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The Effect of Temperature and Relative Humidity on the Survival of Foodborne Viruses during Food Storage

식품 저장 중 온도 및 상대습도가 식중독 바이러스 생존에 미치는 영향

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ABSTRACT

The Effect of Temperature and Relative Humidity on the Survival of Foodborne Viruses during Food Storage

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Millions of people suffer from foodborne diseases throughout the world every year, and the importance of food safety has grown in recent years, worldwide. The aim of this study was to investigate the survival of major foodborne viruses, such as hepatitis A virus (HAV) and human norovirus (HuNoV), during food storage. HAV and two surrogates of HuNoV [MS2 and murine norovirus (MNV)] were inoculated into either oysters or fresh peppers and their survival on these food matrices was measured under various temperature (4°C, 15°C, 25°C, and 40°C) and relative humidity (RH) (50% and 70%) conditions. Inoculated viruses were recovered from food samples and quantified by plaque assay at predetermined time points over 2

weeks (0, 1, 3, 7, 10, and 14 days). Virus survival was the influenced primarily by temperature. On peppers at 40°C and 50% RH, greater than 4- and 6-log reductions of MNV and HAV, respectively, occurred within 1 day. All three viruses survived better on oysters. In addition, HAV survived better at 70% RH than at 50%. The survival data of HAV, MS2, and MNV were fitted to three different mathematical models (liner, Weibull, and biphasic models). Among them, the biphasic model was optimum in terms of goodness-of-fit. The results of this study suggest that major foodborne viruses such as HAV and HuNoV can survive over prolonged periods of time with limited reduction in numbers. Because the persistence of foodborne virus was observed on contaminated foods, precautionary preventive measures should be performed.

Key words: foodborne virus, norovirus, hepatitis A virus, temperature, RH, oyster, paprika, biphasic model

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I. Introduction

Throughout the world, millions of people suffer from foodborne diseases every year, and the number of patients is predicted to increase in proportion to global warming(1-3). Foodborne diseases are transmitted via fecal-oral routes and person-to-person contact. Among the numerous enteric pathogenic viruses, human norovirus (HuNoV) and hepatitis A virus (HAV) are considered to be the most important. In recent reports(4-6), foodborne outbreaks of HuNoV and HAV were closely associated with fresh produce (e.g., leafy greens, fruits, etc.), shellfishes (oysters, clams, etc.), and ready-to-eat foods (e.g., salads, sandwiches, etc.).

HuNoV is considered to be the leading cause of foodborne outbreaks worldwide(7). However, despite the importance of public health, the inability to cultivate HuNoV in vitro makes research difficult(8, 9). Therefore, several viruses including feline calicivirus (FCV), murine norovirus (MNV), tulane virus, and bacteriophage MS2 have been proposed as surrogates of HuNoV(10-13) due to similarities in size and genome structure. The incidence rate of hepatitis A has decreased in most developed countries, but its severity in adults is increasing(14). Massive sporadic outbreaks of hepatitis A from contaminated foods have been reported worldwide, and over two million patients were infected through the consumption of HAV-contaminated oysters in China(15-18).

Viruses cannot replicate in food or water because an appropriate host is required for viral replication. After contamination occurs, the virus is subject to decay. Therefore, the survival of foodborne viruses depends on various factors such as stability of the virus, treatment of the food, and environmental conditions(19). Enteric viruses are reported to be relatively resistant to various environmental factors (e.g., low pH, heat, etc.)(20) and viral persistence has been reported in various environments (e.g., marine and soil environments) and fresh foods(21-23).

Some of the most commonly identified contaminated food items are oysters and fresh vegetables. Oysters are known to be the main food involved in both HuNoV and HAV outbreaks(14, 24, 25). The demand for salads and raw vegetables has increased markedly recent years due to growing interest in healthy foods. However, these foods are commonly consumed without any heat treatment. Therefore, it is essential to characterize the survivability of these viruses on oysters and vegetables under various environmental conditions.

In this study, we investigated the survivability of HAV and two surrogates of HuNoV, MS2 and MNV, under various temperature and relative humidity (RH) conditions. Viral survival was been characterized on either oysters or peppers, which are one of the most widely consumed vegetables. In addition, the survival results of the tested viruses under the various environmental conditions were fitted and explained using three different mathematical models.

II. Materials and methods

1. Preparation of viral stocks.

HAV and MNV were cultivated according to methods described previously(*26*). Briefly, HAV and MNV were propagated in FRhK-4 cells and Raw 264.7 cells, respectively, in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 10 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (Gibco), 10 mM sodium bicarbonate (Gibco), 10 mM minimum essential medium non-essential amino acids (Gibco), and gentamicin (50 g/L) (Gibco). FRhK-4 cells were grown to confluence, and then infected with HAV for 1 h. After 10 days of incubation at 37°C in 5% CO_2, at observation of cytopathic effect (CPE), the HAV-infected cells were treated with three freeze-thaw cycles and purified with chloroform (AMRESCO, Solon, OH, USA), followed by centrifugation at 5,000 × g for 20 min at 4°C. Supernatants were carefully collected and centrifugation was repeated until a sufficient concentration was obtained. MNV was inoculated in confluent Raw 264.7 cells for 1 h, and then incubated for 5 days until CPE was observed. After three freeze-thaw cycles, MNV-infected cells were mixed with chloroform (AMRESCO) and then centrifuged at 5,000 × g for 20 min at 4°C. Bacteriophage MS2 was propagated in *Escherichia coli* C3000. MS2 stocks (0.1 mL) were thoroughly mixed with 0.3 mL of sub-cultured *E. coli* C3000 and 30 mL of

molten tryptic soy agar (TSA) (BD, Sparks, MD, USA), and then the mixtures were poured into plates. After an overnight incubation at 37°C, the surfaces of the agar plates were washed several times with phosphate-buffered saline (PBS) to collect the virus. Each virus-containing suspension was collected in 0.1 mL aliquots and kept frozen at –70°C until use. The titers of virus stocks were quantitated by plaque assay.

2. Evaluation of the recovery efficiency of tested viruses.

In order to find the appropriate recovery methods to each of peppers and oysters, three previously reported recovery methods were systematically evaluated (*27*-*29*). These recovery methods are: *(i)* sonication using non-homogenized pepper samples; *(ii)* centrifugation using homogenized oyster samples; and *(iii)* Polyethylene glycol (PEG) precipitation using both of non-homogenized pepper and homogenized oyster samples. Each method was carried out after a predetermined amount of viral inoculation and then the recovered virus titer was subsequently quantified by either plaque assay or single agar layer. Then, recovery efficiency (%) was calculated using inoculation titer of each of viruses as 100%. All of the recovery tests were performed in triplicate. Followings are detailed procedures of each of three methods used in current experiment.

2.1. Sonication using non-homogenized pepper samples.

Sonication methods were applied only to pepper samples. As similar to described by Mcleod et al. (*27*), a piece of pepper within 20 mL PBS was sonicated for 30min at 4°C and then mixed enough for a minute. Suspensions were subject to viral plaque assay as described below.

2.2. Centrifugation using homogenized oyster samples.

Centrifugation methods were applied only to homogenized oyster samples. After inoculation, the viruses were recovered in accordance with the

modified procedure described by Kingsley et al. (*28*). Two oyster tissues and 20 mL PBS were put in a filter bag and homogenized using stomacher for 30 sec. After transferring 15mL of homogenates to a new conical tube, centrifugation was performed at 10,000 × g for 30min at 4°C. The supernatants were used as virus samples to performing plaque assay.

2.3. PEG precipitation using both of pepper and oyster samples.

Each virus was recovered referring to the literature about PEG precipitation method (*29, 30*). After inoculation step, each of a piece of pepper and two individual tissue section of oyster was put in 20 mL of elution buffer (pH 9.5) supplemented with 3% beef extract (BD), glycine 50 mM (Yakuri Pure Chemical Co., Ltd., Osaka, Japan), and Tris 100 mM (AMRESCO). Subsequently, these were placed in shaking incubator at 120 rpm for 20min at room temperature and then centrifuged at 10,000 × g for 30 min at 4°C. After being neutralized with 5N HCl (final pH was to 7.2 to 7.4), the supernatant obtained 10% PEG 6000 (Fluka, Buchs, Switzerland) and 0.3 M NaCl (AMRESCO) as final concentration. They were then performed second centrifugation at 10,000 × g for an overnight at 4°C and then final supernatants were discarded. Pellets from pepper were dissolved with 1 mL PBS, but pellets from oyster were dissolved with 3 mL. It was because pellets from oyster were much more than one from pepper.

3. Characterization of viral survival on oysters and peppers under various environmental conditions.

Peppers (*Capsicum Annuum*) were purchased from the local grocery market 1 day before the experiments to maintain freshness. Frozen oysters (*Crassostrea gigas*) (shucked) were bought in bulk from the local fish market in advance and stored at –70°C until use. Peppers were washed with running water, then cut into 2-cm squares. Frozen oysters were defrosted at room temperature and then rinsed with running water. Oysters possessing an intact midgut gland were selected as final samples for inoculation. Both peppers and oysters were placed in petri dishes and disinfected with UV radiation for 30 min. After preparation of the food samples, 100 μL of HAV (2×10^6 PFU/mL), MS2 (2×10^7 PFU/mL), or MNV (2×10^7 PFU/mL) were inoculated onto the smooth surface pepper samples. For the oysters, to minimize weight differences between samples, each sample contained two oysters. For each sample, 50 μL of virus were directly injected into a piece of midgut tissue (i.e., 100 μL per sample) using a disposable syringe. The tissue section, called the region of viral bioaccumulation, was dissected from the whole oyster and immersed in 20 mL of PBS. Virus-infected food samples were placed in a temperature- and RH-controlled environmental chamber (TH-TG-300, JEIO TECH, Daejeon, South Korea). To simulate various storage conditions, four different temperature settings (4°C, 15°C, 25°C, and 40°C) and two different RH settings (50% and 70%) were used. While infected food samples were stored in the chamber, viruses were

recovered from samples at six time-points (0, 1, 3, 7, 10, and 14 days). On the day of inoculation (day 0), viruses were recovered immediately after the inoculation step, and then used as a control for viral reduction. Viruses inoculated onto either peppers or oysters were recovered through sonication or centrifugation methods, respectively. Briefly, peppers were immersed in 20-mL PBS, and sonicated for 30 min at 4°C. For oysters, a set of midgut tissues was placed in a filter bag with 20-mL PBS and homogenized using a stomacher (Seward, West Sussex, UK) for 30 s. After homogenization, 15 mL of homogenate were transferred to a conical tube, and centrifugation was performed at 10,000 × g for 30 min at 4°C. Recovered viruses were analyzed by plaque assay. When the virus titer was below the limit of detection (LOD), the experiment was repeated using shorter recovery times (6, 12, and 24 h). The experiment was not performed with MNV at 70% RH due to mold contamination. All experiments were performed in triplicate.

4. Quantification of infectious viruses by plaque assay.

Infectious viral particles were measured by plaque assay (HAV and MNV) and single agar layer (MS2) methods, respectively. To quantify HAV or MNV, confluent cells (FRhK cells for HAV or Raw 264.7 cells for MNV) were cultured in 6-well plates. Viral suspensions recovered from food samples were serially diluted in serum free-DMEM from 10^{-1} to 10^{-5}. The cells were inoculated with 500 μL of virus for 1 h and then covered with 1.5% SeaPlaque agarose (Lonza, Rockland, ME, USA) mixed with an equal volume of plaque assay medium. MNV plaques were counted within 3–5 days. Plates containing HAV were incubated for 7 days at 37°C in 5% CO_2, followed by a second overlay of agarose containing 1% neutral red solution (Sigma-Aldrich, St. Louis, MO, USA). The virus was incubated for another 3 days and then counted. To quantify MS2, the single agar layer (SAL) method was used, following the United States Environmental Protection Agency (EPA) standard protocol(*31*). Serial 10-fold dilutions of MS2 suspensions recovered from food samples were mixed with *E. coli* C3000 and TSA, and the mixtures were poured into 150-mm petri dishes. After an overnight incubation at 37°C, counts were obtained from plates containing between 3 and 300 plaques.

5. Fitting the experimental data to three different models.

To predict the survivability of viruses based on time (days) after inoculation, modeling analysis was performed. We compared three survival models (linear, Weibull, and biphasic models) to find the model that best fit our observed survival data. Survival curves were fitted to the observed data points using SigmaPlot version 10.0 (Systat Software, San Jose, CA, USA), and predictive curves were overlaid upon the survival curves using the formula for each model. Finally, goodness-of-fit of models were evaluated by comparing parameters such as the regression coefficient (R^2) and root mean square error (RMSE). The three models described below were used for this study.

5.1. Linear model

The linear model is a general model for explaining the inactivation of microorganisms at a constant temperature over time(*32*) . The equation for the linear model follows:

$$\log \frac{N_t}{N_0} = -\frac{t}{D}$$

where N_0 and N_t denote the initial virus titer (PFU/mL) and the virus titer after an elapsed time (PFU/mL), respectively; t is exposure time (days); and D is the D-value (decimal reduction time), which is the time required to inactivate 90% of the virus, indicating the thermal resistance of a microorganism(*33*).

5.2. Weibull model

Microbial survival curves may be non-linear, having a sigmoidal shape with a shoulder and tailing. Several models have been proposed for the adequate prediction of survivability of microorganisms. The Weibull distribution is a well-known non-linear model with the following equation(*28, 33*):

$$\log \frac{N_t}{N_0} = -bt^n$$

where *t* is exposure time (days); and *b* and *n* represent scale parameter and shape parameter, respectively. The main advantage of the Weibull model is that is describes both downward concave (shoulder) survival curves (n > 1) and upward concave (tailing) survival curves (n < 1).

5.3. Biphasic model

The biphasic model proposed by Kamau(*34*) describes inactivation of microorganisms in two fractions. This model applies a logistic equation to fit linear and nonlinear survival curves. The model also assumes that microorganism numbers decrease exponentially throughout the two phases, and that the rate of decline of each phase is independent. The equation of biphasic model is as follows:

$$\log \frac{N(t)}{N_0} = \left(log \frac{2f}{1 + e^{b_1 t}} + \frac{2(1-f)}{1 + e^{b_2 t}} \right)$$

where f represents first fraction on the survival curve and b_1 is the specific death rate in the first slope; $(1 - f)$ denotes the second fraction on the survival slope and b_2 denotes the specific death rate in the second slope; and t is exposure time (days)(*35*).

6. Statistical Analysis.

Means and standard deviations of obtained data were calculated using Microsoft Excel 2010 (Microsoft Corporation, USA).

III. Results

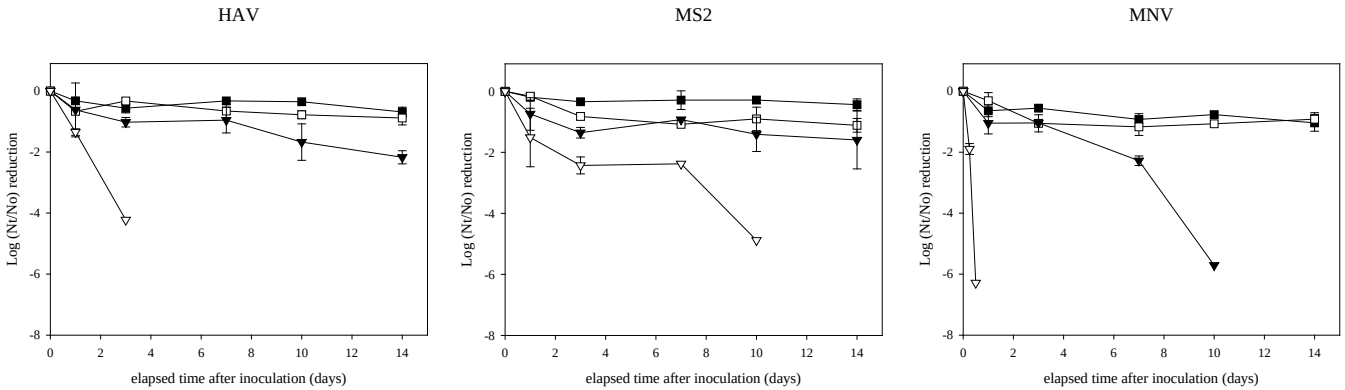
1. Evaluation of recovery efficiency.

As preliminary test, recovery efficiency was evaluated. Among tested method, PEG precipitation produced the best efficiency of viral recovery from peppers (HAV: 390%; MS2: 492%; MNV 198%). Recovery efficiencies on pepper using sonication were 21% (HAV), 45% (MS2), and 61% (MNV). In case of oyster, the recovery efficiencies using PEG were 82% for HAV, 150% for MS2, and 22% for MNV. Recovery efficiencies on oysters using centrifugation were similar to sonication method, which were 21% for HAV, 47% for MS2, and 33% for MNV. The recovery efficiencies of PEG were higher than other two methods but presented a wide difference between oyster and pepper. Differences of recovery efficiency between PEG precipitation and other two methods were due to the presence of concentration step. PEG precipitation method include concentration step after elution unlike the other two methods. Based on the recovery efficiency, time-consuming, the consistency of viral recovery from tested food items, we determined the centrifugation for oyster and sonication for pepper as elution methods for each food.

2. Inactivation curves of viruses under different temperature and RH.

The survival kinetics of HAV, MS2, and MNV on oysters and peppers under different temperature and RH conditions are described in Figures 1 and 2, respectively. Regardless of RH or the inoculated food, all tested viruses survived best at the lowest temperature (4°C) and were inactivated most at the highest temperature (40°C). On oysters, a less than 1-log reduction of both HAV and MNV occurred at 4°C, even after 14 days. However, a greater than 5-log reduction of MNV occurred on peppers at 4°C. MNV showed the shortest survival duration on peppers at all temperatures, compared to the other viruses. Viral survival was better on oysters than peppers, regardless of temperature conditions; all tested viruses, particularly MNV, survived significantly better on oysters. At a given temperature, HAV survived better in higher RH, while MS2 survived better in lower RH. At 40°C, inactivation of HAV was greater than 1 log at 50% RH, but only 0.1 log at 70% RH, at 1 day post-inoculation.

A. 50% RH



B. 70% RH

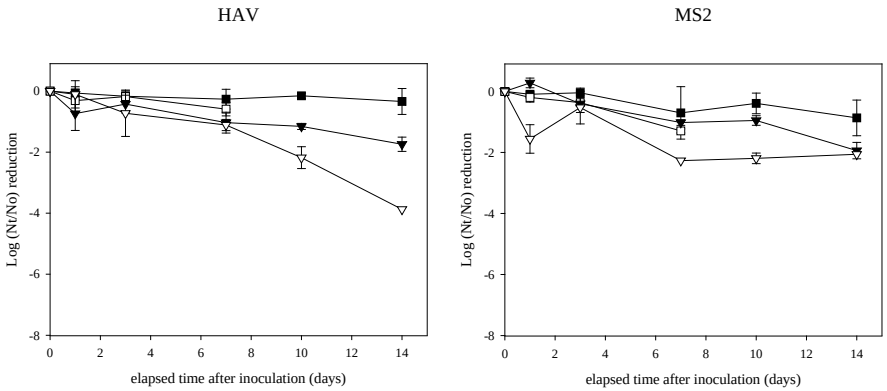
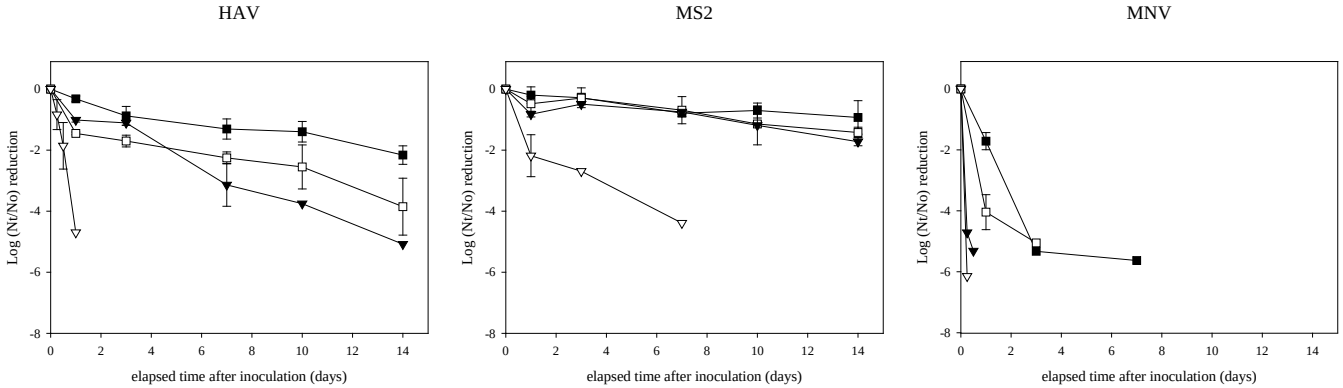


Figure 1. Inactivation curves of foodborne viruses on oysters at 50% RH (A) and 70% RH (B).

Each graph contains four survival curves at the following four temperatures: 4°C (■), 15°C (□), 25°C (▼), and 40°C (▽). The error bars indicate standard deviations. There is no data for MNV at 70% RH due to mold contamination. In the cases of HAV and MS2, molds appeared on foods after 10 days at 70% RH (B) and 15°C (□), at which point the experiments were stopped.

A. 50% RH



B. 70% RH

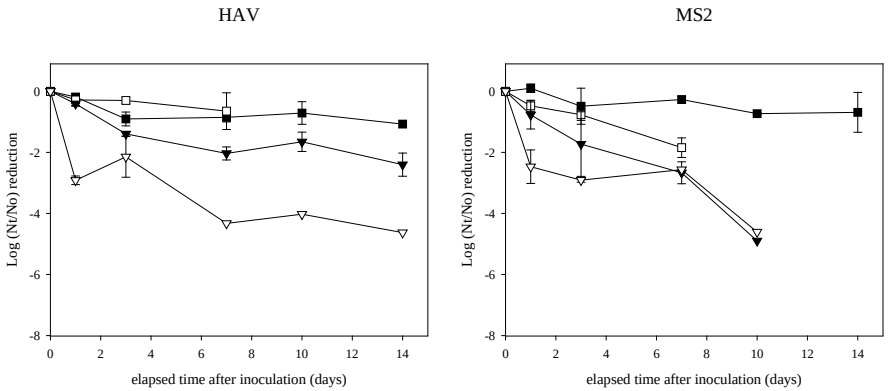


Figure 2. Inactivation curves of foodborne viruses on peppers under 50% RH (A) and 70% RH (B).

Each graph contains four survival curves at the following four temperatures: 4°C (■), 15°C (□), 25°C (▼), and 40°C (▽). The error bars indicate standard deviations. There is no data for MNV at 70% RH due to mold contamination. In the cases of HAV and MS2, molds appeared on foods after 10 days at 70% RH (B) and 15°C (□), at which point the experiments were stopped.

3. Model evaluation of the experimental data.

To assess the survivability of each virus, three different models were applied to the observed data: *(i)* a linear exponential model; *(ii)* the Weibull model as a non-linear model; and *(iii)* the biphasic model as another non-linear model. The goodness-of-fit of each model was evaluated based on R^2 and RMSE, and the results are summarized in Table 1-4. R^2 and RMSE are the correlation coefficient and the average deviation between the observed and predicted data, respectively. Generally, higher R^2 and lower RMSE values indicate a better-fitting model(*32*). At all environmental conditions and for both food types, the linear model showed the poorest fit in terms of both R^2 (0.67) and RMSE (0.55). In contrast, there were slight differences between the Weibull and biphasic models. The mean R^2 for each virus was 0.89 (HAV), 0.93 (MS2), and 0.92 (MNV) for the Weibull model, and 0.94 (HAV), 0.96 (MS2), and 0.99 (MNV) for the biphasic model. The biphasic model gave a lower mean RMSE (0.22) than the Weibull model (0.37).

To further evaluate the goodness of fit of the Weibull model and the biphasic model, the correlations between observed and predicted data were compared (Figure 3). The plot indicates that the biphasic model predicted the data better than the Weibull model. As the biphasic model appeared to be the best model for explaining our data, we used this model for further analysis. Table 5 summarizes the parameters of the fitted biphasic model. Table 6 shows the D-values of the three tested viruses predicted by the biphasic model under different temperature and RH conditions.

Table 1. Summary of estimated parameters for fitted biphasic, Weibull, and linear models using virus experimental data.

	Biphasic distribution		Weibull distribution		Linear distribution	
Virus	$R^{2\,a}$	RMSE [b]	R^2	RMSE	R^2	RMSE
HAV	0.93	0.20	0.89	0.21	0.69	0.36
MS2	0.91	0.29	0.88	0.33	0.68	0.43
MNV	0.99	0.18	0.92	0.57	0.65	0.86

[a] R^2 is the correlation coefficient between the predicted and observed data.

[b] RMSE is the root mean square error.

Table 2. Estimated parameters for fitted biphasic, Weibull, and linear models using experimental data of HAV.

Food	RH (%)	Temperature (℃)	Biphasic distribution		Weibull distribution		Linear distribution	
			R^2 [a]	RMSE [b]	R^2	RMSE	R^2	RMSE
Oyster	50	4	0.72	0.16	0.67	0.15	0.04	0.23
		15	0.86	0.16	0.79	0.17	0.25	0.28
		25	0.96	0.21	0.92	0.25	0.82	0.33
		40	1.00	ND [c]	1.00	0.00	1.00	0.04
	70	4	0.83	0.07	0.80	0.06	0.65	0.07
		15	0.85	0.17	0.76	0.15	0.69	0.14
		25	0.94	0.20	0.86	0.25	0.78	0.29
		40	0.99	0.20	0.98	0.22	0.94	0.36
Pepper	50	4	0.98	0.13	0.97	0.15	0.91	0.23
		15	0.99	0.18	0.93	0.37	0.72	0.67
		25	0.99	0.30	0.98	0.30	0.96	0.37
		40	1.00	0.03	1.00	0.06	0.98	0.30
	70	4	0.90	0.17	0.80	0.22	0.42	0.33
		15	0.98	0.07	0.94	0.08	0.84	0.11
		25	0.97	0.23	0.91	0.31	0.69	0.52
		40	0.92	0.65	0.90	0.61	0.31	1.45

[a] R^2 is the correlation coefficient between the predicted and observed data.

[b] RMSE is the root mean square error.

[c] ND (not determined) showed when the analysis was carried out at less than 2 time point.

Table 3. Estimated parameters for fitted biphasic, Weibull, and linear models using experimental data of MS2.

Food	RH (%)	Temperature (℃)	Biphasic distribution		Weibull distribution		Linear distribution	
			R^2 [a]	RMSE [b]	R^2	RMSE	R^2	RMSE
Oyster	50	4	0.91	0.06	0.87	0.06	0.19	0.13
		15	0.97	0.11	0.87	0.20	0.55	0.32
		25	0.91	0.23	0.87	0.24	0.21	0.52
		40	0.89	0.83	0.86	0.77	0.76	0.87
	70	4	0.80	0.21	0.79	0.19	0.79	0.17
		15	0.99	0.10	0.99	0.08	0.97	0.10
		25	0.92	0.29	0.92	0.26	0.90	0.21
		40	0.72	0.66	0.71	0.58	0.30	0.80
Pepper	50	4	0.95	0.10	0.94	0.10	0.85	0.14
		15	0.95	0.15	0.91	0.18	0.89	0.18
		25	0.94	0.19	0.79	0.30	0.70	0.32
		40	1.00	0.13	0.98	0.31	0.71	0.97
	70	4	0.76	0.22	0.73	0.71	0.70	0.40
		15	1.00	0.08	0.98	0.12	0.98	0.12
		25	0.97	0.50	0.96	0.45	0.96	0.39
		40	0.90	0.75	0.86	0.72	0.38	1.30

[a] R^2 is the correlation coefficient between the predicted and observed data.

[b] RMSE is the root mean square error.

[c] ND (not determined) showed when the analysis was carried out at less than 2 time point.

Table 4. Estimated parameters for fitted biphasic, Weibull, and linear models using experimental data of MNV.

Food	RH (%)	Temperature (℃)	Biphasic distribution		Weibull distribution		Linear distribution	
			R^2 [a]	RMSE [b]	R^2	RMSE	R^2	RMSE
Oyster	50	4	0.93	0.12	0.91	0.12	0.20	0.33
		15	1.00	0.01	0.78	0.25	0.07	0.46
		25	0.96	0.60	0.92	0.71	0.87	0.79
		40	1.00	ND [c]	0.83	1.88	0.94	0.79
Pepper	50	4	1.00	0.00	0.90	1.06	0.66	1.61
		15	1.00	ND	1.00	0.00	0.65	1.58
		25	1.00	ND	1.00	0.00	0.80	1.30
		40	1.00	ND	1.00	ND	1.00	0.00

[a] R^2 is correlation coefficient between the predicted and experimental data.

[b] RMSE is root mean square error.

[c] ND (not determined) showed when the analysis was carried out at less than 2 time point.

Table 5. Summary of estimated parameters for fitted biphasic model using experimental data of viruses

Food	RH (%)	Temperature (℃)	HAV			MS2			MNV		
			f [a]	b1 [b]	b2 [c]	f	b1	b2	f	b1	b2
Oyster	50	4	-0.57	-2.98	0.01	-0.46	-2.07	0.01	0.74	29.32	0.11
		15	-0.67	-67.35	0.02	0.05	-4584.47	1.08	-0.99	-1.19	-0.01
		25	0.76	3.80	0.29	-0.91	-2.89	0.00	2.00	0.00	-0.89
		40	0.02	2.24	3.82	0.05	0.76	30.05	157.14	40.45	207.55
	70	4	-0.28	-1.06	0.02	-0.52	-0.45	0.03	- [d]	-	-
		15	-0.35	-167.27	0.07	-2.20	-0.30	-0.07	-	-	-
		25	1.76	0.02	-30.95	-0.33	48.40	0.38	-	-	-
		40	1.10	0.50	0.33	0.93	33.10	0.24	-	-	-
Pepper	50	4	0.77	1.61	0.28	1.77	0.01	-0.58	0.00	0.08	4.63
		15	1.99	0.00	-4.47	0.54	0.24	36.79	0.00	1.27	41.98
		25	0.80	50.08	0.78	1.74	0.02	-30.43	0.00	-416.17	47.33
		40	1.00	9.94	2.86	0.01	0.92	31.43	0.00	-1586.88	61.11
	70	4	-0.84	-1.38	0.00	0.48	0.91	0.11	-	-	-
		15	0.64	0.22	25.32	0.47	37.98	0.61	-	-	-
		25	-0.97	-1.54	0.00	0.67	41.81	1.01	-	-	-
		40	1.00	31.97	0.42	0.01	0.49	36.17	-	-	-

[a] f is the more resistant fraction of the population.

[b] b1 is the specific death rate corresponding to the first fraction.

[c] b2 is specific death rate corresponding to the second fraction.

[d] MNV was observed under 50% RH only.

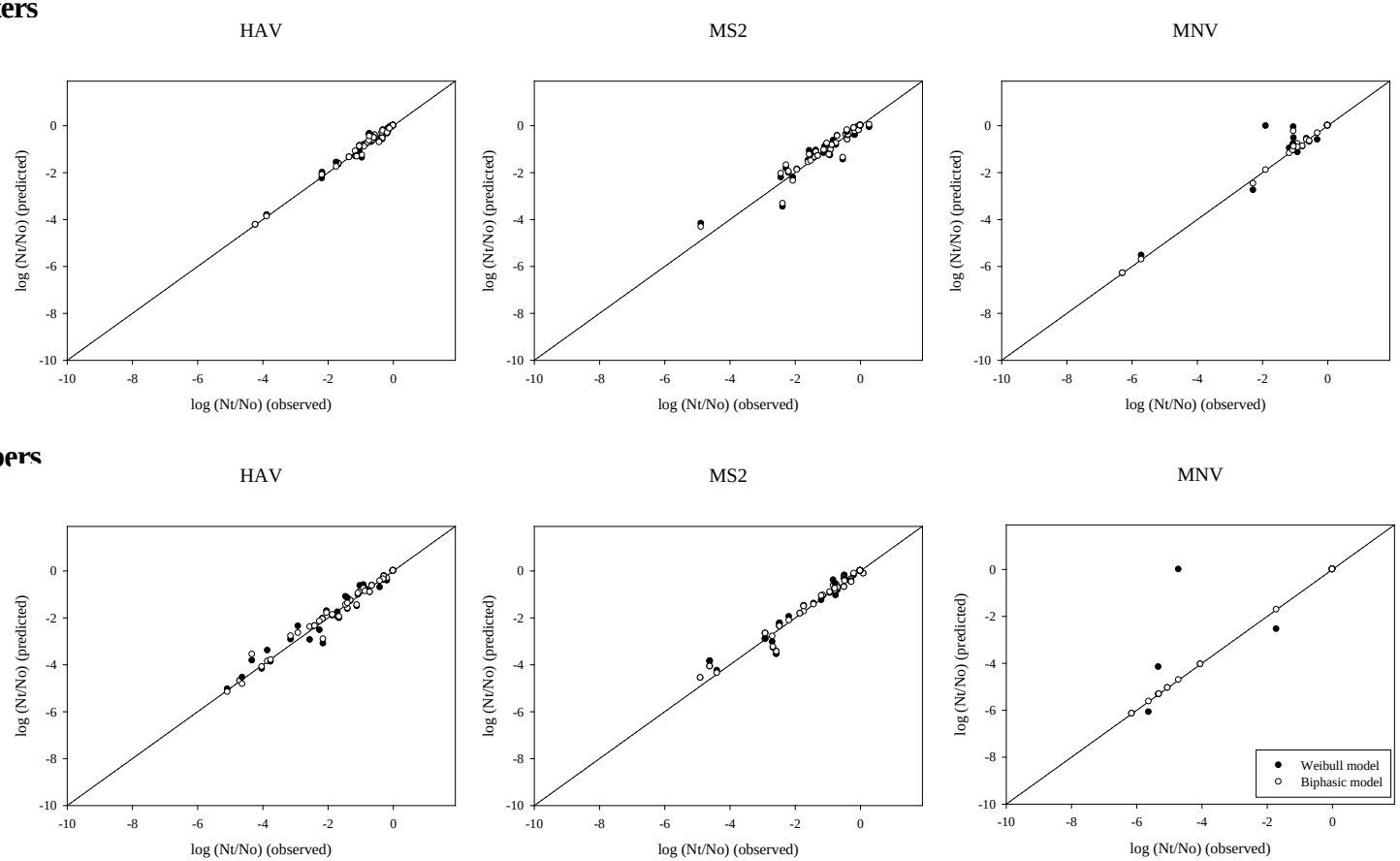


Figure 3. Correlation between the observed and predicted log-reductions of foodborne viruses on oysters (A) and peppers (B).

Closed circles (●) indicate the Weibull model, and open circles (○) indicate the biphasic model. Circles deviating from the line of equivalence indicate discrepancies between the observed and predicted values.

Table 6. Summary of predicted D-values (days to 1-log reduction) predicted by biphasic model under different temperature and RH conditions.

		Oyster		Pepper	
Virus	Temperature (°C)	50% RH	70% RH	50% RH	70% RH
HAV	4	28.9	42.6	4.5	16.8
	15	15.8	11.3	0.7	11.4
	25	4.5	8.8	1.4	2.1
	40	0.8	0.8	0.3	0.1
MS2	4	47.7	15.9	17.2	13.7
	15	5.0	5.8	9.5	3.7
	25	1.5	8.5	9.1	1.7
	40	0.1	0.1	0.1	0.1
MNV	4	13.5	- [a]	0.6	-
	15	2.8	-	0.4	-
	25	1.6	-	0.1	-
	40	0.1	-	0.0	-

[a] MNV was observed under 50% RH only.

IV. Discussion

In this study, we characterized the survival of HAV, MS2, and MNV on either oysters or vegetables under various environmental conditions, and fitted the data to three commonly used inactivation models (linear, Weibull, and biphasic models). Our study indicated that both temperature and food type are major factors influencing inactivation for all of the tested viruses. For example, at 4°C, viruses inoculated onto oysters were predicted to show only 1-log inactivation after 29 days (HAV), 48 days (MS2), and 14 days (MNV) post inoculation. The strong persistence of HAV and MNV on oysters has been reported previously(*23, 36, 37*). Our data support the current epidemiology suggesting that consumption of virus contaminated oysters causes gastroenteritis worldwide. In addition, HAV and MS2 on peppers remained infectious until 14 days post inoculation at 4°C and 15°C. A maximum of 17 days was required for a 1-log reduction of HAV or MS2 at 4°C. However, less than 1 day was required for MNV. The longer survival times at lower temperatures shown by our data are consistent with previous studies. Sun et al.(*4*) investigated the survival of HAV on the surface of green onions from temperatures ranging from 3°C to 24°C. When HAV-contaminated green onions were stored at 3°C, D-values were 29–30 days, indicating even higher survivability than shown by our data. In addition, Croci et al.(*38*) reported that HAV on lettuce remained vital for over 9 days, and the virus was completely inactivated on carrots on day 4,

and on fennel on day 7. While it is not possible to make a direct comparison with previous studies due to differences in experimental conditions, the results are similar, demonstrating that lower temperature conditions decrease the reduction of viruses on foods. Considering that vegetables and shellfish are typically stored under refrigerated conditions, the survival properties of foodborne viruses at 4°C are remarkably important. In addition, under most conditions, infectious viruses were still detectable after 2 weeks.

Inactivation rates of viruses, particularly MNV, were significantly different between oysters and peppers. In general, viruses survived better in oysters than on peppers in this study. For example, given the same quantity of inoculated virus, HAV on oysters survived better by 1.4- to 4.1-log units than on peppers, depending on the temperature conditions. MNV followed the same trend; at 25°C, MNV was undetectable on oysters after 10 days, whereas on peppers, it was undetectable after only 1 day. The exact location of inoculation could be the important factor. Viruses on peppers remained on the surface, while viruses were inoculated into the digestive tract of the oysters. These methods were used because viruses are generally exposed to the outer surfaces of fresh vegetables(*38*) and the digestive tract of shellfish(*39-41*). Even though the porous surface of the pepper provides the shelter to virus, harsh environmental conditions would be much more detrimental to viruses inoculated on vegetable surfaces than those inside of the digestive tract.

In addition, several research groups have proposed another reason as to why viruses can persist for long periods on oysters(*42-44*). Virus-associated diseases can occur when viruses penetrate into the host and then replicated themselves. With regard to the binding site of HuNoV, a certain carbohydrate complex known as histo-blood group antigen (HBGA) plays a major role as a virus receptor in humans and the great apes(*45-47*). HBGA are found widely within body components such as the erythrocytes, saliva, and the surfaces of epithelial cells on various tissues. Other enteric viruses are also considered to use a type of HBGA as a binding receptor(*48*). Interestingly, an HBGA-like complex associated with binding has been verified to exist in oysters. Le Guyader et al.(*43*) found that HuNoV binds to oyster tissue through an A-like carbohydrate structure, which is also used for attachment to carbohydrate on human epithelial cells. Poliovirus (PV) was also shown to attach to shellfish mucus through ionic bonding, which is a similar process of viral attachment to host cells(*49*). Another previous study reported that hemocytes in oysters play an important role in the retention of enteric viruses such as HAV, MNV, FCV, and PV(*23*). The ability of virus to tolerate low pH is required to survive within hemocytes in oysters. Therefore, a specific binding or tolerance of acidic conditions may allow the virus to persist inside the digestive tissue, and viruses can maintain high levels of vitality in oysters.

In the case of vegetables, similar mechanisms were observed in previous studies. Virus-like particles (VLPs) of HuNoV attached to romaine lettuce

(leaves, fresh-cut edges, etc.) by binding cell wall carbohydrates (e.g., GalNAc, GlcNAc, and sialic acid)(*50*). The surface charge of virions is associated with nonspecific binding to lettuce if viruses do not use specific cell surface receptors to attach(*51*). Both specific- and nonspecific interactions between viruses and the surfaces of vegetables can affect binding ability. In addition, the porous characteristics of vegetables could explain viral persistence. In a previous study, influenza virus (H5N1) had greater survival on porous materials (chicken feces, soil) than on nonporous materials (glass, galvanized metal)(*52*). The enteric viruses observed in our study persisted for longer periods on porous wood than on nonporous stainless steel under various environmental conditions, and the type of surface had a significant effect on virus survival(*26*). Inactivation rates of both HAV and human astrovirus were higher on nonporous surfaces than on porous surfaces(*53, 54*). Virus may prolong their survivability by obtaining shelter within porous structures on the surfaces of environmental fomite(*26, 55*). These structures can protect viruses from harsh environmental conditions (e.g., light, RH, temperature, etc.). Therefore, it is likely that enteric viruses do not perish rapidly on the surfaces of peppers because they bind to certain cell wall materials or hide in the holes on the surface.

Water content in food stuffs has been reported to play a significant role in stability and shelf life by influencing on microbial growth(*56, 57*). During long-term storage, water activity reaches equilibrium with RH slowly, depending on factors such as the structure of food components, solvents,

and other properties(*58*). This study was performed on the assumption that the moisture content in virus-infected foods would be affected by the RH maintained in the chamber. However, influence of RH on viral inactivation was obscure in this study, especially in oysters. Survival of viruses on oysters decreased with rising temperatures, but was rarely associated with variations in RH. Virus-infected oysters could retain constantly high moisture contents, as they were stored in PBS. This means that inactivation of viruses in oysters was solely the result of temperature. On the surface of peppers, the D-value of HAV was lower in 50% RH, while the D-value of MS2 was lower in 70% RH. The difference in D-value was clearly greater at lower temperatures. Unlike oysters, peppers were exposed directly to both temperature and RH during the experiment. Therefore, we infer that virus survival was influenced mainly by temperature, and secondary effects occurred through the combination of temperature and RH. Previous studies have also described synergistic effects of temperature and humidity on viral inactivation(*59, 60*).

The results of this study suggest that foodborne viruses may last for over 2 weeks prior to a 1-log reduction at 4°C, which means that viruses could survive under refrigerated conditions until foods are no longer consumable. This implies that the natural decay of viruses on foods might not provide adequate protection from viral pathogens once viral contamination occurs. Therefore, the prevention of viral contamination is the most important factor for the prevention of foodborne outbreaks. Precautionary prevention should

be considered to be more efficient than efforts to reduce viral particles after contamination. Furthermore, it is time to evaluate the current status of food hygiene practices. Although focus on the importance of food safety has grown in recent years, many foods are still produced inappropriately using contaminated irrigation water, unsterilized equipment, or unclean working clothes(*5*). Under such unsanitary surroundings, most foodborne viruses can contaminate both fresh and processed foods throughout the food supply chain, including manufacture, storage, and distribution, and can survive a wide range of temperature and RH(*20, 24*) conditions[20,24]. The universal practice during food production processes must be to maintain clean environments and hygienic conditions.

In conclusion, our study demonstrated the prolonged survival of HAV and MNV on typical foods without heat treatment. These results suggest that careful consideration should be taken during food production and storage processes. This study will provide useful information for the establishment of food safety preventive guidelines.

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국문초록

식품 저장 중 온도 및 상대습도가 식중독 바이러스 생존에 미치는 영향

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이 수 진

노로바이러스 (human norovirus, HuNoV) 및 A형 간염 바이러스 (hepatitis A virus, HAV) 는 가장 중요한 식중독 바이러스로 손꼽힌다. 본 연구에서는 식품 저장 환경에 따른 HuNoV 및 HAV의 생존을 관찰함으로써 식품 안전에 적절한 정보를 제공하고자 하였다.

HAV 및 박테리오파지 MS2, 뮤린노로바이러스 (murine norovirus, MNV) 를 굴 및 파프리카에 각각 감염시킨 후 2가지 상대습도 (50, 70%) 및 4가지 온도 (4, 15, 25, 40˚C) 조건 아래 최대 14일까지 보관하였다. 저장기간 중 정해진 날짜 (0, 1, 3, 7, 10, 14 일) 에 바이러스를 회수하여 정량평가 하였다. 바이러스의 생존은 주로 온도에 의해 영향을 받는 것으로 나타났다. 모든 바이러스는 4˚C 에서 가장 오랫동안 생존하였으며 40˚C 에서 가장 빨리 저감하였다. 또한 바이러스는 파프리카보다 굴에서 장기간 생존하였다. 파프리카에 감염된 HAV는 70% RH에서, MS2 는 50% RH에서 오랫동안 생존하는 것으로 나타났다. 바이러스 생존성을 예측하기 위해 3가지 미생물 생존 모델 (linear model 및 Weibull model, biphasic model)을 비교·평가 하였고, 적합도가 가장 높은 biphasic model 을 최종적으로 선정하였다.

신선식품은 주로 저온에서 저장되므로 본 연구에서 확인된 바이러스의 강한 지속성은 식품 안전에 큰 위협이 될 것이다. 식품에 감염된 바이러스는 쉽게 사멸하거나 제거되지 않기 때문에 바이러스 감염을 사전에 예방하는 것이 가장 중요한 것으로 사료된다.

주요어: 식중독바이러스, 노로바이러스, 간염 A형 바이러스,
온도, 습도, 굴, 파프리카, biphasic model

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