



Persistence of Coronavirus Surrogates on Meat and Fish Products during Long-Term Storage

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ABSTRACT Multiple pathways of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) transmission have been examined, and the role of contaminated foods as a source of SARS-CoV-2 exposure has been suggested. As many cases of SARS-CoV-2 have been linked to meat processing plants, it may be that conditions in live animal markets and slaughterhouses or meat processing plant procedures transfer viral particles to meat, poultry, and seafood during animal slaughter, processing, storage, or transport. Because of the potential for contamination of foods such as beef, chicken, pork, or fish, the goal of this study was to evaluate the survival of a lipid enveloped RNA bacteriophage, phi 6, as well as two animal coronaviruses, murine hepatitis virus (MHV) and transmissible gastroenteritis virus (TGEV), as SARS-CoV-2 surrogates for their survival under various meat and fish cold-storage conditions over 30 days. Viral surrogates differed in survival, depending on food product and temperature, but overall, viruses survived for extended periods of time at high concentrations at both refrigerated and frozen temperatures. The ability of SARS-CoV-2 viral surrogates like Phi 6 and animal coronaviruses to survive for varying extents on some meat and fish products when stored refrigerated or frozen is a significant and concerning finding. Continued efforts are needed to prevent contamination of foods and food processing surfaces, worker hands, and food processing utensils such as knives, and there is a need to better address the lack of or inadequate disinfection of these foods prior to meat packaging.

IMPORTANCE The ability of SARS-CoV-2 viral surrogates like Phi 6 and animal coronaviruses to survive for long periods on meat and fish products at cold temperatures emphasizes the need for rigorous and sustained food sanitation and hygiene in the harvest, transport, processing, and distribution of these foods.

KEYWORDS SARS-CoV-2, coronavirus, coronavirus surrogates, meat, fish

The disease COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was declared a pandemic by the World Health Organization (WHO) in March 2020. Multiple pathways of SARS-CoV-2 transmission have been identified and examined; however, it has been determined that the primary methods of transmission are likely through airborne droplets, direct personal contact, and aerosols (1–3). Recent studies have also shown that fomites, such as high contact surfaces like cell phones, screens, etc., may also be sources of exposure and transmission (4, 5). Low levels of SARS-CoV-2 have been detected on stainless steel and cardboard held at room temperature with constant humidity (6), and this virus has been recovered at both refrigerated (4°C) and frozen (0°C and –80°C) temperatures on both meat and food contact surfaces (7, 8).

The role of contaminated foods as sources of SARS-CoV-2 exposure has been suggested because of the possible emergence of the pandemic in a food market in Wuhan China (9–13). Other reports, particularly in China, have attributed outbreaks of SARS-CoV-2 to contaminated

Editor Nicole R. Buan, University of Nebraska—Lincoln

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The authors declare no conflict of interest.

Received 22 March 2022

Accepted 18 May 2022

Published 7 June 2022

4°C Survival

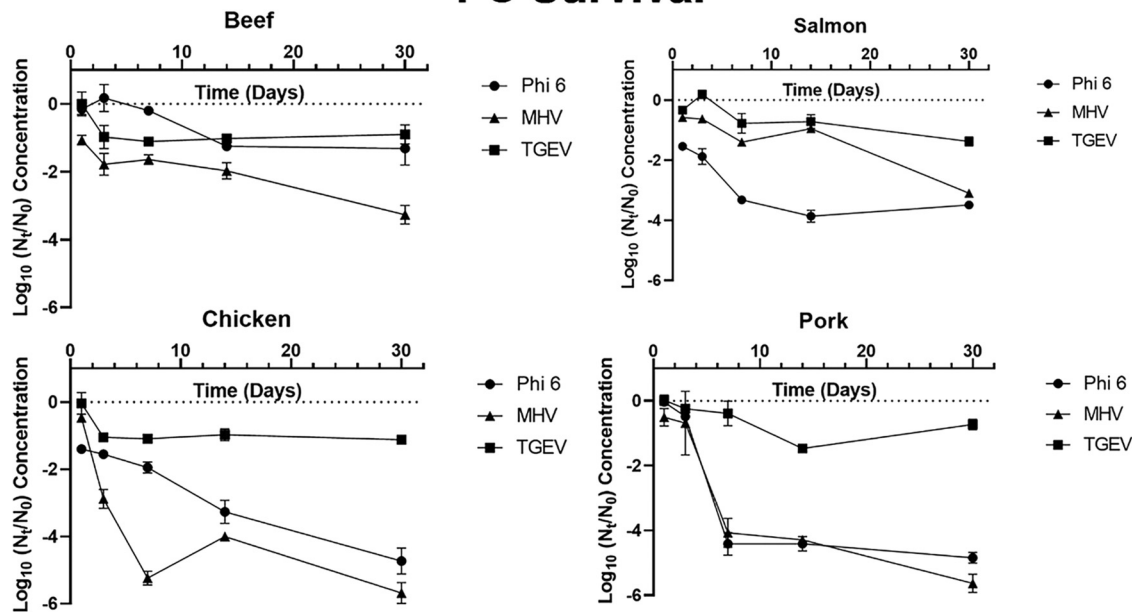


FIG 1 $\text{Log}_{10} (N_t/N_0)$ reductions and 95% confidence intervals of phi 6, MHV, and TGEV on salmon, beef, pork, and chicken at 4°C over 30 days. Phi 6 is measured in PFU/100 mL, and MHV and TGEV are measured in MPN IU/100 mL.

food (14) many months after the end of locally sustained transmission. Similarly, Vietnam and New Zealand have also seen outbreaks at 99 and 102 days after the last identified local transmission (15, 16), suggesting another persistent exposure source.

Because the extent to which SARS-CoV-2 and similar enveloped viruses can survive on foods, such as meat, poultry, and fish, under cold-storage conditions is poorly quantified, we determined the survival of an enveloped bacteriophage, phi 6, a widely used coronavirus surrogate, and two animal coronaviruses during refrigerated and frozen storage of such foods for up to 30 days.

RESULTS

Survival of coronaviruses at refrigerated temperatures. Figure 1 shows the $\text{log}_{10}(N_t/N_0)$ survival of bacteriophage phi 6 and coronaviruses murine hepatitis virus (MHV) and transmissible gastroenteritis virus (TGEV) on meat and fish products stored at 4°C over a 30-day period. All viruses were recoverable above the lower detection limit throughout the full 30-day experiment. The average log_{10} reductions for each virus are summarized by temperature in Table 1. Based on both the trends over time as well as the overall log_{10} reductions at 4°C, MHV was reduced most extensively, followed by phi 6, with the least reductions for TGEV. At 4°C, the 30-day log_{10} reductions of MHV were greatest at 5.7, 5.6, 3.7, and 3.5 on chicken, pork, salmon, and beef, respectively, followed by 30-day log_{10} phi 6 reductions of 4.8, 4.7, 3.5, and only 1.3 on pork, chicken, salmon, and beef, respectively, with the lowest reductions of TGEV, which ranged from 0.7, 0.9, 1.1, and 1.4 on pork, beef, chicken, and salmon, respectively. Thirty-day log_{10} reductions of MHV and phi 6 were more closely related than those of TGEV, with the exception of the relatively low 1.3 log_{10} reduction of phi 6 on beef.

To compare the differences between average log_{10} reduction by virus and across the different types of meat, a one-way analysis of variance (ANOVA) was conducted followed by a Tukey's multiple comparison test (Table 2). Based on this analysis at refrigerated temperatures (4°C), the average 30-day reduction for phi 6 was significantly different across all meats except on pork and chicken; from Table 1, the average log_{10} reductions for these meats are 4.84 and 4.73 log_{10} PFU, respectively. When compared pairwise by food type, MHV reductions were similar when evaluated on salmon and beef and likewise similar on pork and chicken but significantly different for all other pairwise food comparisons. For TGEV at this temperature, all log_{10} reductions were not statistically significantly different on the compared meats.

TABLE 1 Summary of average total log₁₀ virus reductions on salmon, beef, pork, and chicken over 30 days of storage at 4 and −20°C^a

Meat	Avg total log ₁₀ virus reduction					
	4°C			−20°C		
	Phi 6	MHV	TGEV	Phi 6	MHV	TGEV
Salmon	3.49	3.10	1.38	2.03	3.71	1.70
Beef	1.31	3.27	0.90	0.52	5.33	1.54
Pork	4.84	5.63	0.73	0.42	5.91	1.54
Chicken	4.73	5.68	1.12	1.77	6.82	2.28

^aPhi 6 is measured in PFU/100 mL; MHV and TGEV are measured in MPN IU/100 mL.

Survival of coronaviruses at frozen temperatures. As shown in Table 1 and Fig. 2, phi 6 and TGEV log₁₀ reductions were generally similar on all frozen fish and meat samples. Both viruses were reduced the least on beef and pork and had somewhat greater reductions on chicken and salmon, with 30-day log₁₀ reductions ranging from 0.4 to 2.0 for phi 6 and 1.5 to 2.3 for TGEV. The lowest 30-day log₁₀ reductions were 0.4 for phi 6 on pork and 0.5 for phi 6 on beef, and the highest reductions were 2.3 for TGEV on chicken and 2.0 for phi 6 on salmon. In contrast, MHV log₁₀ reductions were much greater on all samples, with 30-day log₁₀ reductions of 3.7, 5.3, 5.9, and 8.8 on salmon, beef, pork, and chicken, respectively.

In the comparison of average log₁₀ reduction across food products, there were no significant differences in the pairwise comparison between salmon and chicken or in the pairwise comparison between beef and pork for phi 6 at −20°C. For MHV, there was also no significant difference between beef and pork and also no difference between the reductions on pork and chicken. As at 4°C, there were no significant differences between average log₁₀ reductions for TGEV in pairwise comparisons of the different food products.

DISCUSSION

We assessed the survival of bacteriophage phi 6 and animal coronaviruses MHV and TGEV on three meats and a fish product stored at both refrigerated (4°C) and frozen (−20°C) temperatures over a 30-day time period. Phi 6 has been previously evaluated as a surrogate for multiple virus pathogens, including ebolavirus (17–19), and various human respiratory viruses (20, 21), including coronaviruses such as SARS-CoV-2 (6, 22, 23). MHV and TGEV have also been used as coronavirus surrogates in virus survival studies due to their similarities in taxonomy and structure to SARS-CoV-2 (24, 25).

In this study, we found that all viruses inoculated onto the meat and fish products survived to varying extents at both 4°C and −20°C for the full 30-day period, with different

TABLE 2 Tukey's multiple comparison test of the relationship between log₁₀ reduction average by virus and type of meat^a

Meat comparison at 4°C or −20°C	Phi 6		MHV		TGEV	
	Avg difference (95% confidence interval)	P value	Avg difference (95% confidence interval)	P value	Avg difference (95% confidence interval)	P value
4°C						
Salmon vs beef	−1.97 (−3.00, −0.93)	0.01*	0.30 (−0.72, 1.32)	0.79	−0.40 (−1.03, 0.23)	0.25
Salmon vs pork	1.38 (0.34, 2.41)	0.01*	2.63 (1.61, 3.66)	<0.01*	−0.48 (−1.11, 0.15)	0.15
Salmon vs chicken	1.37 (0.34, 2.40)	0.01*	2.71 (1.69, 3.74)	<0.01*	−0.11 (−0.74, 0.52)	0.94
Beef vs pork	3.34 (2.31, 4.38)	<0.01*	2.33 (1.31, 3.56)	<0.01*	−0.08 (−0.71, 0.55)	0.98
Beef vs chicken	3.34 (2.30, 4.37)	<0.01*	2.41 (1.39, 3.44)	<0.01*	0.29 (−0.34, 0.92)	0.50
Pork vs chicken	−0.007 (−1.04, −1.03)	>0.99	0.08 (−0.94, 1.10)	0.99	0.37 (−0.26, 0.99)	0.31
−20°C						
Salmon vs beef	−1.53 (−2.04, −1.02)	<0.01*	1.70 (0.38, 3.02)	0.01*	−0.08 (−1.32, 1.16)	1.00
Salmon vs pork	−1.63 (−2.15, −1.12)	<0.01*	2.57 (1.25, 3.89)	<0.01*	−0.02 (−1.26, 1.22)	>0.99
Salmon vs chicken	−0.27 (−0.78, 0.25)	0.40	3.49 (2.17, 4.80)	<0.01*	0.69 (−0.55, 1.93)	0.35
Beef vs pork	−0.10 (−0.62, 0.41)	0.92	0.87 (−0.45, 2.19)	0.23	0.06 (−1.18, 1.30)	1.00
Beef vs chicken	1.26 (0.75, 1.78)	<0.01*	1.79 (0.47, 3.11)	0.01*	0.77 (−0.47, 2.01)	0.27
Pork vs chicken	1.37 (0.85, 1.88)	<0.01*	0.91 (−0.41, 2.23)	0.20	0.71 (−0.53, 1.95)	0.32

^a*Significant at the 0.05 level.

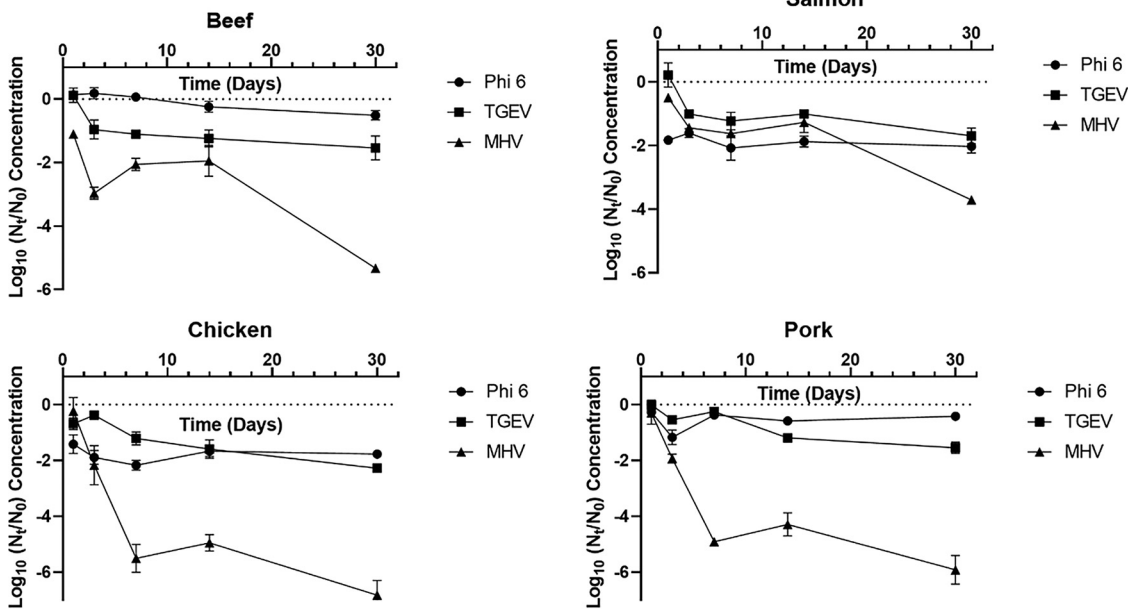
-20°C Survival

FIG 2 $\text{Log}_{10} (N_t/N_0)$ reductions and 95% confidence intervals of phi 6, MHV, and TGEV on salmon, beef, pork, and chicken at -20°C over 30 days. Phi 6 is measured in PFU/100 mL, MHV and TGEV are measured in MPN IU/100 mL.

extents of virus reduction among the four foods and generally greater reductions at the refrigerated temperature than the frozen temperature. However, MHV was appreciably reduced at the frozen temperature, particularly after about 7 days of storage. When comparing the differences in average log_{10} reductions across food products, Phi 6 and MHV were statistically significantly different among these products at both temperatures. However, TGEV reductions were not significantly different at either temperature on any of the foods tested, indicating that this virus is reduced to similar extents between the two temperatures and across all four food products.

Only a few studies have evaluated the survival of coronaviruses and their surrogates on meat and fish products or other foods, such as produce. Mullis et al. studied the persistence and survival of a bovine coronavirus on romaine lettuce based on the detection of viral RNA by real-time PCR methods at 4°C throughout a 30-day study period (26). Also, infectious viruses assayed in cell cultures were detected for at least 14 days, indicating possible zoonotic transmission of coronaviruses from contaminated vegetables (26). Yépez-Gómez et al. studied the survival of human common cold coronavirus (CoV 229E) as well as some nonenveloped viruses on lettuce, strawberries, and raspberries at 4°C (27). They reported that CoV 229E survived up to 4 days on produce, which also indicates the potential risk for transfer of respiratory coronaviruses via hands after cleaning or handling vegetables (27). As some respiratory coronaviruses can replicate in both the respiratory tract and the gut (including severe acute respiratory syndrome coronavirus [SARS-CoV]), this is an important consideration when evaluating risks from viral survival on food products that would be eaten. Similarly, our results show that the enveloped bacteriophage Phi 6 was also detectable for long periods on food products. In a separate study, we detected this bacteriophage for as long as 60 days on these same four food surfaces at these two storage temperatures (data not shown). Because we used high-titer surrogate viruses, we were able to track far greater than the 1 to 2 log_{10} infectivity loss reported in some previous studies, which were constrained by lower virus inoculum levels, shorter storage times, and less sensitive infectivity assays.

One study evaluated the survival of herpes simplex virus 1 (HSV-1) compared to SARS-CoV-2 on apple skin, mushrooms, poultry, and seafood stored at 4°C over a period of only 24 h (28). In this comparison, less infectious HSV-1 was recovered on apple skin and mushrooms compared to poultry and seafood. Less than 1 log_{10} PFU was detectable within 24 h

for HSV-1, and less than 1 log₁₀ PFU SARS-CoV-2 was detectable within 1 h on mushroom samples. Similar to our work, there was an initial decline in virus titer; however, in this study, viruses were not detectable after 24 h. This difference in survival may be due to the use of infectious SARS-CoV-2 or to limitations of the virus recovery methods used, which involved mechanical massage of the samples. In other work evaluating the stability of SARS-CoV-2 in viral transport media, the virus was highly stable at 4°C (7).

In addition to temperature, there are several key factors that may influence the survival of viruses on foods and other surfaces, including meat, poultry, and fish surfaces. One of the primary factors influencing virus survival is biological, in particular, for SARS-CoV-2 and other enveloped viruses, the properties of the outer envelope and surface spikes of the virus itself. In general, environmental survival of viruses is lower for enveloped than nonenveloped viruses and is even lower when compared to other microbes. Enveloped viruses have great susceptibility to the effects of temperature, pH, desiccation, and other environmental stressors (29).

In a recent study examining SARS-CoV-2 adsorption onto meat surfaces, researchers found that an interplay between pH and electrostatic forces was an important contributor to the survival of the virus (30). In particular when the pH of meat surfaces was 5.5 or less, it was found that viruses firmly adsorbed to the surface of the meat due to a protonated amine group and a hydrogen bond formed with the thin layer of water present on the surface of meat products (30).

Theoretical work has also indicated that the structural proteins of SARS-CoV-2 would be less likely to interact with surfaces at high pH, as these strong hydrogen bonds could not form under pH conditions above the virus isoelectric point (31). Although we did not measure the pH or surface electrical potential of the meat products, it is clear that the viruses were stable on the surfaces of both meat and fish products for long periods of time. The surface hydrophobicity of the virus and a surface to which it becomes associated may also influence virus survival (32). The surface hydrophobicity of the viruses and food surfaces studied were not characterized in this study, but they may also contribute to observed differences in virus survival.

As enveloped viruses such as SARS-CoV-2 and other coronaviruses have the ability to survive refrigeration and freezing for long periods of time, there is a potential risk of viral persistence on contaminated food products that could result in subsequent exposure and transmission. Although the World Health Organization (WHO) has stated that transmission of COVID-19 from food or food packaging is unlikely (8, 32, 33), this statement is uncertain and not supported by much available evidence. However, the ability of SARS-CoV-2 viral surrogates like Phi 6 and animal coronaviruses to survive to varying extents for long time periods on some meat and fish products when stored refrigerated or frozen is a significant and concerning finding. It emphasizes the need for rigorous and sustained food sanitation and hygiene in the harvest, transport, processing, and distribution of these foods. Clusters of COVID-19 infection continue to occur in slaughterhouses and meat processing plants. Evidence of virus presence has been detected on some of these foods, despite increased use of personal protective equipment (PPE) and other engineering and administrative prevention and control measures. We provide evidence of SARS-CoV-2 surrogates surviving for relatively long and varying time periods on refrigerated and frozen meat and fish, potentially due to the low temperatures that allow virus survival. Therefore, continued efforts are needed to prevent contamination of such foods and their food processing surfaces, worker hands, and food processing utensils such as knives, and there is a need to better address the lack of or inadequate disinfection of these foods prior to meat packaging (34).

A limitation of this study is the use of surrogate coronaviruses instead of the SARS-CoV-2 virus itself. The primary goal of this study was to examine the persistence of enveloped RNA viruses on food products stored at cold or frozen temperatures. Further research is recommended to include additional viruses, such as SARS-CoV-2 and other human and animal CoVs, and to study their survival on a greater variety of meat, fish, and produce food products. Fresh produce products in particular have previously shown evidence of respiratory virus transmission, including coronavirus 229E and other respiratory adenoviruses

(27). Even though the viruses examined here have been found to be reliable models for the survival of SARS coronaviruses (35–39) under various environmental conditions, the differences in virus survival observed in our results suggest that one indicator virus may not be predictive of the behavior of pathogenic SARS-CoV-2. Further studies of this type that included SARS-CoV-2 are needed, especially studies that better characterize and quantify the effects of physical, chemical, and biological factors, including temperature, water activity, relative humidity, pH, surface electrical potential, hydrophobicity, chemical activities such as enzymes and surfactants, and microbiological activities such as from other microbes on and in foods. As reports continue to emerge about the potential spread of SARS-CoV-2 through contaminated food products and the continued emergence of variants of concern, a greater understanding of the survival of these viruses at low temperatures on meat and fish products as well as other foods is needed and should be encouraged.

MATERIALS AND METHODS

Virus surrogates. Bacteriophage Phi 6 and host *Pseudomonas syringae* were kindly supplied by the Water Institute laboratory of the Gillings School of Public Health at the University of North Carolina at Chapel Hill. Murine hepatitis virus was obtained from the American Type Culture Collection (ATCC) (VR-764) and propagated in NCTC clone 1469 cells (ATCC CCL-9.1). Transmissible gastroenteritis virus (ATCC VR-2384) was kindly provided by Gregory Gray's laboratory at Duke University and was propagated in ST cells (ATCC CRL-1746).

Preparation of viruses. Using prepared 18-h growth of *P. syringae* in 50 mL tryptic soy broth (TSB), bacteriophage phi 6 was propagated by reconstituting with 1 mL of prewarmed (37°C) TSB. Five hundred microliters of reconstituted phi 6 was transferred into 50 mL of fresh TSB with 100 μ L of host *P. syringae* and incubated at 22°C with gentle agitation (100 rpm) for 18 h. The infected cell culture was centrifuged at 1,200 $\times g$ for 15 min at 4°C, and the resulting supernatant was used for the resulting experiments. The phi 6 stock titer was approximately 10⁹ PFU/mL.

For animal viruses, prepared 25-cm² flasks of appropriate cells (NCTC cells for MHV and ST cells for TGEV) were washed with phosphate-buffered saline. Two hundred microliters of virus stock was added to each flask, and they were incubated for 48 h. After 90% of the monolayer was destroyed, the flasks were frozen and thawed completely. All medium in each flask was drawn into a 25-mL centrifuge tube for each virus and spun down at 1,200 $\times g$ for 15 min at 4°C. The supernatant was drawn off and used for inoculation. The MHV stock was approximately 10⁷ most probable number infectious units per milliliter (MPN IU/mL), and the TGEV stock was approximately 10⁵ MPN IU/mL.

Cell culture. Cell culture assays were performed as previously described by Casanova et al. (40). Briefly, NCTC cells were grown in 24-well plates with Dulbecco's modified Eagle's medium (DMEM) (ATCC 30-2002) and supplemented with 10% horse serum (Thermo Fisher; 26050088). ST cells were grown in 24-well plates with Eagle's minimum essential medium (EMEM) (ATCC 30-2003) supplemented with 10% fetal bovine serum (ATCC 30-2020). Cells were incubated for 3 to 5 days at 37°C with 5% CO₂ until at least 80% confluence was attained.

Meat product inoculation and sampling. Samples of beef, pork, chicken, and salmon purchased from a local grocery store were aseptically sliced into small pieces, approximately 0.5 cm thick and 2 cm in diameter, and placed into sterile multiwell plates (Falcon; 353043). Each food slice was inoculated with 100 μ L of diluted phi 6, MHV, and TGEV stock to provide 106 to 108 PFU or MPN IU per food piece. Food slices were allowed to air dry for approximately 10 min and then transferred to a sterile 50-mL conical bottom centrifuge tube. Triplicate sets of food samples (beef, chicken, pork, and salmon) were stored in 50-mL conical tubes at either 4°C or –20°C for 0, 3, 7, 14, and 30 days.

Coronaviruses and phages were dislodged from food pieces in the 50-mL conical tubes using 5 mL TSB. All samples were well mixed using a combination of vortex and rotary mixing for a total of 15 min to elute viruses off food surfaces. The recovered eluate was diluted serially 10-fold in phosphate-buffered saline (PBS).

Phi 6 assay. One hundred microliters of sample were plated with 500 μ L of host *P. syringae* in tryptic soy agar using a double agar layer (DAL) overlay plaque assay method (41). The DAL plates were incubated for 18 to 24 h at 22°C before PFU were counted. PFU per milliliter was calculated based on dilution factor and then log₁₀ transformed. Viral recovery was evaluated on a control sample of each food product and determined to be within 95% of expected spiking concentration.

MPN cell culture assay for MHV and TGEV. Twenty-four-well cell culture plates were prepared as described above and allowed to reach 80 to 90% confluence. One hundred microliters of serially diluted virus eluate was added to five (5) rows of the well plate, while the sixth row was used for PBS controls. Plates were incubated for 1 h at 37°C supplemented with 5% CO₂. Then, 0.5 mL of cell culture medium was added to each well, and plates were incubated for an additional 48 h. The MPN assay of each sample was determined for cytopathic effect (CPE) at 48 h. Each sample well was scored as positive or negative for viral CPE, and the numbers of CPE-positive and -negative wells per sample dilution were used to calculate the most probable number (MPN) virus concentration per milliliter. As with the Phi 6 assay, viral recovery was evaluated on a control sample of each food product and evaluated using the MPN viral concentration assay to be within 95% of expected spiking concentration.

Data analysis. Data analysis and graphical representations were created in GraphPad Prism (version 9). Log₁₀(N_t/N₀) survival values were calculated by taking the log₁₀ of the concentration (N) of virus detected at time (t) divided by the concentration at time zero. Total and periodic log₁₀ reductions over the 30-day experiment were calculated by subtracting the periodic and final 30-day log₁₀ concentrations of virus detected on a

food sample from the initial \log_{10} concentration on that sample. A one-way analysis of variance (ANOVA) test was used to determine if there was a difference among average \log_{10} reductions by virus and food type, and Tukey's multiple-comparison test was used to examine the relationships between average reductions of sample pairs. All statistical significance was evaluated at an alpha level of 0.05.

ACKNOWLEDGMENTS

Funding for this study was provided by the Physicians Committee for Responsible Medicine and the World Health Organization.

We have no conflicts to declare.

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