Research Article

A Case of Hepatitis A Virus Detection in River Water Flowing into Tidal Flats

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Abstract

In 2019, an outbreak of hepatitis A was reported in South Korea due to the ingestion of salted shellfish contaminated with hepatitis A virus. In a survey of the contamination of salted shellfish, hepatitis A virus (HAV) type IA was detected, and it was confirmed that it originated from clams produced in one of the clam farms located in the western parts of the country. The aim of this study was to determine the cycle of human infection by HAV contaminating

the water flowing into the environment. We selected a clam farm that is one of the largest producers of clams in South Korea and tested for the presence of HAV in the river water flowing directly into the vicinity of that farm. An HAV detection test was performed on river water collected from 15 points in three areas of the selected clam farm. HAV was detected in one sample of the collected river water, and its genotype was confirmed as IA, which is the most common genotype causing infection in humans and the same genotype that is prevalent in South Korea. We confirmed that the HAV released via infection in humans contaminated the tidal flats through the river. This result might be a good example of the cycle of infection of HAV in the environment.

Keywords: Hepatitis A virus; Genotype IA; Clam; Shellfish

Abbreviations: HAV-Hepatitis A virus; RT-PCR-Real-time reverse transcription PCR

1. Introduction

Hepatitis A is an infectious liver disease caused by hepatitis A virus. In 2019, an outbreak of hepatitis A was reported in the Republic of Korea due to the ingestion of salted shellfish. Notably, the number of infected individuals dramatically decreased when all individuals were advised to stop ingesting salted shellfish. Hepatitis A is known to spread from one human to another via water and food contaminated with the virus. However, the long incubation period of this virus, which is an average of 4 weeks, makes it difficult to determine the exact route of infection [1]. Different types of food that are directly

consumed without heating, such as vegetables (e.g. lettuce, frozen strawberries) and shellfish (e.g. mussels, clams, and raw oysters), have been identified as sources of infection by hepatitis A [2, 3]. In particular, bivalve shellfish (e.g. clams), which ingest organic matter from the surrounding water, tend to harbor several pathogens in their digestive tracts and are known to be a major infectious agent causing several foodborne diseases, such as norovirus infection [4, 5]. Salted shellfish, such as clams, are a type of salted and fermented food meant for long-term storage and consumption. However, although salting is a food storage method that is used worldwide, few countries consume fermented shellfish, and there have been no reports of any hepatitis A infection from salted shellfish.

Hepatitis A virus was detected while examining the level of contamination of salted shellfish in relation to the 2019 hepatitis A outbreak. Epidemiological investigations revealed that salted shellfish was made from clams produced in a nearby tidal flat in Boryeong. Notably, we detected hepatitis A virus (HAV) in the shellfish purchased by the residents of Boryeong for the production of salted shellfish. The genotype of the virus was determined to be type IA; this is the same genotype prevalent in the Republic of Korea in 2019. Therefore, it was necessary to investigate the river water flowing into this clam-producing tidal flat to investigate the route of pollution of the salted shellfish. The aim of this study was to confirm the cycle of infection by HAV by investigating whether the virus was discharged by the infected population inhabiting Boryeong into the river water without purification, which caused the contamination of clam farms.

2. Materials and Methods

Water samples were first collected from a river close to a clam farm for analysis. A contamination survey was conducted on this farm, which is known to be one of the largest producers of clams in this area. This farm was identified as the source of salted shellfish contaminated with hepatitis A virus. Water samples were collected from points where untreated sewage water flows directly into the river water surrounding the clam farm, from streams flowing directly into the river and from streams connected to nearby farms. Samples were collected from three areas as follows: (i) sewage pipes around the tidal flat, (ii) around a river running through the center of a neighboring city, and (iii) a stream outside the city. In total, 15 points were selected from these three areas (Figure. 1).

Two water samples were collected from each point, placed in a 1-L collection bottle, and transported in separate containers to prevent cross-contamination between samples. Collection was performed three times in November. To confirm the number of infections in the area, the number of monthly reports of patients infected with hepatitis A in Boryeong in 2019 was confirmed using the Infectious Disease Web Statistical System [6].

All collected water samples were first centrifuged at 6,000 rpm for 10 min to remove gross suspended matter, and then the supernatant was collected. Residual suspended matter was removed from the supernatant using a vacuum filter/storage bottle system with a pore size of 0.45 µm (Corning Inc., Corning, NY, USA). The supernatant was then passed through a Concentrating Pipette Select water concentrator (InnovaPrep, Drexel, MO, USA), resulting in a final concentrate volume of 1 mL. Nucleic acids were extracted from the concentrated sample using a QIAGEN commercial viral RNA extraction kit (QIAGEN, Hilden, Germany).

HAV was detected using a custom-made real-time reverse transcription PCR (RT-PCR) system as recommended by ISO 15216-1:2017. The forward primer was TCA TCG CCG TTT GCC TAG, the reverse primer was GGA GAG CCC TGG AAG AAA G, and the probe was FAM-CCT GAA CCT GCA GGA ATT AA-BHQ [7]. A gene detection test was performed with two types of reactions, specifically at 40 and 45 cycles. In addition, nested PCR was performed on the VP1-VP3 (186 bp) region of the hepatitis A virus, and the genotype was confirmed by sequencing the amplified gene product [8]. For a sample in which the HAV gene was identified, reamplification of the VP1-P2B region (315 bp) was performed to analyze the relationship between this HAV and the virus that caused human infection [9]. All genetic analyses were performed using the HAVNET genotyping tool (www.rivm.nl/en/havnet) and MEGA6 program.

The virus recovery rate of the Concentrating Pipette Select (InnovaPrep) water concentrator was examined to indirectly calculate the virus detection rate in the collected water samples. The test sample consisted of norovirus-positive feces with a Ct value of approximately 16.2 by real-time RT-PCR. After inoculating the positive fecal sample (140 µL) into sterile distilled water (500 mL), the virus was concentrated to 1 mL using the Concentrating Pipette Select water concentrator (InnovaPrep). Nucleic

acids were then extracted from the concentrated sample using a QIAGEN commercial viral RNA extraction kit (QIAGEN). A PowerCheck norovirus real-time RT-PCR kit (Kogene, Seoul, Republic of Korea) was used for the gene detection test. The original sample diluted 10-fold up to 1/10,000 was used as the control. The recovery rate was calculated with the gene amplification rate of the concentrated sample based on the step-diluted original sample.
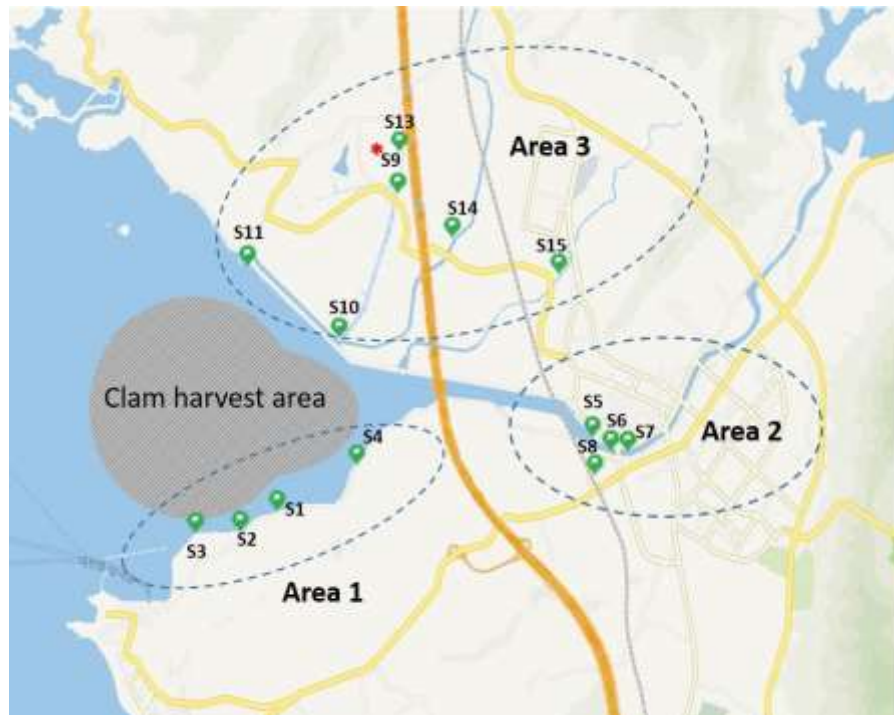

Figure 1: Collection and detection point for surveying the river water flowing into the clam production area. Sample collection was performed over three areas and 15 points to investigate the river water flowing into the tidal flats of the clam production area. The first area is the sewage pipe around the tidal flat experience center, the second area is around the river flowing through the center of Boryeong, and the third area is the streams outside the city. Hepatitis A virus was detected in S9 in the third area.

3. Results

In 2019, most nationwide cases of hepatitis A in the Republic of Korea were reported from May to September. However, in Chungnam and Boryeong, the highest incidences of infections occurred in July and August. In total, more than 2,000 new incidences of infection were reported every month from May to September 2019. However, in the province of Chungnam, more than 200 new cases per month were reported in July and August. In Boryeong, in which the clam farm is located, more than 10 cases per month were reported in July and August (Table 1). Of all these points, at point S9, the region where sewage flows from nearby villages, HAV was det-

ected in one sample. No gene detection was observed under the 40-reaction scenario; however, when the 45-reaction scenario was adopted, gene amplification was confirmed at a Ct value of 39. Hence, the genotype of the virus was confirmed to be type IA, but the HAV gene was not amplified in the experiment to analyze the relationship between this HAV and the virus that caused human infection. Notably, the virus recovery rate of the water-concentrating equipment examined using norovirus was approximately 4.5%.

4. Discussion

In this study, the genotype of the HAV detected in the river water flowing into the clam farm under investigation was found to be type IA. This is the most common genotype found in human infection cases worldwide and is the same as that found to be prevalent in 2019 in the Republic of Korea. However, some studies on river water showed that seven of nine positive cases (77.8%) in the Republic of Korea and 32 of 35 positive cases (91.4%) in South Africa were type IB [10. 11]. Therefore, most of the genotypes detected in the environment are estimated to be type IB. In addition, although no genotyping has been performed, the genotype of the HAV detected in the rivers of Ansan, as well as in various strawberry farms, was also estimated to be type IB [12, 13]. However, the presence of type IA in the water is commonly associated with human infection, proving that the HAV detected in the river water flowing into the clam farm under investigation is mostly due to human infection [14, 15]. Although the HAV detected in the water and the virus that caused human infection were not analyzed and compared, they are estimated to be significantly related.

Boryeong is a shellfish-rich area, especially with respect to clams, because of its tidal flats. The clams produced in this area are consumed not only as salted seafood and raw material, but also as ready-made food in shops and restaurants. The clam harvest area is located at a site where a river flows through Boryeong, with several streams of unpurified sewage treatment water directly flowing close to the river. In other words, most of this unpurified river water flows directly into the clam farm. Clams, the raw material of salted shellfish, were collected from July to August 2019, the time at which the incidence of hepatitis A is highest in Chungnam and Boryeong (Table 1). However, we performed our tests in November, after the outbreak had sharply declined. Notably, it is estimated that the level of contamination in the clam farm had increased from July to August when the clams were harvested.

Normally, because the concentration of HAV is generally low, a detection test is performed after a concentration process to detect the virus in water. This method, which has been recommended by the United States Environmental Protection Agency, is used worldwide and is recommended to detect viruses by concentrating 1,500 L of purified water (e.g. groundwater) and 300 L or more of highly polluted water (e.g. surface water) [16]. However, in practice, there is a limit with regard to concentrating a large amount of water. Therefore, various volumes of water, ranging from 10 to 300 L, were used to concentrate the virus in the water [17-19]. In addition, several reports have shown that the virus recovery rate (45~64%) does not largely differ depending on the properties of water, the amount of water to be concentrated, and the method of con-

centration [20]. In this study, the virus was concentrated using a Concentrating Pipette Select water concentrator (InnovaPrep) after collecting 1 L of water twice from one sampling point. The virus recovery rate of this concentration equipment is allegedly more than 50%; however, our actual experiment showed a low recovery rate of only 4.5%. In addition, in this experiment, the amount of virus recovered was expected to be low as suspended matter was removed twice. Hence, although the amount of virus present in the water was small, the possibility of detecting the viral gene was extremely low because the virus has a low recovery rate during the pretreatment process. Furthermore, in the gene amplification process, no HAV was detected in the 40-reaction scenario, but rather it was confirmed in the 45-reaction scenario. Genetic amplification is generally recommended using the 40-reaction scenario because of the RT-PCR threshold in clinical samples [7], whereas the 45-reaction scenario is sometimes recommended to increase the sensitivity of gene detection in environmental samples [11]. Therefore, even though this viral gene was detected in only one of 15 samples, it is very significant that HAV was detected in the river water.

Region		Total	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Nationwide		17,596	429	588	1,235	1,699	2,271	2,241	2,513	2,745	2,029	1,166	382	298
Chungnam	Subtotal	1,437	34	53	106	139	173	195	211	217	170	97	29	13
Province	Boryeong	58	3	0	2	1	7	8	15	16	2	2	1	1

Table 1: Patients with hepatitis A in 2019 in the region near clam farms.

5. Conclusion

In conclusion, it is difficult to conclude whether the clams harvested from the tidal flats of Boryeong were the direct cause of the hepatitis A outbreak in 2019.

However, the virus transmitted from the population inhabiting this area contaminated nearby tidal flats through rivers, and HAV of the same genotype was detected in salted shellfish and clams harvested from these tidal flats, creating a loop of continuous infection. These results can be seen as a good example of the cycle of infection of hepatitis A virus.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

Deog-Yong Lee was responsible for writing the

original draft and supervision. Seung-Rye Cho was responsible for the methodology and investigation. Su-Jin Chae and Sae Jin Oh were responsible for handling the investigation. Wooyoung Choi was responsible for writing a review and editing. Myung-Guk Han was responsible for the conceptualization of the study.

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