experiments of the implicated oysters, and an identical HAV sequence was detected in sera from all 28 patient serum specimens tested. Ten percent heterogeneity over 315 nucleotides (31 variants) was observed between the outbreak strain (subgenotype 1A) and an HM-175 strain (subgenotype 1B) used in the laboratory where the oysters were processed. To our knowledge, this investigation is the first in the United States to identify an HAV-identical strain in persons with hepatitis A as well as in the food that was implicated as the source of their infections.

Hepatitis A virus (HAV) contains a 7.5-kb-long, positive-sense, single-strand RNA genome. A single open reading frame encodes all proteins, and the P1 region of the HAV genome encodes four structural proteins (VP1, VP2, VP3, and VP4). Regions P2 and P3 encode nonstructural proteins associated with replication. Major antigenic epitopes are located across structural and nonstructural regions of the HAV polyprotein (13), but only a single HAV serotype exists. Analyzing over 150 worldwide strains of HAV, Robertson et al. (22) and Lemon et al. (14) initially classified seven HAV genotypes on the basis of the VP1-2A junction. However, a more detailed analysis showed that genotype VII should be reclassified as a subgenotype of genotype II. Thus, only six HAV genotypes are currently recognized (7, 15, 19).

Primarily, the fecal-oral route via person-to-person contact, contaminated food, or contaminated water transmits HAV. The source of transmission, however, cannot be identified for approximately 50% of the reported hepatitis A cases in the United States (2, 4). Only 2 to 5% (11, 17) of the reported hepatitis A cases each year are attributed to contaminated food. However, foodborne outbreaks of hepatitis A can cause considerable morbidity and even mortality and require a resource-intensive public health response (11). Methods to identify HAV contamination of food would significantly help investigators link individuals with HAV and outbreak clusters whose exposures are uncertain or who are geographically separated from other outbreak clusters.

The HAV genome can be detected in serum specimens taken from persons with hepatitis A, and genetic relatedness analyses have been successfully used in conjunction with epidemiology data in foodborne hepatitis A outbreak investigations (1, 5, 8, 12, 20, 21, 25). Molecular epidemiologic analyses have been used to quickly link persons for whom a common exposure is suspected and to support product traceback data by demonstrating similarities between patient HAV sequences and those from individuals with HAV living in or traveling to the geographic source of the contaminated product (1, 8, 12, 25). A comparison of HAV sequences from individuals with hepatitis A with sequences detected in implicated foods would provide additional evidence to link implicated products with illnesses. However, the chemical complexity of food matrices and likely low concentrations of HAV in food are major obstacles to successful viral detection in the implicated foods. In addition, the onset of hepatitis A symptoms typically occurs 21 to 35 days after exposure, making it difficult to obtain implicated food samples.

The first well-described hepatitis A outbreak associated with the consumption of bivalve molluscan shellfish in the United States occurred during the first 3 months of 1961 (16). In this outbreak, 80 residents of...

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Mississippi and Alabama, in addition to four visitors from northern states, developed hepatitis A after the consumption of raw oysters from Pascagoula Bay, Mississippi. Concurrently, another report documented 459 hepatitis A cases associated with the consumption of raw clams mostly from Raritan Bay, New Jersey (10). Over the next 25 years, shellfish consumption was frequently reported as the cause of HAV outbreaks. In 1988, an outbreak of 61 cases associated with oyster consumption was reported to involve illegal harvest from an unapproved area (9). However, for the next 15 years, there was no large hepatitis A outbreak associated with shellfish consumption in the United States.

In August and September 2005, however, reports from a four-state region identified 39 persons with hepatitis A who had consumed oysters in the previous 2 to 6 weeks. The illness was strongly associated with the consumption of oysters in 1 of 12 unaffiliated restaurants in Alabama, Florida, South Carolina, and Tennessee. The details of the epidemiologic investigation of this outbreak were described elsewhere (3). In the present study, we describe the collection of implicated oysters, the extraction and detection of HAV, and the comparison of a selected 315-nucleotide section of the HAV genome detected in implicated oysters and patients’ sera.

MATERIALS AND METHODS

Extraction and concentration of HAV from oysters. The U.S. Food and Drug Administration (FDA) and state authorities conducted traceback studies. In September 2005, a wholesaler that distributed oysters to restaurants where persons had eaten oysters initiated a voluntary product recall. Frozen shucked oysters, with tags indicating harvest from the same areas and at the same time as the implicated oysters, were found during this recall. These recalled oysters were used for subsequent HAV detection studies. Oysters chosen for these studies had a harvest date at the midpoint of the time interval during which all oysters served to hepatitis patients were harvested. Implicated oysters were thawed once and aseptically separated from icy water and adductors. Portions of 25 g each were prepared and stored in sterile containers in a −70°C freezer until viral analysis. The procedure used to process oysters for virus concentration was published previously (23). In brief, viruses in 25-g portions of oyster meat were adsorbed to oyster-homogenate solids at pH 4.8 ± 0.3 and then eluted with amino acid solutions (glycine and threonine) at pH 7.5. Viruses in sample concentrates were polyethylene glycol precipitated, solvent extracted, and RNA extracted.

One-step RT-PCR and nested PCR examinations of HAV in oyster sample concentrates. The viral target in the RNA sample concentrates was amplified by one-step reverse transcription (RT)-PCR (with an Invitrogen SuperScript III Platinum one-step RT-PCR kit) and then by nested PCR with Platinum Taq (Invitrogen, Carlsbad, Calif.). The reaction conditions recommended by the manufacturer were followed. The following profile was used in the one-step RT-PCR: reverse transcription at 47°C for 25 min, Taq activation at 95°C for 2 min, and then 40 cycles of 95°C for 55 s, 48°C for 45 s, and 72°C for 55 s. One tenth of the first amplicon (5 µl) was utilized as a template for the next (nested) PCR with Platinum Taq under the cycling profile of enzyme activation at 95°C for 2 min and was followed by 40 cycles of 95°C for 45 s, 48°C for 35 s, and then 72°C for 45 s. Primer sequences were 5′-GACAGATTCTACATTGGATTTGGT-3′ and 5′-CCATTCAAAGTCCACACACT-3′ for the first PCR and 5′-CTATTCAGATTGAATTTACAT-3′ and 5′-AACTTCAT TATTTGATGCTCCT-3′ for the nested PCR. Amplicon sizes were 512 and 392 bp, respectively, for the first and nested PCRs. Negative reagent controls were included in each RT-PCR and nested PCR. Extensive precautions were taken to prevent contamination, including physical separation of the area for preparing the reaction mixture from the area for preparing the template or sample.

DNA sequencing and nucleotide sequence analysis. We carried out a blinded trial to sequence HAV amplicons from oysters in order to compare them with sequences of HAV from patients. As an experimental control, we added an unlabeled positive control (HM-175, a laboratory strain) as the fifth amplicon to four 392-bp amplicons of oysters derived from independent experiments. Twenty nanograms of each 392-bp amplicon (without extraction) was sequenced with a Big Dye v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif.). The sequencing reactions were first purified through a Millipore Montage SEQ96 Cleanup Kit (Millipore Corp., Bedford, Mass.) and then run on 3130XL Genetic Analyzer (Applied Biosystems). Sequences of the 315-nucleotide region within the 392-bp amplicons were used for genetic relatedness analysis. Preliminary sequence analysis was performed with the SeqMan and MegAlign programs from the Lasergene DNA & Protein analysis software (version 6.0, DNASTAR Inc., Madison, Wis.). The ACCELERYS GCG Package (Genetic Computer Group, version 10.3, Accelrys Inc., San Diego, Calif.) performed a more complex sequence analysis. The HAV sequences were classified into genotypes by comparing each sequence with the reference sequences from GenBank.

RESULTS

Detection of HAV in implicated oysters. On 28 September 2005, two recalled oyster samples were collected by the FDA from the wholesaler that had shipped oysters to the restaurants. Records illustrated that, after harvest on 1 August, the shellstock oysters from both samples were cold shipped and then dry stored at 4°C for approximately 10 to 12 days before being shucked, packaged, and shipped further to retailers in different states. Sample 1 was sold as a frozen product and remained frozen. Sample 2 was sold as a fresh shucked product and returned frozen. Both samples were frozen at the time of collection. Sample 1 was packaged 2 days later than sample 2.

We detected HAV RNA in the oyster meat of sample 1, while sample 2 was not found to have any detectable viral RNA. An average of five (with a range of three to eight) shellfish were in each 25-g composite of sample 1. HAV RNA was detected in one 25-g portion of the three to four 25-g portions tested in each experiment. The phenomenon was observed consistently in six independent experiments. Results from one of the six (experiment 3) are illustrated in Figure 1. We estimated that 1 in every 15 oysters had HAV contamination, given the estimate that a typical 25-g portion consisted of five oysters. Each 25-g oyster portion, labeled A, B, C, and D, consisted of four, five, six, and eight oysters, respectively (Fig. 1). Approximately 100 U of HAV was detected in sample portion B containing five oysters (Fig. 1). One RT-PCR unit of HAV was defined as the lowest concentration (highest dilution)
of the RNA sample that resulted in a positive amplification of HAV. On average, there were at least four HAV RT-PCR–detectable units per gram of oyster meat in this portion of oyster sample 1. Sample 2 and all negative controls consistently showed an absence of HAV throughout the experiments.

**Comparison of HAV genome sequences in oysters and patients.** For sample 1 (HAV positive), all four amplicons (designated oys-seq a, b, d, and e in Fig. 2) that derived from the six experiments were identical to each other over the 315 nucleotide regions of the VP1-2B region of the HAV genome. Additionally, the multiple 315 nucleotide regions sequenced from oysters were 100% identical to those recovered from 28 of the 31 patients for whom sera were available (identical sequences were found among the 28 (3) and were designated patient 28 in Fig. 2). No HAV RNA could be detected in the other 3 patients of the 31. The HAV nucleotide sequences from oysters differed by 10% (31 variants among the 315 nucleotides) from HM-175, the only laboratory strain of HAV used in our laboratory (designated ampli-seq c in Fig. 2), indicating that the HAV detected in oysters was not the result of laboratory cross-contamination. The HAV strain from the 2005 outbreak was classified as HAV genotype 1, subtype A, while HM-175 is genotype 1, subtype B.

The nucleotide sequences of control ampli-seq c also indicated the accuracy and consistency of our sequencing analyses. The 315 nucleotides of our laboratory strain were 100% identical to those of laboratory strain HM-175 (hm175,43c) that were extracted from GenBank (Fig. 2). Both laboratory strains differed from the wild type of HM-175 (hm175,WT) by 1 nucleotide (position 101) among the 315 in this highly variable region of VP1-2B (Fig. 2).

**Status of oyster-harvest areas and possible contamination source.** All implicated shellstock oysters were from approved harvest areas off eastern Louisiana between late July and mid-August 2005. On 30 August, these shellfish-harvesting areas were closed in response to Hurricane Katrina. According to the Louisiana Department of Wildlife and Fisheries, Hurricane Katrina disrupted and severely damaged oyster beds and farming. Two months later, however, the harvesting areas where samples 1 and 2 came from were reopened. No hepatitis A cases linked to oysters from these areas have been reported since harvesting resumed.

**DISCUSSION**

Since the last large hepatitis A outbreak in the United States linked to oysters in 1988 (9), significant advances in molecular biology have been made. However, identifying the viral etiologic agents in implicated food is difficult, even with the application of advanced molecular techniques and sufficient numbers of viruses. This outbreak was the first to identify a single HAV strain with identical sequences from both patients and implicated food sources. These results strongly supported epidemiologic data implicating oysters and traceback information indicating that oysters harvested from a specific region in Louisiana were the source of the outbreak. In this investigation, the 100% identical HAV sequences from this variable genomic region indicate that a common contamination occurred in shellstock oysters harvested from one state and distributed to four other states. Viral contamination is most likely to have occurred while oysters were in the harvest area rather than during shipping and handling. The most probable sources of contamination were illegal waste discharges from harvest vessels or recreational boats within legal harvest areas and illegal harvesting in closed areas.

All shellstock oysters were cold shipped and dry stored at 4°C (wet storage might deplete or accumulate further viral contaminants in shellfish). If contaminated water had been used to wash oysters during shucking, a larger number of contaminated oysters might have been expected. The rel-
FIGURE 2. The 315 nucleotide sequences of the HAV VP1-2B region from oyster sample 1 (oys-seq a, oys-seq b, oys-seq d, and oys-seq e), 28 patient sera, and three strains of HAV HM-175 (ampli-seq c, hm175WT, and hm17543c).

atively limited number of persons who became ill at each restaurant where implicated oysters were served suggests that relatively few oysters were contaminated. This epidemiologic observation was consistent with our estimate that 1 in every 15 oysters in sample 1 was contaminated. In addition, some persons who ate contaminated oysters might have been immune because of a previous infection or vaccination or might have had an asymptomatic or undiagnosed infection.

In general, the identification of etiologic agents in clinical specimens is faster and easier than in implicated food, because of lower virus levels in food and the complexity of the food matrix. Compared with the method previously used (18) and reported to have a detection limit of 1 PFU of HAV HM-175 initially seeded in 1 g of oyster meat, the current method in this study increases detection sensitivity by two- to threefold by eluting viruses with a higher concentration of threonine in the elution buffer throughout the virus extraction procedure (23). Thus, the method used in this study allows the detection of less than 1 PFU of HAV HM-175 initially seeded in 1 g of oyster meat. Concurrently, the quantitative RT-PCR detection of HAV in sample 1 with a Smart Cycler (Cepheid, Sunnyvale, Calif.) was carried out in our laboratory. Similar to the finding of this report, a high CT of around 40 (indicating low levels of viruses) was observed by quantitative RT-PCR, with most of the reactions being negative. Unlike another report that examined HAV in outbreak-associated clams (6), we analyzed individual oysters (not just diverticula) to derive the HAV frequency in individual oysters (1 positive in every 15 oysters examined), and we identified the HAV strain by sequencing (not just by genotype) to match the HAV strain from patients.

Samples 1 and 2 were harvested on 1 August from growing areas associated with the illnesses, but HAV was only detected in sample 1. Failure to detect HAV in sample 2 might have been because of an insufficient number of HAV in the sample. However, the shellstock oysters of sample 1 were held in cold storage at 4°C for 12 days after harvest, shucked, and frozen at −20°C for 7 weeks before analysis. Maintaining oyster samples at constant freezing temperatures may have reduced the degradation of viral RNA. In contrast, sample 2 was kept at 4°C for weeks (for sale as a fresh product). Enzymes, including RNases, are likely released from oyster meat after shucking, thereby accelerating viral RNA degradation. Other RNA viruses have
also been detected in shellstock oysters despite prolonged refrigeration at 4°C. For example, norovirus GI-4 was detected in shellstock oysters that were in dry storage at 4°C for 24 days before viral analysis (24) during an outbreak investigation. Dry storage of unshucked shellstock at 4°C or a one-time freezing of shucked shellfish meat at −70 or −20°C immediately after shucking may help prevent viral RNA degradation, compared with shucked shellfish meat stored at 4°C.

In some outbreaks of shellfish-borne viral disease, the most probable source of viral contamination was illegal discharge of human waste into harvest waters. In other cases, illegal harvest of shellfish from waters that were not approved for harvest was suspected. In response, the National Shellfish Sanitation Program has established specific requirements in an effort to reduce the number of outbreaks. (i) Harvest vessels are required to have waste containment receptacles. (ii) Harvesters are educated on the risks of overboard discharges of human waste. (iii) Patrol agencies have been asked to increase their patrol efforts in closed areas. For one and a half decades (1989 to 2004), there was no major hepatitis A outbreak reported. Although there has been a consistent downward trend in the occurrence of hepatitis A outbreaks associated with shellfish consumption in the United States, the 2005 outbreak brought renewed attention to the risk of HAV transmission via bivalve molluscan shellfish and underscores the need for continued vigilance and control efforts.

In summary, this article describes crucial elements that enabled us to establish a direct link between patients in the hepatitis A outbreak and implicated seafood for the first time, to our knowledge, in U.S. history. The crucial elements include the use of (i) molecular technology and epidemiology, (ii) an efficient virus extraction protocol, (iii) proper preservation of implicated food samples, and (iv) cooperative partnerships between the federal and state agencies to ensure a rapid and targeted response. To our knowledge, this is the first report in the United States in which HAV sequences amplified from patients’ sera were identical to those in an implicated food and the first to find 100% identity in all implicated food samples and all patients’ sera.

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