Original Article

A Cluster of Hepatitis A Infections Presumed to be Related to Asari Clams and Investigation of the Spread of Viral Contamination from Asari Clams

Ryusuke Tsukada1*, Satoko Ono1, Hiroki Kobayashi2, Yumi Wada1, Kanako Nishizawa1, Masumi Fujii1, Michiko Takeuchi1, Kazuo Kuroiwa2, Yoshikiyo Kobayashi2, Koji Ishii1, and Haruyuki Nakazawa1

1Division of Infectious Disease, Nagano Environmental Conservation Research Institute, Nagano 380-0944; 2Nagano Saku Health and Welfare Office, Nagano 385-8533; and 3Department of Virology II, National Institute of Infectious Diseases, Tokyo 208-0011, Japan

SUMMARY: In a cluster of hepatitis A infections that occurred in Nagano Prefecture in 2017, hepatitis A virus (HAV) was detected in asari clams (reference food) and the patients’ fecal samples. Initially, the asari clams were suspected to be the infection source. However, the exact infection route remained unknown because a patient who had not consumed an asari clam dish also developed the disease. Suspecting a secondary infection originating from the asari clams, we investigated the presence of HAV genomes in water used for washing and soaking the frozen asari clams and detected HAV in the soaking water. These results suggest that soaking water is a risk factor for secondary contamination because of the leakage of HAV accumulated in midgut gland of the asari clam. During the asari clam sand removal process, the water used to clean asari clams spread across a wide area in a concentric fashion, raising concerns that this process may aggravate contamination. In addition to HAV, diarrhea viruses, such as norovirus, have often been detected in bivalves, including asari clams. Thus, handling these foodstuffs requires adequate care.

INTRODUCTION

Hepatitis A is an acute form of hepatitis primarily caused by oral infection by hepatitis A virus (HAV). After approximately one month of incubation, infected patients start experiencing cold-like symptoms, including fever exceeding 38°C and marked sense of malaise and headache, followed by characteristic hepatitis symptoms such as jaundice and hepatomegaly (1,2). In Japan, hepatitis A is classified as a Class IV infectious disease in accordance with the Act on the Prevention of Infectious Diseases and Medical Care for Patients with Infectious Diseases (1). This law effectively obligates medical practitioners to report all instances of HAV infection, including those of asymptomatic carriers.

According to infection surveillance conducted by the Ministry of Health, Labour and Welfare of Japan, the number of notifications regarding hepatitis A patients ranged between 128 and 433 from 2010 to 2016 (3). Furthermore, in statistics from 2010 to 2014, 80% of infections were suspected to have occurred orally via ingestion of food and drink (1). Seafood, including oysters and fish, accounted for 41% of suspected infections (1). However, because of the long incubation period of approximately one month from infection to onset, epidemiological investigations are difficult to conduct (1). In a number of food poisoning cases that occurred in Hamamatsu city in 2001, purple Washington clams imported from China were identified as the source of infection because HAV was detected in a similar product with the same lot number (4).

When infected with hepatitis A, 90% of adults develop the disease and manifest symptoms (1,2). Since an abundance of HAV particles is discharged in feces of patients during the long incubation period, mishandling of food by infected kitchen workers can cause an outbreak (5). Such instances also complicate the identification of infection routes. In this study, we experienced a cluster of HAV infections that occurred in Nagano Prefecture (5). Frozen asari clams were initially suspected to be the primary infection source. However, given that they were heated during the cooking process, and that infections were also confirmed among people who had not eaten fried asari clams, we hypothesized secondary HAV infection originating from asari clams. The dearth of information and reference materials regarding secondary HAV infection proved to be an obstruction in identifying the exact infection route. With a view of determining the secondary infection route originating from frozen asari clams, we investigated the level of contamination on the surface of frozen asari clams and the possibility of viral leakage from soaked asari clams.

In addition to HAV, various viruses have been isolated from the midgut gland of bivalves (6–10). Although not applicable in this case, asari clams generally require a sand removal process. This process could further spread contamination. With this possibility in mind, we also examined the spreading of soaking water during sand removal.
MATERIALS AND METHODS

Detection of HAV in infected cases: Outline of the infected cases concerned: This HAV case came to light after a series of notifications of hepatitis A onset regarding four patients from three different social groups were submitted by medical institutions to multiple health and welfare offices in 2017. The investigation found that all four patients visited the same eating establishment, Restaurant A and developed the disease 32.0 days later, on average (Table 1). Three of the four patients ate asari clam dishes at Restaurant A. The asari clams used for cooking the dish originated as frozen product from China, with instructions on the packaging stating that the asari clams can be cooked without being thawed. Although one of the patients did not order an asari clam dish (Table 1), the person consumed raw vegetables prepared in the same kitchen as the asari clams. No other similar notifications were submitted. After investigation, the health and welfare office determined that Restaurant A had properly prepared the asari clams by heating them at ≥ 85°C for ≥ 1 min, a condition necessary to inactivate HAV (1). However, it emerged that Restaurant A had used to wash raw vegetables in the same stainless-steel pan as used for washing uncooked asari clams. Furthermore, another group of customers ordered asari clam dishes at Restaurant A on the same day as the patient who consumed raw vegetables; however, these customers did not develop the disease. Besides, we could not confirm the cooking process which causes the risk of secondary contamination.

Pretreatment of the samples and extraction of viral RNA: Six fecal samples were collected from four patients as well as two employees at Restaurant A. Additionally, the health and welfare office collected one food sample of 17 frozen asari clams imported from China (from a different package than the one consumed by the patients but with the same expiry date) during inspection of the facility. Seven to eight asari clams were selected from one food sample for inspection. The samples were pretreated in accordance with Pathogen Testing Guidelines (the Guidelines) (11). RNA was extracted from 140 μL of each RNA sample using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) in accordance with the kit’s instruction manual to obtain 60 μL of RNA extraction solution.

DNase treatment and cDNA synthesis by reverse transcription reaction: This process was performed in accordance with the protocol reported by the Guidelines, Yoshida et al., and Nishida et al. (6,11,12).

HAV genome detection by reverse transcription polymerase chain reaction (RT-PCR): This process was performed in accordance with the Guidelines (11), except that the primers were selected based on the report by Ishii et al. (13) (1st PCR: HAV-JCT-2F/HA V-JCT-1R-A; 2nd PCR: HAV-JCT-2F/HA V-JCT-2R). The purified DNA fragments amplified via PCR were used as templates for direct HAV sequencing. The determined base sequence was aligned with MEGA 6 software (https://www.megasoftware.net), and the genotype was determined by phylogenetic analysis based on the neighboring method. For the phylogenetic analysis of HAV nucleotide sequences, we included sequences of several common and/or widespread strains. These strains were: genotype I (Genebank accession numbers: AF512536 for DL3/China, AF357222 for LU38/China, AF485328 for LY6/China, EU131373 for HAV5/Uruguay, AB020564 for AH1/Japan, AB020569 for FH3/Japan, AB643803 for Ch27/Japan, X75215 for GBM/WT/Germany, X83302 for FG/Italy, K02990 for LA/USA, AF314208 for LA/China, M20273 for MBB/Germany, AF268396 for HAF-203/Brazil, M14707 for HM-175/Australia, AB839692 for BaliA03-H29/Indonesia, AB969748 for KagsmP-4HA7/Japan, AB973401 for KobeC-2/Kb/ Japan, and KF182323 for ISS 2.2(Italy); genotype II (AY644676 for CF53/Barne and AY644670 for SLF88/ USA); or genotype III (FJ360735 for IND-HAV-97F/ India, AJ299464 for NOR-21/Norway, EU011791 for PN-IND/India, AB279733 for HA-JNG08-92/Japan, AB973400 for SendaiC-13/Ao/Japan, AB258387 for HA-JNG06-90/Japan, AB279735 for HAJ85-1/Japan, AB300205 for KRM238G59/Japan, and JQ655151 for Kor-HAV-F/Korea).

Inspection of viral leakage into contaminated asari clam washing water and soaking water: Test samples: The possibility of viral leakage into asari clam washing water and soaking water was inspected twice under different sets of conditions. The samples used were collected from a package of asari clams that was different from the one involved in the infection cluster but had the

| Table 1. Epidemiological survey of patients |
|------------------------------------------|
| Patients No.                             |
| Date of eating at the restaurant         |
| Date of onset                            |
| Incubation period (days)                 |
| Clinical symptoms                        |
| Malaise                                  |
| Decreased appetite                       |
| Fever                                    |
| Jaundice                                 |
| Liver dysfunction                        |
| Hepatomegaly                             |
| Temporomandibular pain                   |
| Epidemiological survey on eating         |
| Asari clam dish                          |
| Eating                                   |
| Eating                                   |
| Eating                                   |
| No-eating                                |
| 1): Already described in ref. 5. In ref. 5, Nagano338 is patient a, Nagano340 is patient b, Nagano365 is patient c and Nagano416 is patient g. |
same expiry date.

In the first inspection, eight asari clams were randomly selected from the package (weighing 86 g in total) and placed into a sterile bag along with a small amount of sterile distilled water (DW). For uniform washing, the sample was subjected to ultrasonication at 40 kHz for 5 min using a USD2R ultrasonic generator (As One, Osaka, Japan) until the DW became slightly turbid. The treated frozen asari clams and DW were moved into a sterile bottle to which DW was added for a total volume of 500 mL. After the bottle was shaken several times, the entire volume was collected, and the collected solution was used for testing (Washing Water 1). Additionally, 500 mL of DW were added again to the sterile bottle-containing asari clams, incubated at ≤10°C for 18 h (overnight), and the entire volume was collected the following morning. The collected solution was used for testing (Soaking Water 1).

Similarly, in the second inspection eight frozen asari clams were selected (weighing 86 g in total). DW was added for a total volume of 250 mL as washing water (Washing Water 2). Additionally, 250 mL of DW were added again as soaking water, which was incubated at ≤10°C for 3 h (Soaking Water 2). The midguts of the asari clams used for the two inspections were also used as test samples.

Pretreatment of test samples, viral RNA extraction, and cDNA synthesis by reverse transcription reaction: The virus was concentrated in the washing water and soaking water in accordance with the laboratory’s diagnostic manual of poliovirus infection (14). First, either 500 mL or 250 mL of the collected DW were condensed through absorption on a negatively charged membrane. Then, 5 mL of the condensed solution was mixed with 3 mL of SDS Tris-Glycine buffer (Cosmo Bio, Tokyo, Japan). The mixed solution was incubated for 1 h and then added to a layer of 2 mL 30% sucrose. The solution was then subjected to centrifugation at 4°C and 284,400 × g for 2 h, after which the supernatant was removed. The precipitate was diluted in 200 μL of SDS Tris-Glycine buffer and used for RNA extraction. The asari clams were pretreated as described above. Viral RNA extraction from the RNA extraction sample, cDNA synthesis by reverse transcription reaction, and HAV genome detection were performed as described above.

Quantification of the number of HAV genome copies: Using the samples in which RT-PCR confirmed the amplification of HAV genomes, the number of HAV genome copies was quantified by real-time RT-PCR using a PRISM7500 system (Thermo Fisher Scientifics Inc., Waltham, MA, USA) in accordance with the Guidelines (11). The primers and probe used were selected based on the Infectious Agents Surveillance Report (1) (11). The primers and probe used were selected based on the determined nucleotide sequences of the VP1/2A region. Viral RNA extraction from the RNA extraction sample, cDNA synthesis by reverse transcription reaction, and HAV genome detection were performed as described above.

Assessment of environmental contamination caused by leaked water from the asari clam sand removal process: Water sprayed on the asari clams spread around the tray in a concentric fashion (Fig. 2).

RESULTS

HAV genome detection in fecal and asari clam samples collected from patients and kitchen employees and phylogenetic analysis: HAV genomes were detected in the four patients’ fecal samples and two asari clam samples, whereas no HAV genomes were present in the two employees’ fecal samples (Table 2) (5). Based on the determined nucleotide sequences of the VP1/2A region, the detected HAV strains were classified as genotype IA (Fig. 1) (5). The HAV strain detected from the Nagano338 patient [LC415417], Nagano340 patient [LC415418], Nagano416 patient [LC415420], and two asari clam samples (Nagano3-1_clam) [LC415421] showed 100% nucleotide homology. The Nagano365 [LC415419] patient strain had 1 nucleotide insertion in location 592 (5).

HAV genome detection in asari clam washing water, soaking water, and asari clam samples: HAV genomes were detected in the asari clam samples used in the 1st and 2nd inspections and the soaking water (Table 3). In the 1st inspection, the amount of viral RNA was 3.3 × 10^5 copies per 1 g of midgut gland and 6.0 × 10 copies per 1 mL of soaking water (Table 3). The HAV strain detected in soaking water showed 100% nucleotide homology in the VP1/2A region (619 nt) with that detected in the asari clam sample.

Assessment of environmental contamination caused by leaked water from the asari clam sand removal process: Water sprayed on the asari clams spread around the tray in a concentric fashion (Fig. 2).

DISCUSSION

Here, we investigated the possibility of viral leakage from contaminated asari clams into washing water and soaking water. Consequently, we ascertained that HAV leaks from asari clams after soaking in water for a set period of time, thus indicating that soaking water is a risk factor for secondary HAV infection.

| Sample | Number of samples | Number of positive | Genotype |
|--------|------------------|--------------------|----------|
| Feces  | Patients         | 4                  | 4        | IA       |
|        | Food handlers    | 2                  | 0        | —        |
| Asari clams | 2^1 | 2                  | —        | IA       |

1^1 Asari clams used in this examination had the same expiry date as asari clams consumed by the patients, although the packaging differed.

2 Asari clams used in this examination had the same expiry date as asari clams consumed by the patients, although the packaging differed.

DISCUSSION

Here, we investigated the possibility of viral leakage from contaminated asari clams into washing water and soaking water. Consequently, we ascertained that HAV leaks from asari clams after soaking in water for a set period of time, thus indicating that soaking water is a risk factor for secondary HAV infection.
Also, there is a possibility that viral quantity was low, since no HAV was detected in washing water, it appeared that the surfaces of asari clams used for testing were not contaminated. However, the fact that HAV leaked into soaking water suggests that the asari clams might have thawed during transportation or storage and leaked HAV had contaminated the asari clam surfaces. When washed, the contaminated asari clam surfaces transmitted HAV to washing water and then to soaking water, further increasing the risk for secondary contamination. Therefore, foodstuffs can directly or indirectly transmit virus to patients via cooking utensils and other tools. In our inspections, no HAV was detected in washing water. Moreover, interviews with the kitchen employees confirmed that the asari clams were only washed for about 20 sec. If the asari clams were the cause of the viral contamination, those provided to the patients might have been contaminated on the surface. Because surface contamination and viral leakage from asari clams are likely to vary depending on the frequency of freezing/thawing and the type of washing/soaking water, further investigation is necessary.

Concerning viral leakage into soaking water, although there are no reports on live or frozen asari clams, Kumagai et al. detected norovirus from water contained in a package of commercial raw oysters (15). Moreover, the Food Safety Commission of Japan advises caution during the handling of shell-less oysters because...
internally accumulated virus can be transmitted from contaminated oysters into filler water within the package through absorption and discharge of seawater (16). Various viruses have been isolated from the midgut gland of bivalves (6–10). In fact, the midgut glands of the asari clams used in our inspections carried sapovirus, group A rotavirus, and astrovirus in addition to HA V (data not shown). Viral leakage may determine the life or death of bivalves, quantity of virus accumulated in the midgut gland, washing method and soaking method, and differences in the bonding strength with midgut gland cells (17). Further studies are necessary to clarify the influence of these factors on viral leakage.

Although not applicable in this case because frozen asari clams were involved, the sand removal process, which is indispensable for cooking live asari clams, may spread viral contamination. We have not confirmed reproducibility, but our investigations ascertained that the water sprayed on the asari clams spread over a wide area. If soaking water is contaminated with virus, that contamination can be transmitted to unheated salads and raw vegetables and cooking utensils, which then become the causes of food poisoning. To prevent contamination, performing the sand removal process in a closed space using plastic wrap and similar tools may be an option. However, inappropriate disposal of wraps used for performing the sand removal process may also lead to contamination.

Seroepidemiological surveys suggest that the incidence of HAV in Japan has been low since 1960 (2,18). However, other Asian countries have a perennial presence of HAV. Because most Japanese people aged younger than 50 years do not possess antibodies against HAV (2,13), there is concern that travel to an HAV-endemic area and consumption of contaminated food, including that imported from an HAV-endemic area, are major risk factors for HAV in Japan (2). In fact, widespread outbreaks of hepatitis A occurred in 2010 and 2014 (13,19,20). Therefore, it is crucial to inform the public about the inherent risks of foodstuffs and the risks associated with the handling of foodstuffs. It is also essential to carry out adequate hygiene control measures such as washing hands, heating ingredients (≥ 85°C for ≥ 1 min for HAV), and disinfecting cooking utensils.

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Conflict of interest None to declare.

REFERENCES

1. National Institute of Infectious Diseases and Tuberculosis and Infectious Diseases Control Division, Ministry of Health, Labour and Welfare. Hepatitis A in Japan. 2010–2014, as of November 2014. Infectious Agents Surveillance Report. 2015;36:1-2.
2. Kiyohara T, Sato T, Totsuka A, et al. Shifting Seroepidemiology of hepatitis A in Japan, 1973–2003. Microbiol Immunol. 2007;51:185-91.
3. Ministry of Health, Labor and Welfare. National Epidemiological Surveillance of Infectious Diseases (NESID) Program. Available at <https://www.niid.go.jp/niid/ja/survei/2085-idwry/data/7310-reportja2016-20.html> Accessed February 22, 2018. Japanese.
4. Furuta A, Akiyama M, Kato Y, et al. A food poisoning outbreak caused by purple Washington clam contaminated with norovirus (Norwalk-like virus) and hepatitis A virus. Kansenshogakukai Zasshi. 2003;77:89-94. Japanese.
5. Kobayashi H, Wada Y, Kitano K, et al. An outbreak of hepatitis A in Nagano Prefecture, 2017. Infectious Agents Surveillance Rep. 2018; 39:25-6. Japanese.
6. Nishida T, Nishio O, Kato M, et al. Genotyping and quantitation of noroviruses in oysters from two distinct sea areas in Japan. Microbiol Immunol. 2007;51:177-84.
7. Hansman GS, Oka T, Li TC, et al. Detection of human enteric viruses in Japanese clams. J Food Prot. 2008;71:1689-95.
8. Charles PG, Sagar MG. Detection and Occurrence of Enteric Viruses in Shellfish: A Review. J Food Prot. 1978;41:743-54.
9. Nakagawa-Okamoto R, Arita-Nishida T, Toda S, et al. Detection of multiple sapovirus genotypes and genogroups in oyster-associated outbreaks. Jpn J Infect Dis. 2009;62:63-6.
10. Nishida T, Kimura H, Saitoh M, et al. Detection, quantitation, and phylogenetic analysis of noroviruses in Japanese oysters. Appl Environ Microbiol. 2003;69:5782-6.
11. National Institute of Infectious Diseases. Guideline for the Diagnosis of Hepatitis A Virus (2006). National Institute of Infectious Diseases, Tokyo, Japan. Available at <http://www0.nih.go.jp/niid/reference/HA-manual.pdf> Accessed July 25, 2018. Japanese.
12. Yoshida T, Nakazawa H. An outbreak of gastroenteritis caused by norovirus: suspected due to dust transmission. Kansenshogakukai Zasshi. 2010;84:702-7. Japanese.
13. Ishii K, Kiyohara T, Yoshizaki S, et al. Epidemiological and genetic analysis of a 2014 outbreak of hepatitis A in Japan. Vaccine. 2015; 33:6029-36.
14. National Institute of Infectious Diseases. Laboratory’s diagnostic manual of poliovirus infection. National Institute of Infectious Diseases, Tokyo, Japan. Available at <https://www.niid.go.jp/niid/images/lab-manual/polio.pdf> Accessed July 25, 2018. Japanese.
15. Kumaegi K, Ishikawa K, Mikami T, et al. Investigation of Norovirus Contamination in Commercial Raw Oysters—Targeting Midgut Glands and Filler Water in Packages—. Journal of Aomori University of Health and Welfare. 2008;9:74-5. Japanese.
16. Food Safety Commission of Japan: Risk profiling for food safety impact and future challenges—norovirus in food—. 2010;4. Available at <http://www.fsc.go.jp/sonota/risk_profile/risk_norovirus.pdf> Accessed July 25, 2018. Japanese.
17. Le Guyader F, Loisy F, Atmar RL, et al. Norwalk virus-specific binding to oyster digestive tissues. Emerg Infect Dis. 2006;12:931-6.
18. Kiyohara T, Satoh T, Yamamoto H, et al. The latest seroepidemiological pattern of hepatitis A in Japan. Jpn J Med Sci Biol. 1997;50:123-31.
19. Ishii K, Kiyohara T, Yoshizaki S, et al. Epidemiological and genetic analyses of a diffuse outbreak of hepatitis A in Japan 2010. J Clin Virol. 2012;54:286-1.

Fig. 2. Evaluation of water sprayed from asari clams by using pigment.