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Research Paper

Evaluation of Heating Conditions for Inactivation of Hepatitis E Virus Genotypes 3 and 4

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ABSTRACT

Hepatitis E virus (HEV) is a causative agent of acute hepatitis throughout the world. HEV genotypes 1 through 4 infect humans, whereas genotypes 3 and 4 (Gt3 and Gt4) also infect other animals. In developed countries, the main HEV infection route is by foodborne transmission, resulting from the consumption of undercooked meat. It is important to know the criteria for HEV control in daily cooking. In this study, we assessed the heat conditions required to inactivate HEV Gt3 and Gt4 in culture supernatants and spiked minced pork meat. HEV inactivation was determined by measuring viral RNA amplification in PLC/PRF/5 cell culture. In our cell culture assay, an inoculum containing HEV titer that is equivalent to $>10^5$ genome RNA copies can be determined as infectious. The internal temperature of pork during heating was measured to represent that achieved during cooking. Both HEV Gt3 and Gt4 were inactivated in culture supernatants heated at >65°C for 5 min and at >80°C for 1 min and in minced meat at 70°C for 5 min. Inoculated culture supernatant contained 10^8 HEV genome RNA copies (10^3 infectious units [IU]); therefore, it was indicated that HEV titer decreased >3 log IU after heating. In a comparison of Gt3 and Gt4, Gt4 showed slightly greater heat stability than Gt3. Boiling showed superior heating efficacy compared with roasting, and pork liver was slightly easier to heat than pork loin. Heating for 5 min by both boiling and roasting increased the internal temperature of pork products to more than 70°C. Although our data revealed that HEV Gt4 was slightly more heat stable than Gt3, both genotypes were inactivated by the appropriate heating conditions. Therefore, the risk of HEV foodborne infection could be mitigated by the appropriate cooking of pork meat. It is also important that both the supplier and the consumer are cognizant of the risk of HEV foodborne infection from livestock products.

Key words: Cell culture system; Heat inactivation; Hepatitis E virus; Minced pork meat

Hepatitis E virus (HEV) is one of the viral agents that cause acute hepatitis. There are an estimated 20 million infections and 56,600 hepatitis E–related deaths every year *(21)*. HEV is known to be endemic in the majority of Asian, African, and Latin American regions *(16)*. HEV is mainly transmitted via the fecal-oral route; thus, contaminated water and food can be sources of HEV infection. Several outbreaks have been reported in developing countries *(17, 20, 31)*. HEV infection rarely proceeds to chronic hepatitis and fatal infection, but individuals who are immunocompromised and pregnant women are considered to be at high risk for HEV infection. It has been reported that the HEV mortality rate is 20% in pregnant women in HEV endemic regions *(13)*. The HEV vaccine has been licensed in the People's Republic of China, and it was reported that the vaccine provides immunogenicity against HEV for up to 4.5 years *(30)*. However, the vaccine is unavailable in most countries and also is primarily applicable to HEV genotype 4 (Gt4), with data for the other genotypes being limited *(28)*; thus, avoiding infection is important to prevent HEV disease.

Human HEV has four genotypes (Gt1 to Gt4) and one serotype *(23)*. Gt1 and Gt2 infect only humans, whereas Gt3 and Gt4 are detected in humans and other animals. The main circulating genotype in developing countries is Gt1, which has been associated with hepatitis E outbreaks, whereas Gt2 has been reported in a few outbreaks in Mexico and some African countries *(10)*. Gt3 is the main genotype in developed regions, such as Europe, North America, and Japan, and Gt3 infections in developed countries are often asymptomatic and clinical infections normally present as single cases *(25)*. Gt3 is associated with foodborne infection resulting from the consumption of undercooked meat *(29)*. Gt4 is mainly detected in East Asian regions, and comparable fatality rates to Gt1 and Gt2 have been reported previously *(25)*.

Pork products and wild animal meats are known infection sources of various pathogens, including HEV, if consumed raw or undercooked. In fact, HEV RNA was detected in commercial pork in Japan, the United States, and

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Germany *(7, 27, 29)*. Some HEV cases in Japan were thought to be related to the consumption of undercooked meat in a barbeque restaurant *(12)*. HEV has also been detected in other animals, such as wild boar and deer, and in some acute hepatitis E cases, the source of infection was demonstrated to be the consumption of these meats *(18, 24)*.

Because commercial pork and game meats contain various pathogens, including HEV, disinfection in daily cooking is important for the safe consumption of animal meats. To prevent foodborne infections resulting from the consumption of undercooked meat, food safety guidelines for cooking meat have been established in various countries; for example, heating at 63°C for 30 min in Japan *(26)*, 71°C for 20 min in France *(1)*, 145°F for 3 min in the United States *(5)*, and pan frying for 5 min in Hong Kong *(4)*. However, these conditions are based on a variety of reports, and most are not considered practical for daily cooking.

There are several reports of HEV stability during heating. For example, HEV RNA showed a 2-log decrease by heating at 80°C for 1 min and a 3-log decrease at 90°C for 1 min *(22)*. HEV infectivity was decreased 2 to 3.5 log by heating between 65 and 75°C for 1 min, and HEV was completely inactivated by heating >80°C for 1 min *(14)*. HEV heated at 56°C was able to replicate in cell culture, whereas HEV subjected to frying at 191°C for 5 min or boiling for 5 min was inactivated *(8)*. In an experiment in which pigs were inoculated with heated HEV, some of the animals excreted HEV into the feces after inoculation with HEV subjected to milder heat treatment conditions than 71°C for 20 min *(2)*. Although these reports provide the conditions of HEV inactivation, additional data are required to fully establish the criteria for HEV inactivation by heating.

As mentioned above, several reports focusing on the heat inactivation of HEV are available; however, further experimental data are required to more fully determine the practical guidelines for cooking. In this report, we tested various conditions for HEV inactivation and evaluated HEV infectivity in PLC/PRF/5 cells by using the virus cell culture method. Finally, we examined the internal temperature of meat upon heating.

MATERIALS AND METHODS

Cell line and virus preparation. The human hepatoma cell line PLC/PRF/5 was prepared. Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and penicillin-streptomycin and medium 199 with 2% FBS, penicillin-streptomycin, and 10 mM $MgCl_2$ were used as a growth medium and a maintenance medium, respectively. The HEVs used in the study were Gt3 (strain 83-2, accession no. AB740232) and Gt4 (strain 121-12) isolated from pig liver. Next, the virus was propagated in PLC/PRF/5 cells and concentrated about 500 to 1,000 times by ultracentrifugation at 134,000 × *g* at 4°C for 2 h.

Heat inactivation of HEV. Typically, 300 μL of the virus culture supernatant was heated in a water bath for inactivation; however, a thermal cycler with PCR tubes was used to heat samples rapidly for 1 min. The concentrations of virus culture supernatants used were 1.74×10^8 copies per mL for Gt3 and 1.83×10^8 copies per mL for Gt4. Heating conditions for HEV Gt3 were 60 min at 56, 58, 60, and 62°C; 30 min at 58, 60, and 63°C; 10 min at 60°C; 5 min at 60, 63, 65, and 70°C; and 1 min at 63, 65, 70, 75, and 80°C. Heating conditions for HEV Gt4 were 30 min at 58, 60, and 63°C; 5 min at 63, 65, and 70°C; and 1 min at 60, 63, 65, 70, 75, and 80°C. In a comparative experiment between Gt3 and Gt4, 1 mL of each virus culture supernatant was heated at 65°C for 1, 2, and 3 min.

Food sample processing and thermal treatment. Minced pork meat (general protein and fat content was 17.7 and 17.2 g in 100 g of meat, respectively, according to the Ministry of Education, Culture, Sport, Science and Technology, Japan) was purchased at a grocery store. Five grams of minced pork meat was mixed with 3 mL of purified virus solution (10^{10} copies) to obtain the spiked minced pork meat. Each sealed food sample was incubated under controlled conditions in a water bath at three temperatures: 63, 65, and 70°C. For 63°C, incubation times of 1, 5, and 30 min were applied. For 65 and 70°C, incubation times of 1 and 5 min were applied. After heating, the samples were immediately cooled using a cold-water bath containing ice. After the various heat treatments, the samples were homogenized in phosphate-buffered saline (PBS; pH 7.2) to prepare the viral suspensions as follows: 150 mg of the spiked meat was homogenized on ice with 500 μL of 4°C PBS. After centrifugation at 12,600 × *g* for 15 min at 4°C, the supernatant was collected, aliquoted, and stored at –20°C until inoculation.

Virus infection and recovery of virus RNA. Confluent PLC/PRF/5 cells in six-well plates were used for virus inoculation. The growth medium was replaced with maintenance medium, and then the cells were inoculated with the viruses. Virus incubation was performed in duplicate for each condition. Infected plates were incubated for more than 1 h at 36.5°C, washed with PBS(–) several times, and then new maintenance medium was added. Plates were incubated at 36.5°C for about 4 weeks; during incubation, supernatants were collected and the medium was changed every 3 or 4 days. RNA extraction was performed with 140-μL supernatants by using a QIAmp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Finally, RNA was eluted with 30 μL of buffer AVE (RNase-free water with 0.04% sodium azide).

Virus quantification. The HEV RNA concentration in the medium was quantified every week. One-step quantitative real-time PCR was performed using a TaqMan Virus 1-step kit (Life Technologies, Carlsbad, CA). The Universal primer pair for HEV (5′ to 3′), JVHEVF (GGTGGTTTCTGGGGTGAC) and JVHEVR (AGGGGTTGGTTGGATGAA), and probe JVHEVP (FAM-TGATTCTCAGCCCTTCGC-TAMRA) *(15)* were used. Thermal cycles were 50°C for 5 min, 95°C for 20 s, and then 40 cycles of 3 s at 95°C and 30 s at 60°C. The transcribed RNA containing the target sequence was used as the standard for quantification. The quantification limit was 10 copies per μL, which is equal to 2,143 copies per mL in the virus culture supernatant.

Heating experiments. Pork loin, liver, and minced meat were purchased at a supermarket and cut into 2- to 3-cm cubes. Minced pork meat was shaped into meatballs (ca. 3 cm in diameter) for boiling and pork patties (ca. 1.5 cm thick) for roasting. The tested meat was heated by boiling or roasting for 5 min in this study. The meat sample was roasted by turning over every minute. During heating and 5 min after reaching the peak temperature, the internal temperature was measured by inserting a thermometer every minute. Additionally, 300 μL of maintenance medium was heated

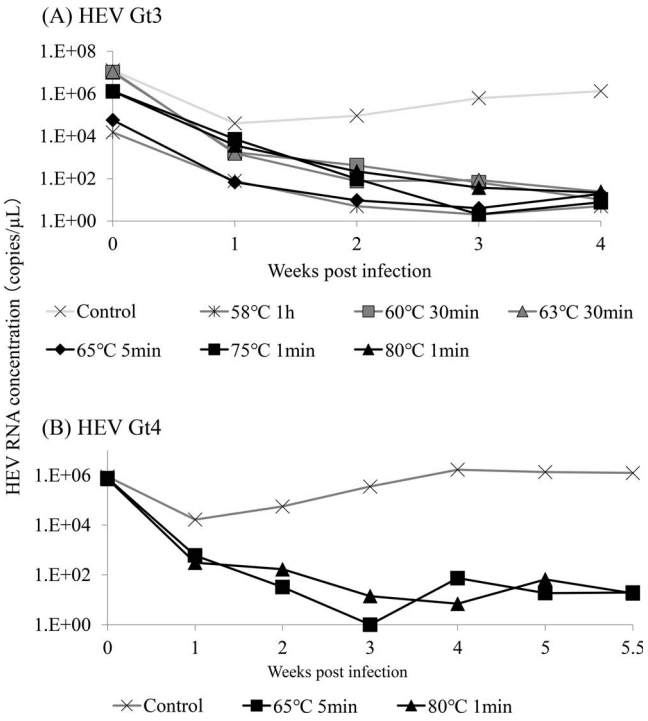

FIGURE 1. *HEV heat inactivation. Concentrations of HEV RNA in the culture supernatant during incubation are shown for HEV Gt3 (A) and Gt4 (B). HEV was heated at each condition and inoculated to PLC/PRF/5 cells. Over an ca. 4-week postinoculation period, the culture medium was collected and HEV RNA was quantified.*

at 65, 70, 75, and 80°C for 5 min, and then the temperature was measured to determine the virus supernatant temperature during heating.

RESULTS

Heat inactivation of virus. The infectivity of HEV was determined by HEV RNA titration by using cell culture and quantitative PCR. To determine the detection limit of the cell culture method, HEV was sequentially diluted 10-fold from 1×10^8 to 1×10^4 copies per mL and inoculated to PLC/PRF/5 cells. Over an ca. 4-week period, the culture medium was collected and HEV RNA was quantified. Amplification of HEV was observed after infection of 1×10^5 copies per mL of HEV, whereas amplification was not observed after infection of 1×10^4 copies per mL. These results suggested that the limit of detection in this cell culture method was between 1×10^5 and 1×10^4 copies per mL. The infectious units (IU) of tested virus culture supernatants were 1.74×10^3 IU/mL (Gt3) and 1.83×10^3 IU/mL (Gt4). Then, various heating conditions were tested. The results are shown in Figure 1, and all tested conditions are shown in Table 1. HEV Gt3 was inactivated at 58°C for 60 min, 60°C for 10 min, 65°C for 5 min, and >70°C for 1 min. HEV Gt4 was inactivated at 63°C for 30 min, 65°C for 5 min, and >75°C for 1 min. Thus, Gt3, but not Gt4, was inactivated by heating at 70°C for 1 min. A comparison of the infectivity of Gt3 and Gt4 after heating at 65°C is shown in Figure 2A. Gt4, but not Gt3, was capable of infecting

TABLE 1. *Heating conditions and results of inactivation in culture supernatant*[a]

HEV genotype and heating temp (°C)	Incubation time (min): 1	5	10	30	60
Gt3					
56					×
58				×	○
60		×	○	○	○
62					○
63	×	×		○	
65	×	○			
70	○	○			
75	○				
80	○				
Gt4					
60	×				
63	×	×		○	
65	×	○			
70	×	○			
75	○				
80	○				

[a] ○, inactivated; ×, not inactivated.

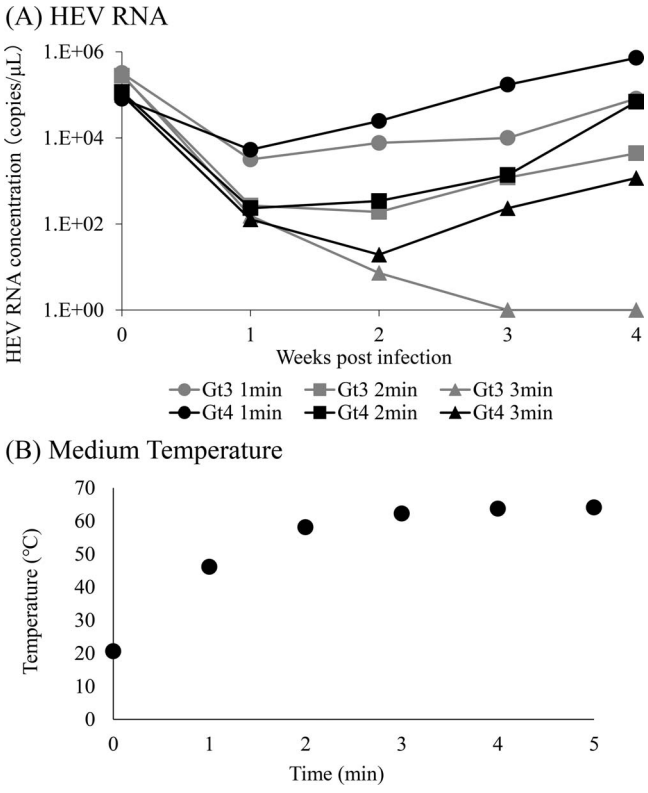

FIGURE 2. *Comparison of stability between HEV Gt3 and Gt4. (A) HEV RNA concentrations of Gt3 and Gt4 in the cell culture assay. HEV Gt3 and Gt4 were heated at 65°C for 1, 2, and 3 min, and the samples were then inoculated to PLC/PRF/5 cells. Concentrations of HEV RNA in the culture supernatant were quantified over a 4-week postinoculation period. (B) Temperature of 1 mL of medium during heating at 65°C.*

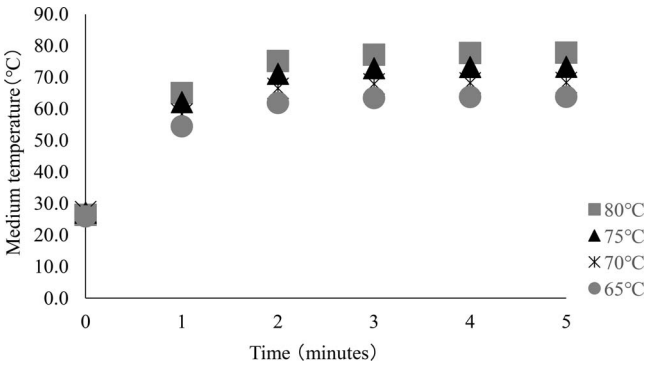

FIGURE 3. *Medium temperature during heating. The temperature of 300-μL aliquots of the medium during heating. Each symbol indicates the heating temperature by using a water bath. The 300-μL aliquots were heated for 5 min at 65, 70, 75, or 80°C.*

cells after heating for 3 min at 65°C. Changes in the medium temperature during boiling are shown in Figures 2B and 3.

Infectivity of HEV after heat treatment of minced meat. The initial contamination of the minced pork meat was estimated to be ca. 2.88×10^8 HEV genome RNA copies per g. The recovery rates were calculated to be 6.46% (Gt3) and 2.95% (Gt4) from the initial contamination and the supernatant from the untreated spiked meat. The infectious units of HEV contained in untreated meat samples were determined as 1.86×10^2 IU/g (Gt3) and 8.49×10^1 IU/g (Gt4) by detection limit with cell culture method. For 63°C, three incubation times (1, 5, and 30 min) were applied. For 65 and 70°C, two incubation times (1 and 5 min) were applied (Table 2). For HEV Gt3, inactivation was achieved by heating at 63°C for 30 min or heating at 65 and 70°C for 5 min. For HEV Gt4, inactivation was achieved by heating at 63°C for 30 min or heating at 70°C for 5 min. Thus, Gt3, but not Gt4, was inactivated by heating at 65°C for 5 min.

Heating medium. One milliliter of medium was heated at 65°C and 300 μL of medium was heated at 65, 70, 75, and 80°C in a heating block, and the change in temperature was recorded every minute up to 5 min (Figs. 2 and 3). The temperature of 300 μL of the medium seemed to almost

TABLE 2. *Heating conditions and results of inactivation in minced pork meat*[a]

HEV genotype and heating temp (°C)	Incubation time (min): 1	5	30
Gt3			
63	×	×	○
65	×	○	
70	×	○	
Gt4			
63	×	×	○
65	×	×	
70	×	○	

[a] ○, inactivated; ×, not inactivated.

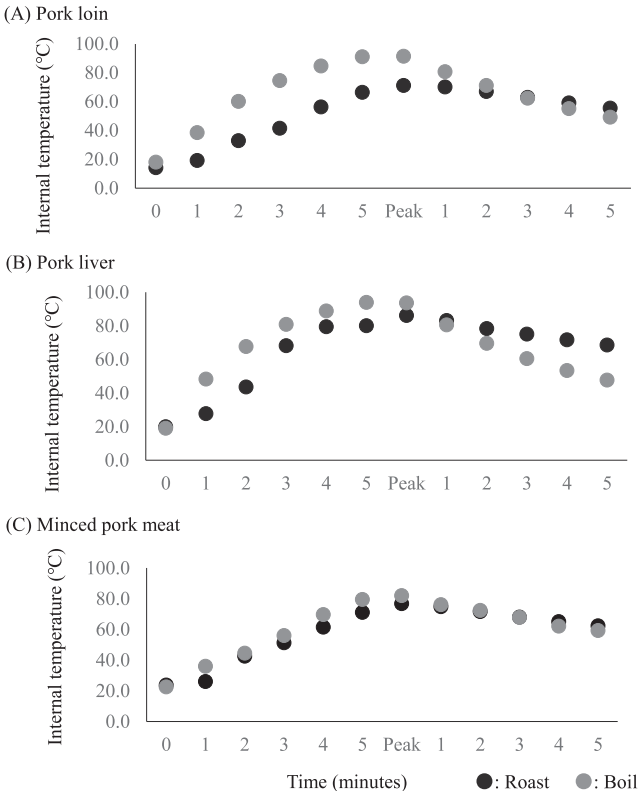

FIGURE 4. *Internal temperature of pork products during roasting and boiling. Internal temperatures of pork loin (A), pork liver (B), and minced pork meat (C) are shown. Meats were cooked by roasting or boiling for 5 min. The temperature was measured during heating and for 5 min after reaching the peak.*

plateau after heating for 3 min. The temperature of the medium heated at 75 or 80°C reached more than 60°C after 1 min. Heating at 65°C for 3 min increased the temperature of 1 mL of medium to more than 60°C.

Heating of pork products. Internal temperatures of meats during roasting and boiling are shown in Figure 4. The internal temperature continued to increase after heating was stopped; the temperature was measured for 5 min after the peak. The time from when heating was stopped to the peak temperature was within 2 min. The internal temperatures were increased using both methods to >70°C as the peak temperature. Even for the roasted pork loin, the internal temperature was maintained at >70°C for at least 1 min. In a comparison of roasting and boiling, the boiled meat reached a higher temperature than the roasted meat with 5 min of heating. However, the roasted meat maintained a high internal temperature for a long time compared with the boiled meat. Meanwhile, in a comparison of pork loin and liver, liver was easier to heat than pork loin. Finally, part of the cross section of pork loin was still red after 5 min of heating. The peak temperature of minced pork meat subjected to boiling was lower than that of the other pork products in this experiment.

DISCUSSION

According to the results, inactivation of HEV Gt3 was accomplished by conditions >65°C for 5 min and >75°C

for 1 min in the culture supernatant and HEV-contaminated pork meat. In contrast, inactivation of Gt4 in minced meat required >70°C for 5 min. Gt4 was also inactivated in the culture supernatant by heat treatment at 80°C for 1 min. In a previous report, HEV RNA decreased 2 log at ≥80°C and 3 log at ≥90°C for 1 min *(22)*. Others have reported the absence of viral infectivity by heating at 60°C for 1 h, >80°C for 1 min, and 70°C for 2 min *(6, 14)*. In an inoculation experiment with pigs, HEV was inactivated by heating at 71°C for 20 min, but not at 71°C for 5 min *(2)*. Although HEV Gt3 and Gt4 were inactivated at 70°C for 5 min, our results are generally consistent with these data. The experiment involving heating of the medium showed that the temperature of the medium needed to reach ca. 60 to 65°C for HEV inactivation. This implied that heating virus particles at greater than 60 to 65°C was needed to inactivate HEV in a short time.

Differences in the thermal stability of HEV Gt3 and Gt4 were indicated in this study. Previously, the stability of several strains belonging to Gt1 and Gt2 at 56°C for 1 h showed little difference *(6)*. It is possible that differences in the stability of viral particles among genotypes and strains affect HEV heat resistance. In addition, it seems that Gt4 can infect cells at a low titer, because there was a difference in the detection limit of our cell culture assay between Gt3 and Gt4. However, as mentioned above, HEV Gt4 was also inactivated by heating at 65°C for 5 min and at 80°C for 1 min in the culture supernatant and at 70°C for 5 min in the minced meat. As these conditions are not considered extreme, it is not likely that differences in strain stability and infectivity are critical for daily cooking.

In the experiment with spiked minced pork meat, heating at 65°C for 5 min was not sufficient to inactivate HEV Gt4. There are two possible reasons for this result: one reason is that the internal temperature did not reach a sufficient level, and the other reason is that the surrounding viral environment (e.g., fat content) might affect virus stability.

With regard to the internal temperature of pork products, boiling for 5 min increased the temperature to >80°C and maintained it at >75°C for longer than 1 min. Even the internal temperature of a meatball reached 82.0°C. In contrast, roasting for 5 min increased the temperature up to 74.5°C (pork loin), 76.1°C (liver), and 76.7°C (pork patty). The peak internal temperature with roasting was lower than that with boiling; however, the temperature of roasted meat was maintained for a longer time compared with that for boiled meat, and the temperature was >65°C for at least 3 min. In reference to the virus inactivation experiment with supernatant, these temperatures are likely sufficient to inactivate HEV. Moreover, Feagins et al. *(8)* reported that frying and boiling for 5 min inactivated HEV in an inoculation experiment with pigs. Even after heating for 5 min, some red parts were observed inside the meat in this experiment. If complete heating of the inside of the meats is confirmed by color observation, the temperature should reach levels sufficient to inactivate HEV. The color of the cross section of meat can be used as a meat safety indicator in households where a proper thermometer is not available.

We observed temperature differences in the different kinds of meat and with the two cooking methods. Because the shape of the minced pork meat was different and it was difficult to make comparisons with other pork meat, we focused on differences in the internal temperatures of pork loin and liver. The fat contents of pork loin (12.96 g/100 g) and liver (3.65 g/100 g) can be found on the U.S. Department of Agriculture's Web site (https://ndb.nal.usda.gov/ndb/search/list). It has been reported that fat increases the stability of hepatitis A virus in skim milk and cream against heat treatment *(3)*. In addition, our results suggested that it is likely to be more difficult to heat pork loin than liver. However, the specific heat values of pork with different proportions of fat were reported, and the fat content did not increase the specific heat of pork *(19)*. The factors responsible for differences in heating difficulty among different kinds of meat remain unknown, and the effect of fat on virus stability in foods is also unclear. Clarification of these effects on virus stability would have safety benefits with respect to food consumption.

There are some limitations in this study. First, our experiment determined HEV infectivity by using HEV RNA concentrations in cell culture for 4 weeks. With increasing an incubation period, very small amounts of infectious viral particles can be amplified to a detectable level. Second, we tested only one strain each for Gt3 and Gt4, and these viral strains were cell culture–adapted strains. It is possible that the viruses used in this study were not representative of wild-type strains of these genotypes. However, results using high-temperature treatment, for example 80°C, produced almost the same inactivation result among strains compared with heating at low temperatures such as 60°C. In addition, it is necessary to investigate the infectivity of HEV by using actual spiked pork meat to evaluate the effect of the surrounding environment on HEV in meat.

Recently, HEV has been considered to be an important pathogen associated with foodborne infections. It was reported that HEV was detected not only in pork meat but also cow's milk *(11)*. These kinds of popular foods, even at low pathogen contamination rates, occasionally cause large foodborne infections because they are consumed over a wide area and at a high frequency. Heating is an effective way to eliminate pathogens; however, focusing solely on the heat treatment of pork is insufficient to prevent foodborne infection. The risk remains that viruses can be transferred through cooking tools, such as cutting boards and knives, used in preparing contaminated foods *(9)*. Cooking tools should be cleaned or changed after coming into contact with raw meat to prevent infection. To ensure the safe consumption of food products from livestock, it is necessary that the supplier make every effort to prevent foodborne infections. Moreover, it is important that individual households are informed of the risks associated with the consumption of livestock products.

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