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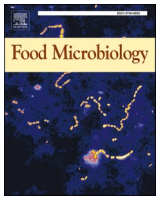

Persistence of hepatitis E virus (HEV) subtypes 3c and 3e: Long-term cold storage and heat treatments

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ABSTRACT

Hepatitis E virus (HEV) is the causative agent of foodborne infections occurring in high income countries mainly by consumption of undercooked and raw pork products. The virus is zoonotic with pigs and wild boars as the main reservoirs. Several studies proved the presence of HEV-RNA in pork liver sausages, pâté and other pork by-products. However, the detection of HEV nucleic acids does not necessary correspond to infectious virus and information on the persistence of the virus in the food is still limited. To which extent and how long the virus can survive after conventional industrial and home-made conservation and cooking procedures is largely unknown. In the present study, we investigated the persistence of two subtypes of HEV-3, by measuring the viral RNA on cell supernatant of infected A549 cells, after long-term storage at +4 °C and −20 °C and after heating for short or long-time span. Results confirmed that either low temperature storage (+4 °C) or freezing (−20 °C) do not influence the survival of the virus, and only a moderate reduction of presence of its RNA after 12 weeks at +4 °C was observed. To the other side, heating at 56 °C for long time (1 h) or at higher temperatures (>65 °C) for shorter time inactivated the virus successfully.

1. Introduction

The hepatitis E virus (HEV) is one of the main cause of hepatitis in humans worldwide. Depending on the genotypes, the symptoms and sequelae can be different, with high mortality rate in pregnant women, mainly in low income countries, and with chronic hepatitis and extra-hepatic manifestations in high-income countries. The genotypes HEV-1 and HEV-2 only infect humans and circulate in low-income countries. Zoonotic genotypes HEV-3 and HEV-4 infect both humans and several animal species, including pigs, wild boars and other less frequent hosts, such as deer and rabbits (Ruggeri et al., 2013; Smith et al., 2020). In the European countries, the HEV-3 genotype is prevalent in both domestic pigs and wild boars, causing most human sporadic cases and small clusters, which have been reported in several countries (Ricci et al., 2017; Dalton and Izopet, 2018). In several cases, molecular and epidemiological investigations accounted for the foodborne origin of the infections establishing a link between the infection and the consumption of pork liver sausages (e.g. figatelli), undercooked food containing swine liver, deer sashimi and wild boar meat (Tei et al., 2003; Adlhoch et al., 2016; Rivero-Juarez et al., 2017). The link between the infections and food consumed was confirmed by the detection of the same viral genome nucleotide sequence from patients and food (Colson et al., 2010). Furthermore, HEV RNA was detected in food at the point of sale including liver pâté, liver sausages and less frequently in meat sausages (Di Bartolo et al., 2012; Pavio et al., 2014; Moor et al., 2018; Boxman et al., 2019; Pallerla et al., 2021; Mykytczuk et al., 2017; Chatonnat et al., 2023). In the food safety risk ranking pork liver sits frequently at the top (Muller et al., 2017; Moro et al., 2021), which is to be expected since it is the organ of HEV replication, being present raw and/or dry in sausages, or partially cooked, as in pâté and other food preparations. The consumption of muscle may represent a lower risk of HEV infection, even if undercooked, since HEV does not replicate in muscles. So far, a complete foodborne risk analysis has not been conducted for HEV, given the scarce report of human cases, the unknown infectious dose, and the lack of estimation of the risk associated with the detection of the viral genome in food.

Due to the lack of an efficient cell culture system for virus cultivation and to ethical limitations for use of *in vivo* experiments, little

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information is available on HEV resistance and persistence on food after conventional industrial treatments, such as storage at room temperature, refrigeration, freezing, cooking, and other procedures involved in food preparation, such as salting, acidification and curing (Johne et al., 2016, 2021; Wolff et al., 2020a, 2020b, 2022). Some studies were conducted on HEV-3 and HEV-4 strains, proving their resistance to long storage within the temperature range of 4–10 °C (Stunnenberg et al., 2023) and up to 37 °C (Johne et al., 2016; Tanaka et al., 2007; Schielke et al., 2011). The virus can be either totally or partially inactivated by heating depending on temperature and holding time.

In vivo experiments conducted on heating effect on HEV-3 reported that depending on food preparation, if the virus was either in liver used for pâté suspensions or in liver itself, at least 72 °C for 15 min and 71 °C for 5 min, respectively were needed for total inactivation (Barnaud et al., 2012; Feagins et al., 2008). Some differences were also observed between the HEV-3 and HEV-4 genotypes, with the latter being slightly more resistant and requiring a treatment at 80 °C for 1 min for the inactivation (Imagawa et al., 2018). No data are available on differences among the different HEV-3 subtypes. A recent study observed some differences on the residual infectivity after heating between HEV-3c and -3e, but the authors highlighted the difficulty to measure little variations of residual viral infectivity (Stunnenberg et al., 2023).

In the present study, the persistence of HEV-3 during long storage at 4 °C and −20 °C was assayed, and the effect of heating at different combinations of time and temperature was evaluated on two different subtypes of HEV-3 (subtypes -3c and -3e) originated from pigs. The scope was to add a piece of information on the ability of the HEV-3 strains to persist after conventional storage or cooking conditions, assessing if any differences in the ability to survive would exist among different subtypes.

2. Materials and methods

2.1. Viral stocks, cell lines and virus propagation

Viral stocks of HEV-3 strains belonging to subtypes 3e (strain IT-12 Acc. No. OP558160) and 3c (IT-13; Acc. No. OP558157) isolated as previously described (Schemmerer et al., 2019; Ianiro et al., 2023) in Minimum Essential Medium (MEM) without FBS (MEM-M), were used in this study.

Lung carcinoma epithelial A549 cells (ATCC® CCL-185TM, Manassas, Virginia, USA) were propagated in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Life Technologies, Grand Island, NY, USA), 2 mM L-glutamine, 1% non-essential amino acids (NEAA), 100 U/mL of penicillin, and 100 mg/mL of streptomycin (P/S; Gibco Life Technologies), at 37 °C in a 5% CO_2 atmosphere.

The growth medium was removed, and cell monolayers were inoculated with treated and untreated HEV viral stocks and incubated at 34.5 °C in 5% CO_2.

The medium was removed and changed every 3–4 days (Ianiro et al., 2023).

2.2. Thermal treatments and long storage

Five hundred μL aliquots of the viral stocks of strains IT-12 (HEV-3e subtype) and IT-13 (HEV-3c subtype) containing 2.54×10^5 and 1.02×10^6 genome copies per mL (GC/mL), respectively, were conserved in 0.5 mL tubes (Eppendorf, Hamburg, Germany) and held at room temperature for 30 min. For heat treatments, a pre-warmed heat block (Thermomixer Comfort 1.5-mL, Eppendorf, Germany) filled with water for rapid heat transfer was used. The viral stocks were subjected to temperatures of 56 °C, 65 °C, and 72 °C for different treatment times (3, 6, 12 and 60 min). Heating at 93 °C was conducted for 1 and 3 min. The temperature was checked and registered using a conventional digital thermometer in control tubes containing virus free medium (Fig. 1). Two aliquots of each viral stock were added to the preheated heat block and incubated for the aforementioned time. Subsequently, they were promptly cooled in ice prior to cell infections.

For long-term storage, the virus aliquots were stored in a light protected box either in the 4 °C fridge or in the −20 °C freezer (KW, Monteriggioni, Siena, Italy). After 3, 6 and 12 weeks the aliquots were removed and stored at room temperature for 30 min and used for cell infection. For each experiment, aliquots of viral stocks not subjected to any treatments were used as positive controls (NT control virus).

Monolayers of A549 (ATCC® CCL-185TM, Manassas, Virginia, USA) in T12.5 cm^2 vented flasks were infected either with aliquots of control virus (untreated) at MOI 0.1 GC/cells or with an equal volume (approximately 0.5 mL) of treated viral stocks. After the inoculum, monolayers were rinsed and filled with MEM-M and incubated at 34.5 °C

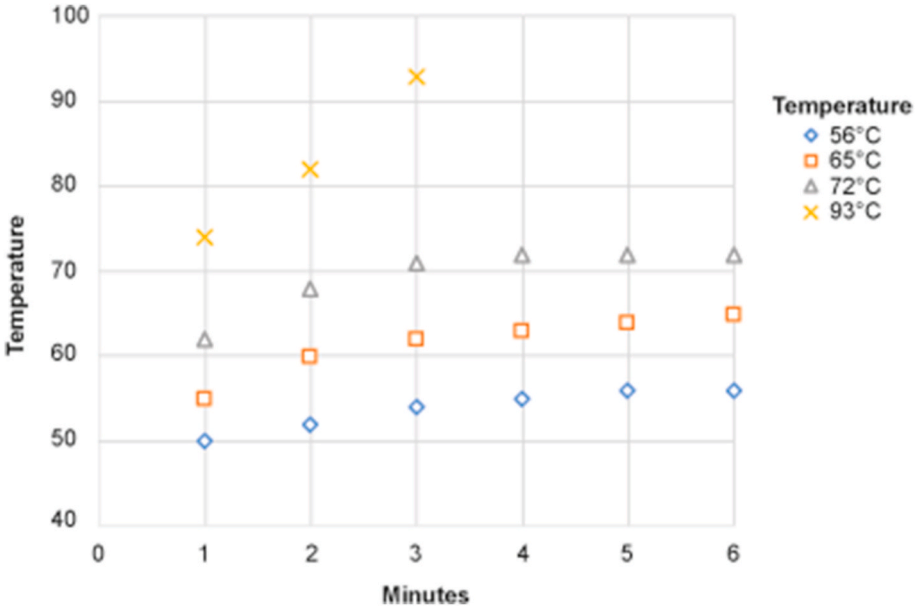

Fig. 1. Monitoring of heating of samples. It was achieved using a digital thermometer (ThermoPro) in a negative control (growth medium) simultaneously during heating of viral stocks.

in 5% CO_2 for 3–7 weeks depending on the experiments, as described below. The cell supernatants collected at 0, 1 and 3 weeks post-infection (p.i.), were centrifuged at 10,000×*g* for 30 min and stored at −80 °C, or immediately used for RNA extraction and Real-Time RT-PCR.

2.3. HEV-RNA quantification in the cell supernatant

After each thermal treatment, RNA was extracted from treated and untreated control viruses and subjected to quantitative Real-Time RT-PCR to establish the HEV GC/mL. Afterward, the HEV-RNA was also quantified in the cell supernatant of cells infected with treated and untreated viruses after 1 week, and 3 weeks and, if negative results were obtained at 3 weeks p. i., monolayers were maintained and further tested at 7 weeks p. i.

RNA was recovered using the Qiamp-Viral mini kit with the QIAcube automated sample prep platform (Qiagen, Monza, Italy), 5 μL of viral RNA were used to quantify the HEV GC/mL by Real-Time qRT-PCR (RNA UltraSense One-Step qRT-PCR System, Thermofisher Scientific, Waltham, MA, USA) on the AriaMx real-time PCR system (Agilent, Santa Clara, CA). The primers (JVHEVF; 5′-GGTGGTTTCTGGGGTGAC-3′; JVHEVR; 5′-AGGGGTTGGTTGGATGAA-3′), and probe (JVHEVP; 6-carboxyfluorescein [FAM]–5′-TGATTCTCAGCCCTTCGC-3′–6-black hole quencher [BHQ-1]) were those described previously (Jothikumar et al., 2006). For quantitative estimation of GC/mL, a standard curve was built as previously described (De Sabato et al., 2020a).

To evaluate the effect of treatments on the reduction of HEV RNA, the viral log GC reduction was calculated as follows: Log reduction = $\log_{10}$(A) - $\log_{10}$(B) where A is the number of RNA GC/mL of the control (untreated NT viral stocks) and B is the number of RNA GC/mL after treatment, collected from the cell supernatants 3 weeks p. i.

Cold treatments at 4 °C and −20 °C and heating at 93 °C for 3 min were performed in duplicate. For the other treatments 3 replicates were conducted.

2.4. Statistical analysis

The Kolmogorov-Smirnov (K–S) test for goodness of fit was used to verify the normality of the distribution of mean log10 reduction values. According to the results of the K–S test, the Mann-Whitney *U* test was used to compare the mean $\log_{10}$ reduction values between NT and each treatment duration in which HEV RNA was still present.

3. Results

3.1. Effect of long-term cold storage on HEV

Viral stocks of HEV-3 strains belonging to subtypes 3e (strain IT-12) and 3c (strain IT-13) were stored at +4 °C for 3 and 6 weeks, and a decrease of the viral RNA was only observed for HEV-3c strain IT-13 after 6 weeks ($\log_{10}$ reduction: <1.00). The reduction was more relevant when the viruses were incubated at 4 °C for 12 weeks, showing $\log_{10}$ reduction 1.42 for IT13 and $\log_{10}$ reduction 2.05 for IT12 (Fig. 2A). In all these cases, the $\log_{10}$ reduction was not statistically significant ($p > 0.05$).

No reduction of HEV RNA was observed at −20 °C after 12 weeks of storage (Fig. 2C and D).

3.2. Effect of heat treatments on HEV at 56 °C, 65 °C and 72 °C

Heating at temperatures of 56 °C, 65 °C, and 72 °C for durations of 3, 6, 12, and 60 min resulted in progressively higher levels of inactivation, measured as reduction of viral RNA in the cell supernatant 21 days p. i., proportionally with higher temperature and longer incubation times (Figs. 3, 4A and 4B).

Heat treatment at 56 °C for 3, 6 and 12 min did not influence the *in vitro* infectivity of HEV-3c strain IT-13 (Fig. 3B), as indirectly proved by the presence of viral RNA 21 days p. i., while a decrease of viral RNA was

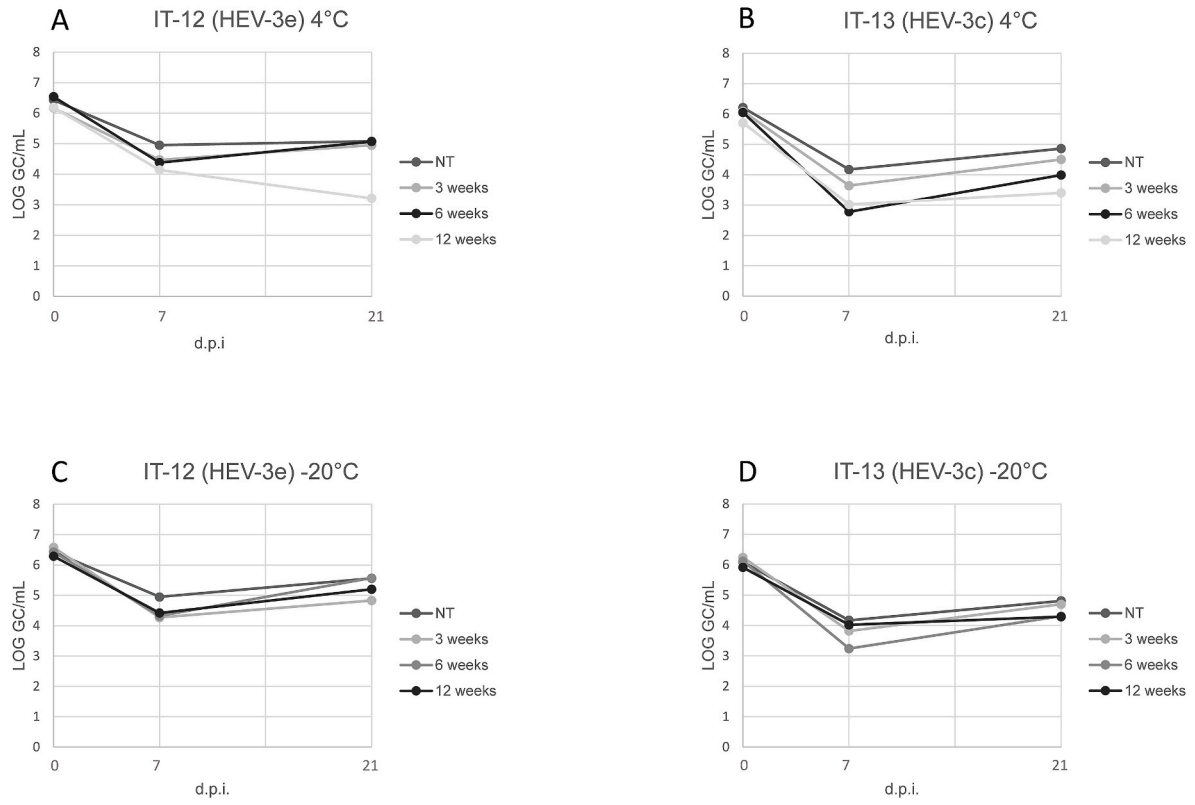

Fig. 2. Quantification of viral RNA levels in the supernatants of A549 cells inoculated with HEV after long-term cold storage at 4 °C (panel A for strain IT-12 and panel B for strain IT-13) and −20 °C (panel C for strain IT-12 and panel D for strain IT-13).

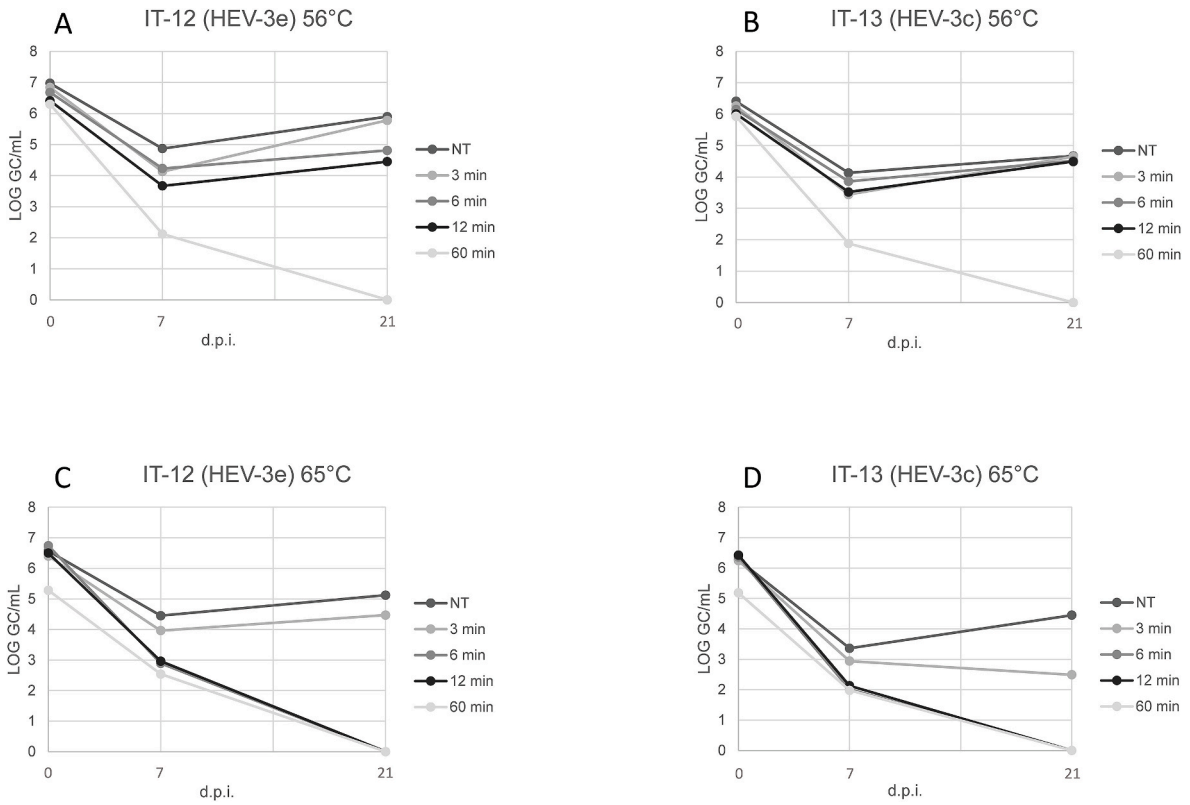

Fig. 3. Quantification of viral RNA levels in the supernatants of A549 cells inoculated with HEV after heat treatment at 56 °C (panel A for strain IT-12 and panel B for strain IT-13) and 65 °C (panel C for strain IT-12 and panel D for strain IT-13).

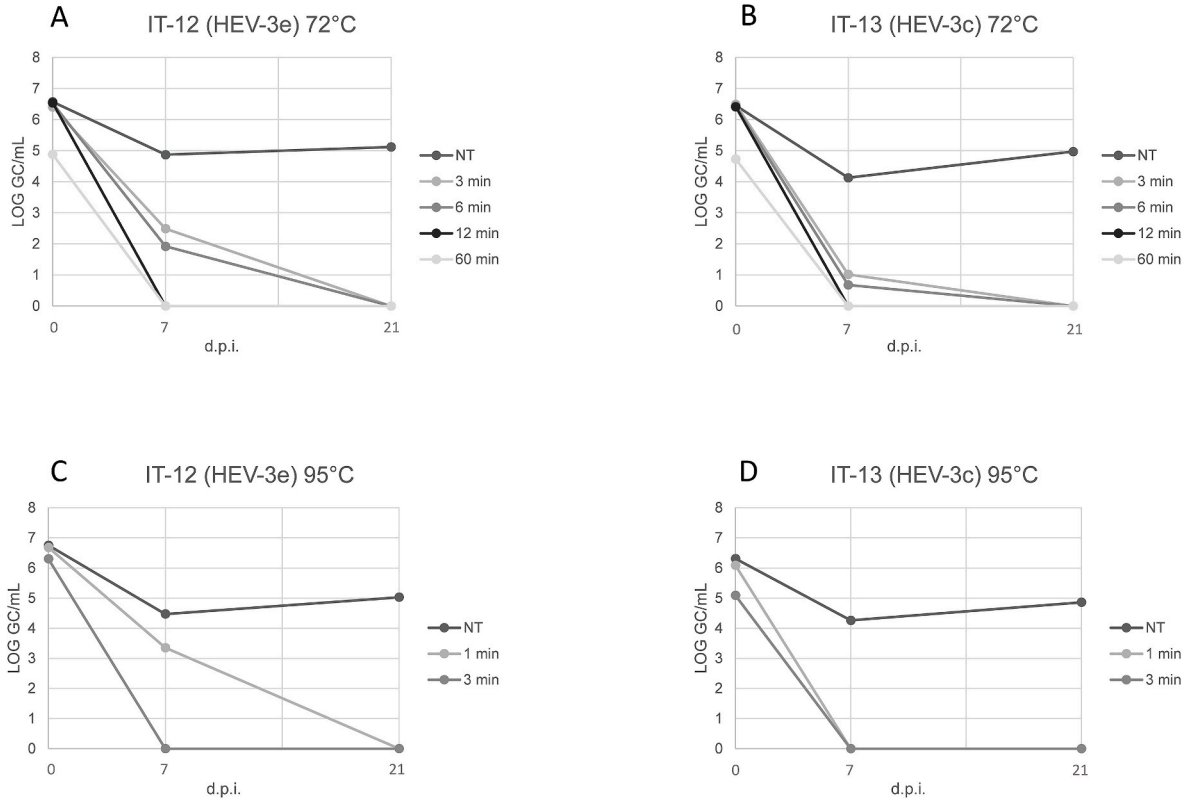

Fig. 4. Quantification of viral RNA levels in the supernatants of A549 cells inoculated with HEV after heat treatment at 72 °C (panel A for strain IT-12 and panel B for strain IT-13) and 93 °C (panel C for strain IT-12 and panel D for strain IT-13).

only observed for the HEV-3e strain IT-12 when treated at 56 °C for 6 min (log_{10} reduction: 1.09; $p > 0.05$) and 12 min (log_{10} reduction: 1.45, $p = 0.016$; Fig. 3A).

Treatments at 65 °C showed a significant log_{10} reduction of 0.72 ($p = 0.008$) and 1.97 ($p = 0.016$) of HEV-3e and -3c strains, respectively, when heated for 3 min, confirmed by the presence of viral RNA after the heat treatment. Conversely, both strains resulted totally inactivated when treated at 65 °C for 6 and 12 min (Table 1; Fig. 3C and D), as proven by the absence of detectable viral RNA in the supernatants of the infected cells even after 7 weeks of *in vitro* cultivation.

Heat treatment of viral stocks at 72 °C resulted in the total inactivation of both strains after 3, 6 and 12 min, as revealed by absence of detectable HEV-RNA in the cell supernatant 3 weeks p. i. (Table 1; Fig. 4A and B). To confirm the complete inactivation of the virus, when no residual HEV-RNA was observed on cell culture supernatants harvested 3 weeks p. i., additional test on cell supernatants was conducted at 7 weeks p. i. (data not shown) always confirming the absence of detectable HEV-RNA.

As showed in Table 1, no residual viral RNA was observed either for HEV-3e or HEV-3c after incubation at 56 °C, 65 °C and 72 °C for 60 min (log_{10} reduction range: 4.46–5.90), neither after 7 weeks p. i. (data not shown).

3.3. Heat inactivation of HEV at 93 °C

The viral strains were also subjected to heating at 93 °C for 1 and 3 min (Fig. 4C and D). No residual HEV-RNA was observed from both strains treated for 1 and 3 min at 93 °C (log_{10} reduction: 4.86 for IT-13 strain and 5.03 for IT-12). The assays further confirmed absence of detectable viral RNAfor both strains even at 7 weeks p. i..

3.4. Effect of heat treatments on HEV-RNA

HEV-RNA was also estimated as GC/mL in all samples after treatments before inoculation on cell monolayers (Figs. 2–4, 0 days p. i.) and from cell supernatants harvested 7 days p. i. (Figs. 2–4). The quantity of HEV-RNA was stable (no differences with the NT control virus) after each treatment before inoculation (Figs. 2–3A, 3B; 0 days p. i), only after 1 h at 65 °C and 72 °C and after 3 min at 93 °C a reduction of the HEV-RNA was observed, ranging between 1 and 2 log_{10} reduction (Fig. 3C and D, 4).

Data on HEV-RNA estimation at 7 days p. i. showed a decrease in the HEV-RNA quantity (GC/mL in the cell supernatants) in the first 7 days of cultivation (Figs. 2–4; 7 days p. i). The reduction observed at 7 days post-infection was evident in both the control virus and cells infected with treated viruses. This reduction was either followed by a subsequent decrease, resulting in the absence of detectable HEV-RNA in the infected cell supernatant at 21 days p. i. (i.e. 93 °C for 1 and 3 min, as shown in Fig. 4C and D), or by an increase in viral RNA levels measured in the cell supernatant at 21 days p. i., indicating viral growth (Figs. 2–3).

4. Discussion

HEV-3 has recently been recognized as an emerging virus causing foodborne infections in high-income countries. The wide detection of HEV-RNA in pork and pork preparations, like pâté and liver sausages (Muller et al., 2017; Moro et al., 2021), poses the serious questions on the ability of the virus to persist in food, subsequently representing a risk for consumers.

The main question is how long and to which extent the virus persists in/on contaminated food after conventional food processing procedures applied by food industry and at home, mainly concerning cooking and refrigeration. Pork is mostly consumed cooked and its by-products, such as liver sausages or salami, are stored for long time under refrigeration and have mostly long expiration date. So far, little information is available on the HEV persistence in food since the scientific studies have been hampered by the lack of an efficient cell culture system to cultivate the virus. Recently, some studies reported survival of the viruses at +4 °C for a long period of time of up to 4 weeks (Stunnenberg et al., 2023) and even longer as assayed by measuring virus encapsidated RNA (Schielke et al., 2011).

In the present study, the ability of HEV-3e and -3c strains to survive for long time at +4 °C was confirmed with only moderate decrease of viral titer after 12 weeks storage at +4 °C. These findings are in line with the hypothesis that HEV could persist for long time in foods such as salami because the virus after salting is still stable (Wolff et al., 2020a).

In this study, no reduction in HEV- RNA level was observed at −20 °C even after long storage (12 weeks). This was expected since it is well known that viruses without envelope can persist even for longer time under freezing conditions, and, in general, low-temperature storage may not significantly decrease viral loads in food (Schemmerer et al., 2019).

This study showed that heating has a different effect on HEV-3 survival depending on combinations of time and temperature. Results obtained in this study highlighted how the increasing of temperature during heating proportionally reduces the time required to achieve the inactivation of HEV. As expected, the effect of heating on the reduction of HEV-RNA level of both virus post cultivation on cells is higher compared to refrigeration, and increases with temperature, or with the treatment duration. In our study, total inactivation was observed heating up to 93 °C, even after 1 min, as previously reported (Tanaka et al., 2007; Schielke et al., 2011).

Comparison with results from previous studies on the effects of heating resulted variable, probably due to different experimental methods to measure residual infectivity, including measurements of nucleic acids, infectivity via immunostaining, or nucleic acid measurements following cell culture. Despite these variations in outcomes, it appears that at least 55–56 °C for 60 min is needed for virus inactivation, shorter time can be also suitable at 71 °C but 20 min are the time

Table 1
Measures of viral RNA after thermal treatments by RT-qPCR on infected cell supernatants 3 weeks p.i.

	Temp °C	Viral RNA Log GC ± SD (Log GC reduction)[a]					
		Time (min)					
		NT[b]	1	3	6	12	60
IT-12 HEV-3e	56	5.90 ± 0.21	np[b]	5.78 ± 0.04 (0.12)	4.81 ± 0.10 (1.09)	4.46 ± 0.10 (1.45)	0 (5.9)
	65	5.15 ± 0.16	np	4.44 ± 0.13 (0.72)	0 (5.15)	0 (5.15)	0 (5.15)
	72	5.14 ± 0.11	np	0 (5.14)	0 (5.14)	0 (5.14)	0 (5.14)
	93	5.03 ± 0.03	0 (5.03)	0 (5.03)	np	np	np
IT-13 HEV-3c	56	4.65 ± 0.18	np	4.68 ± 0.04 (0)	4.52 ± 0.01 (0.13)	4.49 ± 0.12 (0.15)	0 (4.65)
	65	4.46 ± 0.18	np	2.49 ± 0.30 (1.97)	0 (4.46)	0 (4.46)	0 (4.46)
	72	4.97 ± 0.05	np	0 (4.97)	0 (4.97)	0 (4.97)	0 (4.97)
	93	4.86 ± 0.03	0 (4.86)	0 (4.86)	np	np	np

[a] The logarithmic reduction was determined according to follow formula: Log reduction = log_{10} (number of RNA GC/mL of the control virus) − log_{10} (number of RNA GC/mL after treatments).
[b] NT: control, virus untreated; np: not performed.

recognized as safer for total inactivation (Ricci et al., 2017). In our experiments, total inactivation of HEV was obtained with a treatment at 56 °C for 60 min and at 65–72 °C for 3 and 6 min, respectively, consistent with previous studies (Rogee et al., 2013), and demonstrating that this temperature can reduce the viral RNA, but may not achieve total inactivation if applied for shorter time (i.e. 56 °C for <12 min and 65 °C for 3 min). Conversely, in previous studies it was observed that, after exposure to 56 °C for 60 min, HEV-1 remained infectious in cell culture (Emerson et al., 2005) and in liver homogenate (Schielke et al., 2011), similarly during *in vivo* experiments HEV-3 was only partially inactivated on pâté-like preparation at 62 °C for 120 min, at 68 °C for up to 20 min (Barnaud et al., 2012). Thus far confirming that the effect of temperature is influenced by the matrix where the virus is embedded, being more resistant in the presence of fat or in the liver (Imagawa et al., 2018; Stunnenberg et al., 2023).

In this study, two subtypes of HEV-3e and -3c were cultivated and subjected to analyses. In Italy, differently from other European countries, HEV-3f is predominant in humans and pigs, followed by HEV-3e and HEV-3c (De Sabato et al., 2020b). So far, the meaning in terms of infectivity or pathogenicity between subtypes is still controversial (Li et al., 2020; Minosse et al., 2020) and we aimed to examine if any difference in their resistance to thermal treatments existed.

Differences between HEV-3e and 3c in our study were limited and did not lead one of the subtypes as more resistant. Indeed, after heating at 65 °C for 3 min the titer of HEV-3c was reduced and HEV-3e was not, confirmed by replicating 5 times the experiments. On the other side, treatments at 56 °C for 12 min showed the HEV-3c strain as more resistant with respect to HEV-3e. Instead, after other treatments, the effect on the two subtypes was similar. Overall, the fluctuating results in differences between the two subtypes do not allow us to determine if any difference among them exists.

As observed in our previous research (Ianiro et al., 2023), the immunostaining of infected cells succeed if at least log 6 GC/mL viral RNA is present in the cell supernatant. However, to achieve this titer, 9-week growth period for infected cells is needed, making the experiments impractical. Additionally, the observed marginal differences of 1–2 logs in GC/mL between supernatants of cells infected with treated or untreated (NT) viruses are insufficient for reliable distinctions in the immunostaining of cells.

This study has some limits. The virus stocks used were recovered from cell culture supernatants collected without FBS, which could have diminished the resistance of the virus, but at the same time avoided to interfere with heating by the presence of the protein fraction of serum. Nevertheless, it is important to note that the results from our experiments could represent a relatively conservative scenario. In fact, the resistance of the virus in the liver or food could potentially be higher than observed. The temperatures assayed had been achieved by heating samples which resembles cooking procedures, but which may be different if longer or shorter time is needed to reach the same temperatures which largely depends on instruments used or volumes assayed (Gamble et al., 2021).

Future experiments could be designed to explore factors linked to procedures used to assess heat effects in more detail, to achieve a more comprehensive understanding of the impact of different treatment conditions.

For each treatment, the quantity of viral RNA remained unvaried immediately after treatment before inoculation on cells, despites its absence at 3 weeks p. i.. However, three weeks post-infection, residual HEV-RNA was undetectable in the cell supernatants of cells infected with the heated virus. This could be overcome by RNAse treatments before testing, that could allow to measure encapsidated RNA, as already observed (Schielke et al., 2011). However, this result confirms that the viral RNA does not correspond to infectious virus and assessing growth of the virus on cells is more suitable for establishing the HEV-3 ability to persist.

The viral RNA present in the supernatant of cells infected with the virus does not totally correspond to the viral titer, meaning that a genome copy (GC) is not necessary equivalent to a viral particle. FFUs (Focus-Forming Units) would be the appropriate measure of infectivity. However, 21 days after the virus inoculation onto the cells and subsequent washes, RNA is still found in the cell supernatant, suggesting presence of viral replication. The Real-Time RT-PCR Ct values can be regarded as a surrogate marker for the viral load, given the limitation described above of immunostaining, favoring the use of HEV RNA measures which are easily standardized and highly sensitive. The cultivation methods need to be implemented to be less troublesome and allowing to reach higher viral titer in a shorter time.

5. Conclusions

The correct manipulation of food plays a central role in the prevention of foodborne infections. Concerning HEV, the main risk, in terms of foodborne transmission, is represented by pig liver and pork products containing liver, and the manipulation procedure (Ricci et al., 2017; Moro et al., 2021). At the basis of the risk reduction, the use of the correct cooking time and temperature to inactivate adequately the infectious virus play an important role. Understanding the effect of temperature on virus inactivation is also important to evaluate HEV environmental persistence and consequently its effect on virus transmission.

CRediT authorship contribution statement

Marina Monini: Conceptualization, Data curation, Investigation, Writing – review & editing. **Giovanni Ianiro:** Data curation, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. **Luca De Sabato:** Investigation, Software, Validation, Writing – review & editing. **Marta Bivona:** Formal analysis, Methodology, Software. **Fabio Ostanello:** Data curation, Methodology, Software, Writing – review & editing. **Ilaria Di Bartolo:** Writing – original draft, Methodology, Conceptualization, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Adlhoch, C., Avellon, A., Baylis, S.A., Ciccaglione, A.R., Couturier, E., de Sousa, R., Epstein, J., Ethelberg, S., Faber, M., Feher, A., Ijaz, S., Lange, H., Mandakova, Z., Mellou, K., Mozalevskis, A., Rimhanen-Finne, R., Rizzi, V., Said, B., Sundqvist, L., Thornton, L., Tosti, M.E., van Pelt, W., Aspinall, E., Domanovic, D., Severi, E., Takkinen, J., Dalton, H.R., 2016. Hepatitis E virus: assessment of the epidemiological situation in humans in Europe, 2014/15. J. Clin. Virol. 82, 9–16. https://doi.org/10.1016/j.jcv.2016.06.010.

Barnaud, E., Rogee, S., Garry, P., Rose, N., Pavio, N., 2012. Thermal inactivation of infectious hepatitis E virus in experimentally contaminated food. Appl. Environ. Microbiol. 78, 5153–5159. https://doi.org/10.1128/AEM.00436-12.

Boxman, I.L.A., Jansen, C.C.C., Hagele, G., Zwartkruis-Nahuis, A., Tijsma, A.S.L., Vennema, H., 2019. Monitoring of pork liver and meat products on the Dutch market for the presence of HEV RNA. Int. J. Food Microbiol. 296, 58–64. https://doi.org/10.1016/j.ijfoodmicro.2019.02.018.

Chatonnat, E., Julien, M., Jubinville, E., Goulet-Beaulieu, V., Pavio, N., Julie Jean, J., 2023. High occurrence of hepatitis E virus in raw pork liver and pork liver pâté produced in the Canadian province of Quebec. Front. Sustain. Food Syst. 7 https://doi.org/10.3389/fsufs.2023.1163507.

Colson, P., Borentain, P., Queyriaux, B., Kaba, M., Moal, V., Gallian, P., Heyries, L., Raoult, D., Gerolami, R., 2010. Pig liver sausage as a source of hepatitis E virus transmission to humans. J. Infect. Dis. 202, 825–834. https://doi.org/10.1086/655898.

Dalton, H.R., Izopet, J., 2018. Transmission and epidemiology of hepatitis E virus genotype 3 and 4 infections. Cold Spring Harb. Perspect Med. 8 https://doi.org/10.1101/cshperspect.a032144.

De Sabato, L., Amoroso, M.G., Ianiro, G., Esposito, C., De Grossi, L., Fusco, G., Barone, A., Martini, E., Ostanello, F., Di Bartolo, I., 2020a. Detection of hepatitis E virus in livers and muscle tissues of wild boars in Italy. Food Environ. Virol. 12, 1–8. https://doi.org/10.1007/s12560-019-09405-0.

De Sabato, L., Di Bartolo, I., Lapa, D., Capobianchi, M.R., Garbuglia, A.R., 2020b. Molecular characterization of HEV genotype 3 in Italy at human/animal interface. Front. Microbiol. 11, 137. https://doi.org/10.3389/fmicb.2020.00137.

Di Bartolo, I., Diez-Valcarce, M., Vasickova, P., Kralik, P., Hernandez, M., Angeloni, G., Ostanello, F., Bouwknegt, M., Rodriguez-Lazaro, D., Pavlik, I., Ruggeri, F.M., 2012. Hepatitis E virus in pork production chain in Czech Republic, Italy, and Spain, 2010. Emerg. Infect. Dis. 18, 1282–1289. https://doi.org/10.3201/eid1808.111783.

Emerson, S.U., Arankalle, V.A., Purcell, R.H., 2005. Thermal stability of hepatitis E virus. J. Infect. Dis. 192, 930–933. https://doi.org/10.1086/432488.

Feagins, A.R., Opriessnig, T., Guenette, D.K., Halbur, P.G., Meng, X.J., 2008. Inactivation of infectious hepatitis E virus present in commercial pig livers sold in local grocery stores in the United States. Int. J. Food Microbiol. 123, 32–37. https://doi.org/10.1016/j.ijfoodmicro.2007.11.068.

Gamble, A., Fischer, R.J., Morris, D.H., Yinda, K.C., Munster, V.J., Lloyd-Smith, J.O., 2021. Heat-treated virus inactivation rate depends strongly on treatment procedure: illustration with SARS-CoV-2. https://doi.org/10.1101/2020.08.10.242206 bioRxiv, 2020.2008.2010.242206.

Ianiro, G., Monini, M., Ammendolia, M.G., De Sabato, L., Ostanello, F., Vaccari, G., Di Bartolo, I., 2023. In vitro replication of swine hepatitis E virus (HEV): production of cell-adapted strains. Animals (Basel) 13. https://doi.org/10.3390/ani13020276.

Imagawa, T., Sugiyama, R., Shiota, T., Li, T.C., Yoshizaki, S., Wakita, T., Ishii, K., 2018. Evaluation of heating conditions for inactivation of hepatitis E virus genotypes 3 and 4. J. Food Protect. 81, 947–952. https://doi.org/10.4315/0362-028X.JFP-17-290.

Johne, R., Trojnar, E., Filter, M., Hofmann, J., 2016. Thermal stability of hepatitis E virus as estimated by a cell culture method. Appl. Environ. Microbiol. 82, 4225–4231. https://doi.org/10.1128/AEM.00951-16.

Johne, R., Wolff, A., Gadicherla, A.K., Filter, M., Schluter, O., 2021. Stability of hepatitis E virus at high hydrostatic pressure processing. Int. J. Food Microbiol. 339, 109013 https://doi.org/10.1016/j.ijfoodmicro.2020.109013.

Jothikumar, N., Cromeans, T.L., Robertson, B.H., Meng, X.J., Hill, V.R., 2006. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. J. Virol Methods 131, 65–71. https://10.1016/j.jviromet.2005.07.004.

Li, S., He, Q., Yan, L., Li, M., Liang, Z., Shu, J., Zhang, F., Wang, L., Wang, L., 2020. Infectivity and pathogenicity of different hepatitis E virus genotypes/subtypes in rabbit model. Emerg. Microb. Infect. 9, 2697–2705. https://doi.org/10.1080/22221751.2020.1858178.

Minosse, C., Biliotti, E., Lapa, D., Rianda, A., Marchili, M., Luzzitelli, I., Capobianchi, M. R., McPhee, F., Garbuglia, A.R., D'Offizi, G., 2020. Clinical characteristics of acute hepatitis E and their correlation with HEV genotype 3 subtypes in Italy. Pathogens 9. https://doi.org/10.3390/pathogens9100832.

Moor, D., Liniger, M., Baumgartner, A., Felleisen, R., 2018. Screening of ready-to-eat meat products for hepatitis E virus in Switzerland. Food Environ. Virol. 10, 263–271. https://doi.org/10.1007/s12560-018-9340-x.

Moro, O., Suffredini, E., Isopi, M., Tosti, M.E., Schembri, P., Scavia, G., 2021. Quantitative methods for the prioritization of foods implicated in the transmission of hepatititis E to humans in Italy. Foods 11. https://doi.org/10.3390/foods11010087.

Muller, A., Collineau, L., Stephan, R., Muller, A., Stark, K.D.C., 2017. Assessment of the risk of foodborne transmission and burden of hepatitis E in Switzerland. Int. J. Food Microbiol. 242, 107–115. https://doi.org/10.1016/j.ijfoodmicro.2016.11.018.

Mykytczuk, O., Harlow, J., Bidawid, S., Corneau, N., Nasheri, N., 2017. Prevalence and molecular characterization of the hepatitis E virus in retail pork products marketed in Canada. Food Environ. Virol. 9, 208–218. https://doi:10.1007/s12560-017-9281-9.

Pallerla, S.R., Schembecker, S., Meyer, C.G., Linh, L.T.K., Johne, R., Wedemeyer, H., Bock, C.T., Kremsner, P.G., Velavan, T.P., 2021. Hepatitis E virus genome detection in commercial pork livers and pork meat products in Germany. J. Viral Hepat. 28, 196–204. https://doi.org/10.1111/jvh.13396.

Pavio, N., Merbah, T., Thebault, A., 2014. Frequent hepatitis E virus contamination in food containing raw pork liver, France. Emerg. Infect. Dis. 20, 1925–1927. https://doi.org/10.3201/eid2011.140891.

Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Fernandez Escamez, P.S., Herman, L., Koutsoumanis, K., Lindqvist, R., Nørrung, B., Robertson, L., Ru, G., Sanaa, M., Simmons, M., Skandamis, P., Snary, E., Speybroeck, N., Ter Kuile, B., Threlfall, J., Wahlström, H., Di Bartolo, I., Johne, R., Pavio, N., Rutjes, S., van der Poel, W., Vasickova, P., Hempen, M., Messens, W., Rizzi, V., Latronico, F., Girones, R., 2017. Public health risks associated with hepatitis E virus (HEV) as a food-borne pathogen. EFSA J. 15, e04886 https://doi.org/10.2903/j.efsa.2017.4886.

Rivero-Juarez, A., Frias, M., Martinez-Peinado, A., Risalde, M.A., Rodriguez-Cano, D., Camacho, A., Garcia-Bocanegra, I., Cuenca-Lopez, F., Gomez-Villamandos, J.C., Rivero, A., 2017. Familial hepatitis E outbreak linked to wild boar meat consumption. Zoonoses Public Health 64, 561–565. https://doi.org/10.1111/zph.12343.

Rogee, S., Talbot, N., Caperna, T., Bouquet, J., Barnaud, E., Pavio, N., 2013. New models of hepatitis E virus replication in human and porcine hepatocyte cell lines. J. Gen. Virol. 94, 549–558. https://doi.org/10.1099/vir.0.049858-0.

Ruggeri, F.M., Di Bartolo, I., Ponterio, E., Angeloni, G., Trevisani, M., Ostanello, F., 2013. Zoonotic transmission of hepatitis E virus in industrialized countries. New Microbiol. 36, 331–344.

Schemmerer, M., Johne, R., Erl, M., Jilg, W., Wenzel, J.J., 2019. Isolation of Subtype 3c, 3e and 3f-Like Hepatitis E Virus Strains Stably Replicating to High Viral Loads in an Optimized Cell Culture System. Viruses 11. https://doi.org/10.3390/v11060483.

Schielke, A., Filter, M., Appel, B., Johne, R., 2011. Thermal stability of hepatitis E virus assessed by a molecular biological approach. Virol. J. 8, 487. https://doi.org/10.1186/1743-422X-8-487.

Smith, D.B., Izopet, J., Nicot, F., Simmonds, P., Jameel, S., Meng, X.J., Norder, H., Okamoto, H., van der Poel, W.H.M., Reuter, G., Purdy, M.A., 2020. Update: proposed reference sequences for subtypes of hepatitis E virus (species Orthohepevirus A). J. Gen. Virol. 101, 692–698. https://doi.org/10.1099/jgv.0.001435.

Stunnenberg, M., Huizen, S.C.V., Swart, A., Lodder, W.J., Boxman, I.L.A., Rutjes, S.A., 2023. Thermal inactivation of hepatitis E virus in pork products estimated with a semiquantitative infectivity assay. Microorganisms 11. https://doi.org/10.3390/microorganisms11102451.

Tanaka, T., Takahashi, M., Kusano, E., Okamoto, H., 2007. Development and evaluation of an efficient cell-culture system for Hepatitis E virus. J. Gen. Virol. 88, 903–911. https://doi.org/10.1099/vir.0.82535-0.

Tei, S., Kitajima, N., Takahashi, K., Mishiro, S., 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet 362, 371–373. https://doi.org/10.1016/S0140-6736(03)14025-1.

Wolff, A., Gunther, T., Albert, T., Johne, R., 2020a. Effect of sodium chloride, sodium nitrite and sodium nitrate on the infectivity of hepatitis E virus. Food Environ. Virol. 12, 350–354. https://doi.org/10.1007/s12560-020-09440-2.

Wolff, A., Gunther, T., Albert, T., Schilling-Loeffler, K., Gadicherla, A.K., Johne, R., 2020b. Stability of hepatitis E virus at different pH values. Int. J. Food Microbiol. 325, 108625 https://doi.org/10.1016/j.ijfoodmicro.2020.108625.

Wolff, A., Gunther, T., Johne, R., 2022. Stability of hepatitis E virus after drying on different surfaces. Food Environ. Virol. 14, 138–148. https://doi.org/10.1007/s12560-022-09510-7.