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Persistence of murine norovirus, bovine rotavirus, and hepatitis A virus on stainless steel surfaces, in spring water, and on blueberries

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ABSTRACT

The viability of murine norovirus (MNV-1), bovine rotavirus (boRV), and hepatitis A virus (HAV) was evaluated at 21 °C, 4 °C, and −20 °C on stainless steel surfaces, in bottled water, and on blueberries for up to 21 days. After 14 days of incubation at 21 °C on stainless steel, a viability loss > 4 log for MNV-1, > 8 log for boRV, and > 1 log for HAV was observed. Losses were observed for MNV-1 (> 1 log) and HAV (> 2 log) incubated in water at 21 °C for 21 days. No significant loss was detected for MNV-1 and HAV at 4 °C and −20 °C and for boRV at 21 °C, 4 °C, and −20 °C. On blueberries incubated at 4 °C and −20 °C, they all maintained their infectivity. After 7 days at 21 °C, a loss > 2 log, a loss of 3 log, and no loss were observed for boRV, MNV-1, and HAV, respectively. After RNase pretreatment, the detection of extracted RNA from infectious and noninfectious samples suggested the protection of RNA inside the capsid. Even though they all are enteric viruses, their persistence varied with temperature and the nature of the commodity. It is therefore important to use more than one viral surrogate, during inactivation treatments or implementation of control measures.

1. Introduction

Foodborne illnesses caused by enteric viruses occur after ingestion of contaminated food or water, person-to-person contact, or exposure to contaminated aerosols or surfaces. Viral contamination of food can occur both before and after harvest (D'Souza and Joshi, 2016). Even though the illness is usually self-limiting, such contamination remains an important issue. Enteric viruses are generally shed in large numbers by infected individuals, and just a few virus particles can lead to disease (Carter, 2005). In the United States, 48 million foodborne illnesses are estimated to occur each year (Gould et al., 2013). It is therefore important to not only detect enteric viruses, but also adequately evaluate the viral infectivity of samples intended for human consumption as part of a strategy to control and mitigate the viral presence and to better manage the risk.

Human noroviruses (HuNoVs) are recognized as the main cause of sporadic infections and outbreaks of acute gastroenteritis (D'Souza and Joshi, 2016; Lim et al., 2016; WHO, 2015). In Canada, HuNoVs were responsible for most domestically acquired foodborne illnesses during the period from 2000 to 2010 (Thomas et al., 2013). Those viruses are usually excreted in high concentrations, are highly contagious, and are present in the environment for extended periods of time. Since no efficient culture method is available, HuNoV detection is routinely achieved using molecular methods. Murine norovirus (MNV-1) is widely used in viability studies as a cultivable surrogate for HuNoVs. Hepatitis A virus (HAV) is responsible for hepatitis, one of the most common viral foodborne illnesses (WHO, 2018). Also transmitted via the fecal–oral route (Casteel, 2016), HAV is persistent in the environment and is able to withstand low-pH and high-temperature conditions (Carter, 2005). Human rotaviruses (HuRVs) are the main cause of severe diarrhea in infants and young children (Desselberger, 2014). However, with the onset of vaccination programs, the incidence rate has decreased (Donato et al., 2017). Less often transmitted by food than other enteric viruses are, HuRVs can still cause outbreaks after food has been in contact with contaminated surfaces (Donato et al., 2017).

The persistence of viruses on food surfaces is influenced by many factors, including temperature, relative humidity (RH), the nature of the fomites, and the nature of the virus itself (Carter, 2005; D'Souza and Joshi, 2016; Vasickova and Kovarcik, 2013). Because of its resistance to corrosion, its hardness, and its ease of cleaning, stainless steel is widely used for food manipulation surfaces in food-processing industries (Schmidt, 2012). Improper cleaning of contaminated surfaces where ready-to-eat foods are handled can contribute to an increased risk of the spread of foodborne illnesses. Some common foods associated with gastrointestinal illnesses are leafy vegetables (salad, green onions) and berries (raspberries, strawberries), all of which are often eaten fresh

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(Calder et al., 2003; D'Souza and Joshi, 2016). These foods may have rugged surfaces and crevasses that offer protection from wash solutions, providing a high-relative-humidity environment that, when combined with the presence of proteins, contributes to greater virus stability. The contamination of fresh produce can occur during handling by a contaminated food worker as well as in the field from contaminated soil, organic fertilizers, and irrigation water (Brassard et al., 2012; D'Souza and Joshi, 2016). The presence of enteric viruses has been reported in irrigation water (Randazzo et al., 2016), groundwater (Kauppinen et al., 2018), river water (Wyn-Jones et al., 2000), marine water (Gonçalves et al., 2018), lake water (Aslan et al., 2011), and bottled water (Blanco et al., 2017). In aquatic environments, the type of virus and the water temperature are the main general factors that influence virus persistence (Seymour and Appleton, 2001). The presence of organic material may influence virus survival by either increasing the stability of viruses or inactivating them by the action of virucidal substances, if present (Vasickova and Kovarcik, 2013).

Since enteric viruses may be difficult to grow in cell culture, molecular tools can be used to detect food contamination of viral origin. Means of evaluating the infectious potential of these particles are limited, but available approaches that have been used include enzymatic pretreatment before extraction and amplification of protected RNA from intact particles. In heat-treated samples, the use of proteinase K with sodium dodecyl sulfate and nucleases (RNase or DNase) to remove exposed viral RNA or DNA if the viral capsid is damaged has been applied with varying success in order to quantify nucleic acids of intact viral particles from MNV-1, HuNoV, adenovirus, and HAV (Baert et al., 2008; Marti et al., 2017) and from HAV, feline calicivirus, and poliovirus (Nuanualsuwan and Cliver, 2002, 2003). In all cases, the results reflect the extent of damage to the viral capsid associated with the cumulative treatments. Given the possibility that pretreatment such as the use of proteinase K may affect the capsid of intact viral particles (Topping et al., 2009), in our study only RNase treatment before RNA extraction and quantification was used.

In this study, three representative enteric viruses were selected: MNV-1, bovine rotavirus (boRV), and the attenuated laboratory-cultivable HAV strain. It has been argued that other strains may react differently, but information on the persistence of surrogates may provide indications that can be tested further and applied to other strains. The objective of the present study was to evaluate and compare, under the same conditions, the infectivity and RNA integrity of these three RNA viruses on three different commodities: stainless steel coupons, bottled spring water, and blueberries over a 21-day incubation period at domestic use temperatures of 21 °C, 4 °C, and −20 °C. The results would reflect the risk associated with the presence of viable enteric viruses.

2. Materials and methods

2.1. Viruses and cell lines

Murine norovirus strain 1 (MNV-1) and the MA-104 cell line (ATCC CRL-2378.1) were kindly provided by Dr. Y. L'Homme from the Canadian Food Inspection Agency, Saint-Hyacinthe, Quebec, Canada. The RAW 264.7 cell line (ATCC TIB-71), the FRhK-4 cell line (ATCC CRL-1688), and bovine rotavirus (boRV) strain C486 (ATCC VR-917) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Hepatitis A virus (HAV) cytopathic strain HM-175 was kindly provided by Dr. S. Bidawid, Bureau of Microbial Hazards, Health Canada, Ottawa, Ontario, Canada. The MNV-1 was propagated on RAW 264.7 cells (Gonzalez-Hernandez et al., 2012), the HAV HM-175 was propagated on FRhK-4 cells (Bozkurt et al., 2015), and the boRV was propagated on MA-104 cells (Arnold et al., 2009). Working viral suspensions were prepared as follows: MNV-1 was prepared at $10^{6.1}$ plaque-forming units (PFU)/ml in Dulbecco's Modified Eagle Medium (DMEM) (Wisent Bioproducts, St-Bruno, QC, Canada); boRV strain C486 was prepared at $10^{8.6}$ PFU/ml in Medium 199 (Wisent Bioproducts), and HAV HM-175 was prepared at $10^{7.1}$ PFU/ml in DMEM/F12 (Wisent Bioproducts).

2.2. Treatment of stainless steel surfaces, bottled water, and blueberries

Viral suspensions as well as positive and negative controls were included in each set of samples to be analyzed. Positive controls constituted values at time 0. Working viral suspensions of MNV-1, boRV, and HAV were also prepared as previously in DNase/RNase-free water (Wisent Bioproducts) and DMEM and were first incubated at 21 °C, 4 °C, or −20 °C for 1 h, 3, 7, 14 or 21 days, after which viability of the suspensions was assessed by plaque assay.

2.2.1. Stainless steel surfaces

A 100-μl aliquot of each working viral suspension was dropped on each of the stainless steel surfaces, which were coupons measuring 10 cm by 10 cm that had been cleaned and sterilized beforehand. The liquid was spread on the surfaces with a sterile inoculating loop and left to dry for 1 h at 21 °C in biosafety level 2 cabinets. All coupons were then transferred to plastic containers and incubated at 21 °C for 1 h, 3, 7, 14 or 21 days. After incubation, 532-ml-capacity Whirl-Pak Speci-Sponge bags (Fisher Scientific, Montréal, QC, Canada) were humidified with 5 ml of DMEM. Each surface was wiped with the humidified sponge and the sponge was returned to the bag. A total of 15 ml of DMEM was added to the bag, which was manually manipulated for 15–30 s to resuspend viral particles. The contents were transferred into an Amicon Ultra-15 centrifugal filter unit (EMD Millipore, Etobicoke, ON, Canada) for virus concentration and centrifuged for 15 min at 4000 × *g*. The concentrate (150 μl for MNV and HAV and 300 μl for boRV) was used as a viral suspension for viability determination and genomic copy quantification.

2.2.2. Bottled spring water

Working viral suspensions (250 μl/250 ml of water for MNV-1 and HAV or 500 μl/500 ml for boRV) were added to bottled drinking water bought at a local store and were then incubated at 21 °C (1 h, 3, 7, 14 or 21 days), at 4 °C (14 or 21 days) in glass bottles, or at −20 °C (14 or 21 days) in polypropylene plastic bottles. Viruses were recovered according to the method of Brassard et al. (2005). Briefly, the total contents of the water bottles were filtered on positively charged Zeta Plus 60S filter membranes, 0.45 μm (Cuno, Meriden, CT, USA), that had been humidified beforehand with 5 ml of sterile deionized water. Virus recovery was carried out by adding 10 ml of 2.9% tris-phosphate buffer supplemented with 6% glycine at pH 9.0 to the filter and incubating it at 21 °C with agitation for 30 min. The pH of the elution buffer was adjusted to between 7.0 and 7.4 with 1 M HCl. The elution buffers were concentrated (150 μl for MNV and HAV and 300 μl for boRV) using Amicon Ultra-15 centrifugal filter units (EMD Millipore) by centrifugation for 15 min at 4000 × *g*. The concentrate was used as a viral suspension for viability determination and genomic copy quantification.

2.2.3. Blueberries

Aliquots of 100 μl of working viral suspensions were dropped as small droplets of approximately 10 μl onto 25 g of organic blueberries. The fruits were incubated in a biosafety level 2 cabinet for 1 h to allow the viral suspension to dry and then incubated at 21 °C (1 h, 3 or 7 days), 4 °C (1 h, 3, 7, 14 or 21 days), or −20 °C (1 h, 3, 7, 14 or 21 days). After the incubation period, the blueberries were transferred into a sampling plastic bag (Whirl-Pak; Nasco, Newmarket, ON, Canada), and 20 ml of DMEM at 21 °C was added. The blueberries were gently swirled in the medium for 1 min. The medium was then transferred into 50-ml sterile tube and centrifuged at 5000 × *g* for 5 min. To remove remaining particles, the supernatants were filtered with 0.45-μm GD/X polyethersulfone filters and transferred in two consecutive steps into Amicon Ultra-15 centrifugal filter units (EMD Millipore) for concentration. The units were centrifuged for 15 min at 4000 × *g*. The concentrate (150 µl for MNV and HAV and 300 µl for boRV) was used as a viral suspension for viability determination and genomic copy quantification.

2.3. Viral plaque assay

2.3.1. MNV-1

Virus viability was determined according to Gonzalez-Hernandez et al. (2012). Briefly, six-well plates were seeded with 2×10^6 RAW 264.7 cells per well and then incubated in DMEM-10 (DMEM supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, penicillin at 100 U/ml, streptomycin at 100 µg/ml, 1 mM nonessential amino acids, and 2 mM L-glutamine) (Wisent Bioproducts) for 24 h at 37 °C with 5% CO_2. Before infection, 10-fold dilutions of the samples in DMEM-5 (DMEM supplemented with 5% FBS, 10 mM HEPES, penicillin at 100 U/ml, streptomycin at 100 µg/ml, 1 mM nonessential amino acids, and 2 mM L-glutamine) were prepared. The medium was removed from the plates, and 500 µl of the sample was added. The plates were incubated with gentle agitation at 21 °C for 1 h to ensure constant contact between the sample and the cell layer. Afterwards, the samples were removed, and 2 ml of melted warm overlay medium (Eagle's Minimum Essential Medium [EMEM] without phenol red with 5% FBS, 5 mM HEPES, penicillin at 50 U/ml, streptomycin at 50 µg/ml, 2 mM L-glutamine, and 1.5% SeaPlaque agarose [Lonza, Mississauga, ON, Canada]) was added to each well. The plates were placed at 21 °C for 10 min and then incubated at 37 °C with 5% CO_2 for 48–72 h. For plaque visualization, 2 ml of 0.33% neutral red solution in PBS was added to each well, and the plates were incubated at 37 °C with 5% CO_2 for 3 h. The neutral red solution was removed before plaque counts were performed.

2.3.2. boRV

Virus quantification by plaque assay was carried out according to the method of Arnold et al. (2009) for rotaviruses. Briefly, in six-well plates, 3×10^5 MA-104 cells/well were grown in Medium 199 supplemented with 5% FBS, penicillin at 100 U/ml, and streptomycin at 100 µg/ml, and the plates were incubated at 37 °C with 5% CO_2 for 3 days. To activate the viruses, 400 µl of the samples containing boRV were incubated with 4 µg of trypsin (Sigma, Oakville, ON, Canada) in PBS at 37 °C for 1 h. After 10-fold dilutions of 300 µl of the samples were prepared in serum-free Medium 199, 1 ml was used to infect cells. After removal of the samples, 3 ml of melted EMEM overlay containing 1% SeaPlaque agarose and trypsin at 0.5 µg/ml was added to each well. The plates were incubated at 37 °C with 5% CO_2 for 4 days. Plaque visualization was possible after incubation for 4–24 h following the addition of a second overlay containing EMEM supplemented with 1% agarose and neutral red at 25 µg/ml.

2.3.3. HAV

The viability of HAV was determined as described by Bozkurt et al. (2015) and modified for plaque visualization. Each well of the six-well plates was seeded with 3×10^5 FRhK-4 cells/well in DMEM/F12 medium supplemented with 10% FBS, penicillin at 100 U/ml, and streptomycin at 100 µg/ml, and the plates were incubated at 37 °C with 5% CO_2 for 3 days. A total of 500 µl from the 10-fold dilutions of samples in DMEM/F12 supplemented with 2% FBS, penicillin at 100 U/ml, and streptomycin at 100 µg/ml was used to infect cells. The plates were incubated at 37 °C with 5% CO_2 for 90 min. After sample removal, 2 ml of melted overlay medium containing EMEM, 4 mM L-glutamine, 0.22% sodium bicarbonate, 0.20% nonessential amino acids, penicillin at 100 U/ml, streptomycin at 100 µg/ml, and 1% SeaPlaque agarose was poured into each well. The plates were incubated at 37 °C with 5% CO_2 for 7 days. For plaque visualization, 2 ml of 0.33% neutral red solution in PBS was added to each well, and the plates were incubated at 37 °C with 5% CO_2 for 1 h. The neutral red solution was removed. The plates were further incubated at 37 °C with 5% CO_2 for 4–24 h before plaque counts were performed.

2.4. RNase pretreatment, RNA extraction, and molecular detection

A total of 140 µl of concentrated virus suspension was mixed with RNase A (10 µg/ml; Thermo Scientific, Walthan, Massachusetts, USA) and incubated at 37 °C for 15 min. Aliquots of 140 µl from virus samples treated and not treated with RNase A were extracted using a QIAamp Viral RNA Mini Kit (Qiagen Inc., Mississauga, ON, Canada). RT-qPCR detection of viral RNA was carried out in an Mx3005P thermocycler (Agilent Technologies, Santa Clara, CA, USA) according to the conditions reported in previous studies, as follows: Baert et al. (2008) for MNV-1, Zeng et al. (2008) for boRV, and Houde et al. (2007) for HAV. Briefly, viral RNA was detected in 5-µl samples using a TaqMan RT-qPCR assay in a 25-µl final volume of Stratagene Brilliant II QRT-PCR Core Reagent Kit (Agilent Technologies). Incubation was performed at 50 °C for 30 min (for MNV and HAV) or 42 °C for 60 min (for boRV), and the PCR conditions for all samples were 95 °C for 10 min for initial denaturation, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Standard curves were included on each plate for quantification of all samples. All runs included a no-sample extraction control and no-template controls.

2.5. Statistics

Experiments were carried out in triplicate. Student's *t*-test was used to compare viability results from samples (with and without incubation) and to compare genomic content for the same sample (RNase and no-RNase treatments). Values of $P < 0.05$ were considered significant. Statistical analyses were performed using Microsoft Excel enhanced with the Analysis ToolPak.

3. Results

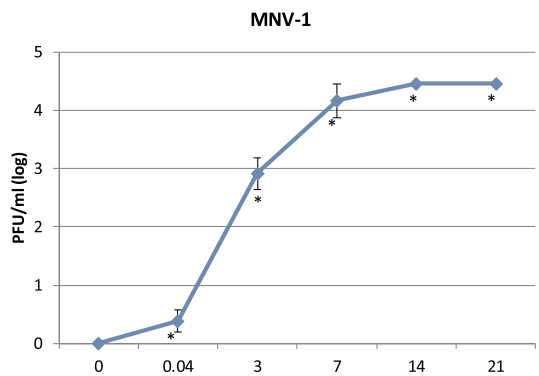
3.1. Effect of time and temperature on virus stability in culture medium and water

The first step consisted of evaluating the stability of viral infectivity over selected incubation times (1 h, 3, 7, 14, and 21 days) and at selected temperatures (at 21 °C, 4 °C, and −20 °C) (Supplementary Material 1) when the viruses were directly diluted in medium or water. These results reflect viral stability in the tested liquid diluents excluding the loss due to the recovery and concentration steps. After 21 days at 21 °C, MNV-1 showed a drop in viability of 3.94 $\log_{10}$ PFU/ml in DMEM and 2.92 $\log_{10}$ PFU/ml in water. At 4 °C, MNV-1 was stable in DMEM, and a loss of 2.55 $\log_{10}$ PFU/ml was observed in water. No loss was observed at −20 °C. In water or medium, boRV showed no loss of infectivity at 21 °C, 4 °C, or −20 °C. At 4 °C and −20 °C, HAV showed no loss of infectivity, but at 21 °C, the infectivity of HAV was reduced by 2.64 $\log_{10}$ PFU/ml in DMEM and 3.09 $\log_{10}$ PFU/ml in water.

3.2. Viral persistence after contact with stainless steel surfaces

For each sample, viral infectivity evaluated by plaque assay and genomic copies of viral RNA were determined. To determine the effect of the stainless steel on viability, virus recovery data (PFU/ml) after the incubation period were compared to the positive control (PFU/ml) used for the experiment (time 0), where viral suspensions were immediately mixed in DMEM with the Speci-Sponge.

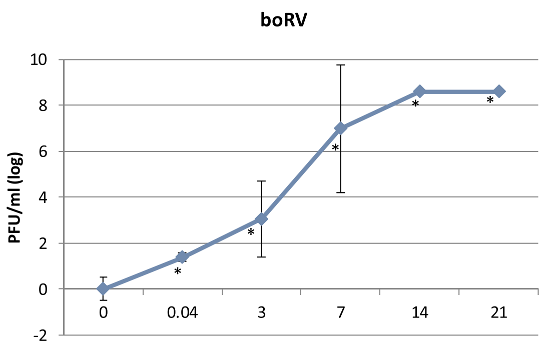
For MNV-1, a 2.91 $\log_{10}$ decrease in viability was observed after 3 days of incubation, and no viability was detected on days 14 and 21 (Fig. 1A). The infectivity of boRV dropped by 1.39 $\log_{10}$ after 1 h, by 3.05 $\log_{10}$ after 3 days, and by 6.99 $\log_{10}$ after 7 days and was absent after 14 days (Fig. 1C). The infectivity of HAV was maintained after 7 days, but a loss of 1.45 $\log_{10}$ was observed after 21 days of incubation (Fig. 1E).



A

B

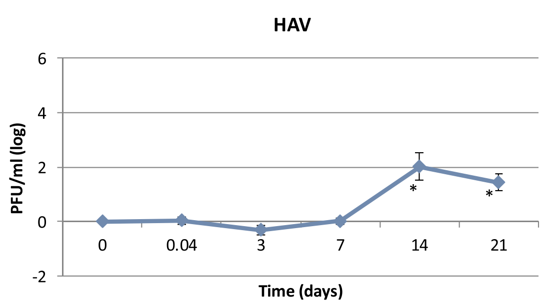
Days	MNV-1 RNase - GC/ml	sd	RNase + GC/ml	sd
0	**9.44**	*0.23*	**8.99**	*0.03*
0.04	**8.93**	*0.28*	**8.01**	*0.82*
3	**8.31**	*0.29*	**7.62**	*0.18*
7	**8.08**	*0.26*	**7.71**	*0.12*
14	**6.87**	*1.34*	**6.79**	*1.11*
21	**6.96**	*0.74*	**6.51**	*1.56*



C

D

Days	boRV RNase - GC/ml	sd	RNase + GC/ml	sd
0	**8.00**	*0.27*	**7.93**	*0.31*
0.04	**7.31**	*0.49*	**7.03**	*0.68*
3	**7.32**	*0.71*	**7.24**	*0.64*
7	**6.23**	*1.74*	**6.36**	*1.01*
14	**7.05**	*1.28*	**6.83**	*0.89*
21	**7.48**	*0.41*	**7.22**	*0.32*



E

F

Days	HAV RNase - GC/ml	sd	RNase + GC/ml	sd
0	**7.50**	*0.65*	**7.28**	*0.72*
0.04	**7.73**	*0.01*	**7.40**	*0.26* *
3	**7.90**	*0.09*	**7.63**	*0.12*
7	**7.84**	*0.12*	**7.71**	*0.13*
14	**6.84**	*0.16*	**6.46**	*0.42*
21	**6.58**	*0.08*	**6.11**	*0.35*

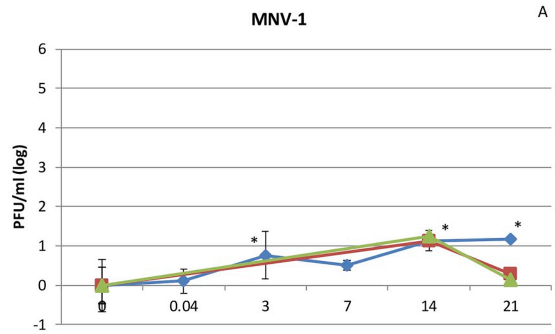
Fig. 1. Persistence of MNV-1 (A, B), boRV C486 (C, D), and HAV (E, F) dried on stainless steel coupons incubated at 21 °C. A, C, and D: Infectivity reduction of recovered viruses (PFU/ml [log]) in comparison with time 0, where each dot averages values from three experiments. An asterisk (*) indicates a statistically different result. B, D, and F: Genomic copies (GC) (log/ml) of each sample without (RNase −) and with RNase treatment (RNase +). The abbreviation "sd" represents the standard deviation from three experiments. An asterisk (*) indicates that the results from RNase − and RNase + samples are statistically different.

For MNV-1, a 21-day incubation resulted in RNA decreases of 2.57 $\log_{10}$ and 2.48 $\log_{10}$ in samples without (RNase −) and with (RNase +) enzymatic RNase pretreatment, respectively (Fig. 1B). For boRV, the loss in RNA was under 1 $\log_{10}$ for RNase − and RNase + samples over the entire duration of the experiment (Fig. 1D). The amounts of HAV RNA were similar up to 7 days of incubation, but over the 21-day period of the experiment, losses of 0.71 $\log_{10}$ (RNase −) and 0.84 $\log_{10}$ (RNase +) were observed (Fig. 1F). For MNV-1, boRV, and HAV samples dried on stainless steel coupons at 21 °C for 1 h up to 21 days, no correlation was observed between total PFUs per milliliter in samples and the detected viral genomic copies per milliliter with or without RNase pretreatment. No significant difference in the quantification between RNase-treated and -untreated samples could be observed over the 21 days of incubation.

3.3. Stability of viral infectivity in bottled spring water

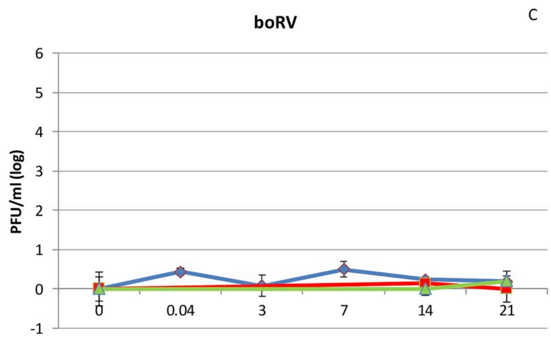
A significant progressive viability loss of 1.18 $\log_{10}$ was observed for MNV-1 incubated for 21 days at 21 °C (Fig. 2A). At 4 °C and −20 °C, viability decreased by 0.28 $\log_{10}$ and 0.14 $\log_{10}$, respectively. BoRV diluted in bottled water and incubated for 21 days showed no detectable infectivity loss at 21 °C, a loss of 0.17 $\log_{10}$ at 4 °C and of 0.68 $\log_{10}$ at −20 °C (Fig. 2C). At 21 °C, 4 °C, and −20 °C, HAV HM-175 showed viability losses of 1.99 $\log_{10}$, 0.58 $\log_{10}$, and 0.49 $\log_{10}$, respectively (Fig. 2E).

For MNV-1, the slight decrease in genomic copies per milliliter was observed only at 21 °C, with losses of 0.20 $\log_{10}$ (RNase −) and 0 $\log_{10}$ (RNase +) (Fig. 2B). The detected boRV viral nucleic material remained constant with no significant drop over 21 days at 21 °C (RNase − and RNase +), 4 °C (RNase −: 0.25 $\log_{10}$; RNase +: 0.12 $\log_{10}$), and −20 °C (RNase −: 0.24 $\log_{10}$; RNase +: 0.18) (Fig. 2D). A decrease in genomic copies per milliliter for HAV was observed over 21 days at 21 °C



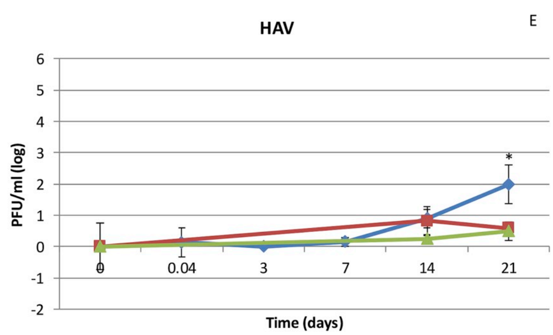
B

	MNV-1				
	Days	RNase -		RNase +	
		GC/ml	sd	GC/ml	sd
	0	**8.72**	*0.33*	**7.73**	*0.47*
21°C	0.04	**8.81**	*0.35*	**8.12**	*1.05*
	3	**8.12**	*0.35*	**7.45**	*0.59*
	7	**8.58**	*0.36*	**7.86**	*0.61*
	14	**8.24**	*0.22*	**7.71**	*0.12*
	21	**8.19**	*0.07*	**8.07**	*0.21*
4°C	14	**8.75**	*0.16*	**8.52**	*0.28* *
	21	**9.13**	*0.11*	**9.08**	*0.15*
-20°C	14	**8.97**	*0.20*	**6.88**	*0.93*
	21	**9.30**	*0.04*	**8.73**	*0.07* *



D

	boRV				
	Days	RNase -		RNase +	
		GC/ml	sd	GC/ml	sd
	0	**6.96**	*0.16*	**6.81**	*0.17*
21°C	0.04	**6.92**	*0.07*	**7.14**	*0.55*
	3	**7.17**	*0.12*	**6.76**	*0.31* *
	7	**7.11**	*0.23*	**6.84**	*0.45* *
	14	**7.16**	*0.19*	**7.33**	*0.23*
	21	**6.98**	*0.18*	**6.85**	*0.14*
4°C	14	**6.95**	*0.23*	**6.80**	*0.36*
	21	**6.71**	*0.12*	**6.69**	*0.18*
-20°C	14	**6.88**	*0.20*	**6.75**	*0.25*
	21	**6.72**	*0.09*	**6.63**	*0.13*



F

	HAV				
	Days	RNase -		RNase +	
		GC/ml	sd	GC/ml	sd
	0	**7.04**	*0.40*	**6.73**	*0.12*
21°C	0.04	**6.79**	*0.19*	**6.73**	*0.18*
	3	**6.47**	*0.47*	**6.29**	*0.49*
	7	**6.40**	*0.17*	**6.41**	*0.30*
	14	**6.40**	*0.27*	**6.28**	*0.19*
	21	**6.24**	*0.37*	**nd**	*nd*
4°C	14	**6.62**	*0.24*	**6.49**	*0.25*
	21	**5.94**	*0.81*	**5.83**	*0.55*
-20°C	14	**7.24**	*0.09*	**6.97**	*0.02* *
	21	**6.71**	*0.13*	**6.10**	*0.30*

Fig. 2. Persistence of MNV-1 (A, B), boRV C486 (C, D), and HAV) (E, F) in bottled drinking water incubated at 21 °C (◆), 4 °C (■), or −20 °C (▲). A, C, and D: Infectivity reduction of recovered viruses (PFU/ml [log]) in comparison with time 0, where each dot averages values from three experiments. An asterisk (*) indicates a statistically different result. B, D, and F: Genomic copies (GC) (log/ml) of each sample without (RNase−) and with RNase treatment (RNase+). The abbreviation "sd" represents the standard deviation from three experiments. An asterisk (*) indicates that the results from RNase− and RNase + samples are statistically different.

(RNase−: 0.80 $\log_{10}$; RNase+: 0.45 $\log_{10}$), 4 °C (RNase−: 1.10 $\log_{10}$; RNase+: 0.90 $\log_{10}$), and −20 °C (RNase−: 0.33 $\log_{10}$; RNase+: 0.63 $\log_{10}$) (Fig. 2F).

3.4. Stability of viral infectivity on fresh blueberries

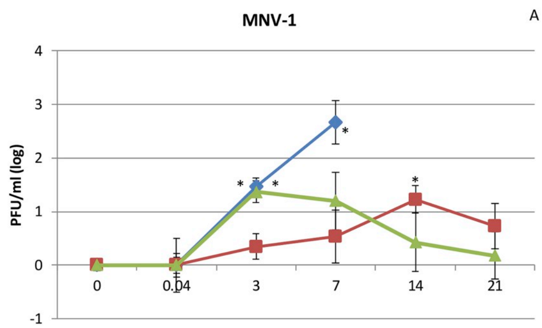
Viability and nucleic acid quantification were evaluated for MNV-1, boRV, and HAV on inoculated organically grown blueberries that were incubated at 21 °C, 4 °C, or −20 °C (Fig. 3). At 21 °C, blueberries kept their integrity up to 7 days, but deterioration and mold growth were considerable thereafter, and the fruits were then discarded.

The infectivity of MNV-1 dropped by 2.74 $\log_{10}$ after 7 days at 21 °C, by 0.66 $\log_{10}$ after 21 days at 4 °C, and by 0.09 $\log_{10}$ after 21 days at −20 °C (Fig. 3A). After an incubation of 7 days on blueberries, boRV lost 2.14 $\log_{10}$ in infectivity at 21 °C, and lost 0.73 $\log_{10}$ at 4 °C and 0.80 $\log_{10}$ at −20 °C after 21 days of incubation (Fig. 3C). After 21 days on blueberries incubated at 4 °C or −20 °C, HAV did not show any significant loss in infectivity (Fig. 3E).

After the last incubation times of 7 days at 21 °C and 21 days at 4 °C, the losses in genomic copies per milliliter for MNV-1 were minimal at 21 °C (RNase−: 0.39 $\log_{10}$; RNase+: 0.23 $\log_{10}$) and 4 °C (RNase−: 0.56 $\log_{10}$; RNase+: 0.65 $\log_{10}$) and remained similar at −20 °C (Fig. 3B). For boRV, genomic copies per milliliter declined after 7 days at 21 °C (RNase−: 0.39 $\log_{10}$; RNase+: 0.38 $\log_{10}$) and 21 days at 4 °C (RNase−: 0.23 $\log_{10}$; RNase+: 0.15 $\log_{10}$) (Fig. 3D). No loss was observed after 21 days at −20 °C. As was the case for HAV, no loss of genomic copies per milliliter was detected in the samples without RNase treatment, and the only loss, 0.33 $\log_{10}$, was observed in the samples with RNase treatment incubated for at 21 °C (Fig. 3F).

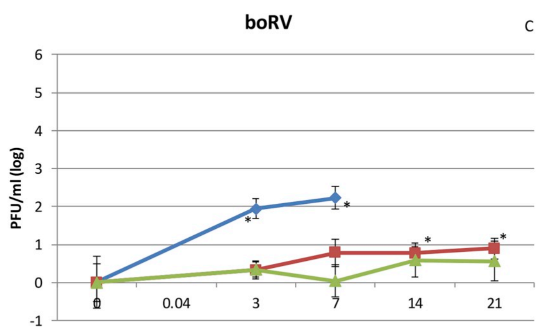
4. Discussion

The persistence of enteric viruses has been widely reported in different food commodities and on different surfaces and has been studied



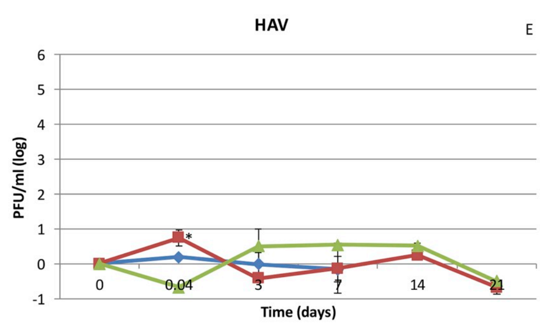
B

	MNV-1					
	Days	RNase -		RNase +		
		GC/ml	sd	GC/ml	sd	
	0	**9.32**	*0.25*	**8.78**	*0.32*	
21°C	0.04	**9.73**	*0.64*	**8.48**	*1.08*	
	3	**8.29**	*0.69*	**8.11**	*0.94*	
	7	**8.93**	*0.34*	**8.55**	*0.38*	
4°C	0.04	**9.46**	*0.26*	**8.88**	*0.38*	
	3	**9.54**	*0.23*	**9.40**	*0.21*	
	7	**9.34**	*0.43*	**8.95**	*0.33*	
	14	**8.33**	*0.24*	**7.75**	*0.39*	*
	21	**8.76**	*0.59*	**8.13**	*0.47*	
-20°C	0.04	**9.17**	*0.54*	**8.48**	*0.13*	
	3	**8.48**	*0.73*	**8.19**	*0.51*	
	7	**8.84**	*0.26*	**8.32**	*0.23*	
	14	**9.36**	*0.13*	**9.11**	*0.33*	
	21	**9.62**	*0.04*	**9.04**	*0.82*	



D

	boRV					
	Days	RNase -		RNase +		
		GC/ml	sd	GC/ml	sd	
	0	**8.6**	*0.08*	**8.37**	*0.10*	
21°C	3	**8.22**	*0.16*	**7.97**	*0.11*	
	7	**8.21**	*0.25*	**7.99**	*0.28*	
4°C	3	**8.53**	*0.30*	**8.22**	*0.24*	
	7	**8.41**	*0.08*	**8.28**	*0.13*	*
	14	**8.60**	*0.38*	**8.43**	*0.38*	
	21	**8.37**	*0.08*	**8.22**	*0.15*	*
-20°C	3	**8.78**	*0.30*	**8.26**	*0.06*	
	7	**8.75**	*0.24*	**8.55**	*0.25*	
	14	**8.37**	*0.12*	**8.04**	*0.16*	
	21	**8.83**	*0.10*	**8.53**	*0.09*	*



F

	HAV					
	Days	RNase -		RNase +		
		GC/ml	sd	GC/ml	sd	
	0	**7.16**	*0.35*	**6.78**	*0.43*	
21°C	0.04	**7.03**	*0.26*	**6.56**	*0.35*	
	3	**8.20**	*0.08*	**7.95**	*0.18*	*
	7	**7.61**	*0.40*	**6.45**	*0.83*	
4°C	0.04	**6.56**	*0.18*	**5.13**	*0.70*	
	3	**7.93**	*0.40*	**7.43**	*0.35*	
	7	**7.92**	*0.61*	**7.69**	*0.56*	
	14	**7.76**	*0.40*	**7.84**	*0.14*	
	21	**7.93**	*0.53*	**7.11**	*0.61*	
-20°C	0.04	**8.33**	*0.74*	**7.36**	*1.82*	
	3	**7.75**	*0.75*	**7.03**	*0.95*	
	7	**7.62**	*0.19*	**7.45**	*0.16*	
	14	**7.96**	*0.32*	**7.48**	*0.20*	
	21	**8.55**	*0.17*	**8.10**	*0.14*	

Fig. 3. Persistence of MNV-1 (A, B), boRV C486 (C, D), and HAV (E, F) dried on blueberry surfaces incubated at 21 °C (◆), 4 °C (■), and −20 °C (▲). A, C, and D: Infectivity reduction of recovered viruses (PFU/ml [log]) in comparison with time 0, where each dot averages values from three experiments. An asterisk (*) indicates a statistically different result. B, D, and F: Genomic copies (GC) (log/ml) of each sample without and with RNase treatment. The abbreviation "sd" represents the standard deviation from three experiments. An asterisk (*) indicates that the results from RNase-untreated (RNase−) and RNase-treated (RNase+) samples are statistically different. No results are available after 7 days at 21 °C because of the presence of mold.

with different viral strains under different conditions and with different extraction methods (Cook et al., 2016; D'Souza and Joshi, 2016; Vasickova and Kovarcik, 2013), all of which makes comparison difficult. For the agri-food and food-processing industries, identification of a viral surrogate that provides the best persistence under production conditions that are close to real life and throughout the food's shelf life would be very useful for evaluating potential control measures. When a viral surrogate has low persistence, the efficacy of treatments and mitigation measures could be overestimated. It is therefore important to compare the persistence of several laboratory-cultivable viral surrogates under the same conditions, with the same methods, and in the same matrices. In this study, experiments were carried out under the same conditions for the three enteric viruses from different virus families, thus making comparison possible.

At first, the effect of incubation time on the infectivity of the three viruses diluted in water and medium and incubated at three temperatures was evaluated. The residual infectivity was immediately assessed to determine viral survival. Subsequently, the viruses needed to be recovered from the stainless steel surfaces, bottled water, and blueberries and concentrated causing some losses generated through the recovery processes. Therefore, our results show the cumulative impact of time, the environment, and recovery processes on infectivity. Incubation of MNV-1 diluted in DMEM for 21 days at ambient temperature (21 °C) caused a loss of 3.94 $\log_{10}$ (Supplementary Material 1A), and when the virus was diluted in medium spread on stainless steel coupons the loss was 4.46 $\log_{10}$. Time, temperature and the incubation on stainless steel all played a role in the decline of MNV-1 viability. Previous studies have reported that MNV loses viability on stainless steel (Cannon et al., 2006;

Kim et al., 2014; Takahashi et al., 2011; Warnes et al., 2015). The duration of incubation and the elution and concentration approaches in those studies vary, but all described a significant decline in viability. The viral load of the inoculum, the virus recovery method used, the incubation time, the temperature and relative humidity, the presence of food residues that may protect virus viability, and the composition and degree of roughness of the stainless steel are all factors that influence virus recovery and may explain discrepancies in results (Turnage and Gibson, 2017; Vasickova et al., 2010).

In this study, boRV incubated for 21 days in water or medium showed a loss in infectivity of only 0.57 log (Supplementary Material 1D), but when boRV dried on stainless steel, viability was not detected after 14 days. Rotaviruses are usually more stable in the environment. They can remain infectious for 2 months on surfaces (Vasickova et al., 2010). The presence of organic matter may preserve rotavirus infectivity. In a previous study, rotavirus SA-11 diluted in water and in 10% stool diluted in water was used to inoculate countertop surfaces by spraying. Following an incubation of 45 min, a loss greater than 3 $\log_{10}$ was measured in the sample diluted in water, and the same loss took twice the time in the presence of feces, which was shown to increase virus survival (Keswick et al., 1983). In our study, the total loss in infectivity may be linked to the viral strain itself and to the fact that the viruses were diluted in culture medium, limiting the protective effect of organic matter.

Previous studies have reported that HAV is resistant from 28 days to approximately 2 months on inanimate surfaces (Bae et al., 2014; Kramer, 2006; Kramer and Assadian, 2014). When 0.05 M glycine buffer with 0.14 M NaCl was used for virus recovery, the viability of HAV tested on various food contact surfaces, including stainless steel, and incubated for 28 days at 21 °C showed a 2.3 $\log_{10}$ loss of PFUs per coupon, and the viruses were still infectious at the end of the incubation (Bae et al., 2014). Similarly, in our study, HAV viability was reduced by 1.48 $\log_{10}$ after 21 days of incubation on stainless steel coupons. Of the three viruses under study, HAV is the only one that remained viable on stainless steel after a 21-day incubation period, but after 3 days, MNV-1 and boRV were still present in a sufficient number for transmission by cross-contamination. Without proper sanitation, the presence of these remaining viable viruses may lead to contamination of fresh food.

Another study reported the detection of HAV in experimentally seeded mineral water after 300 days of incubation at ambient temperature (Biziagos et al., 1988). Bovine rotavirus has been detected in experimentally seeded raw water, tap water, and 0.22-μm filtered tap water after incubation at 4 °C or 20 °C for 64 days (Raphael et al., 1985). According to Biziagos et al. (1988), HAV survived a 1-year incubation period in mineral water incubated at 4 °C and completely disappeared after 1 year of incubation at ambient temperature. The presence of proteins may increase virus stability in mineral waters (Biziagos et al., 1988). In our study, in aqueous suspensions as well as in bottled spring water, 21 days of incubation at 21 °C, 4 °C, or −20 °C did not result in a significant loss of boRV infectivity (Supplementary Material 1D, E, and F and Fig. 2C). Both MNV-1 and HAV showed a decline in infectivity at 21 °C (Fig. 2A and E). Although the follow-up was carried out over a short period, and that some virus absorption to the bottles cannot be ruled out, these results corroborate those of other studies that brought to light that lower temperatures contribute to greater persistence of infectivity (Seymour and Appleton, 2001; Vasickova and Kovarcik, 2013).

Since enteric viruses have been detected in irrigation water, their presence is possible on berries, leafy greens, and produce. In recent years, many cases of food poisoning have been linked to the consumption of fresh fruits and vegetables as well as frozen berries (Brassard et al., 2012; Calder et al., 2003; Marti et al., 2017). Contaminations were not only related to irrigation but also due to hand-picking. Therefore, fruits can be contaminated during harvest and before marketing. In our study, after 7 days of incubation at ambient temperature (21 °C), the blueberries started to deteriorate and the presence of contamination intensified, making the fruits less attractive to consumers. For MNV-1 and boRV, infectivity started to drop (> 1.5 $\log_{10}$) after 3 days, but they were both still infectious after 7 days. Since viability dropped by 2.16 $\log_{10}$ when MNV-1 was incubated at 21 °C for 7 days in medium alone (Supplementary Material 1A), viability loss on the blueberries may have been attributed mostly to the duration of incubation. No loss of infectivity was observed for HAV after 7 days. At ambient temperature, the infectivity of the three viruses under study exceeded the shelf life of the blueberries. At 4 °C or −20 °C, the blueberries were still firm. Our results show that MNV-1, boRV, and HAV were all infectious after a 21-day incubation period when dried on refrigerated or frozen blueberries. Infectivity loss was less than 1 $\log_{10}$. These results show that contaminated blueberries may pose a threat if they are ingested after being kept at any of these three storage temperatures. These results are in line with those of Butot et al. (2008), who reported that storage of contaminated blueberries under freezing conditions for 90 days had limited impact on the infectivity of HAV but who observed a reduction of 1.2 $\log_{10}$ for rotavirus after 2 days. In another study, MNV-1 spiked on raspberries and strawberries and incubated at 4 °C, 10 °C, or 21 °C yielded similar results. Infectivity was maintained at 10 °C and −20 °C, but at 21 °C, reductions of 1.1 log and 1.4 log were observed on raspberries and strawberries, respectively (Verhaelen et al., 2012). For consumers, eating fresh, refrigerated, or frozen blueberries contaminated with enteric viruses may lead to illness. As reported by Butot et al. (2008), washing HAV- and HuRV-infected blueberries with tap, warm, or chlorinated water had some limited effect. Other avenues for virus inactivation are being explored for berries, namely, exposure to 405-nm monochromatic light (Kingsley et al., 2018a), UV-C light (Butot et al., 2018), pulse light (Huang et al., 2017), gaseous ozone (Brie et al., 2018), gaseous chlorine dioxide (Kingsley et al., 2018b), oxidizing disinfectants (Girard et al., 2016), high hydrostatic pressure (Huang et al., 2016), and heat-treated lysozyme (Takahashi et al., 2018).

According to our results, the use of HAV and boRV as models for virus inactivation processes for food would probably be appropriate since those viruses demonstrated greater persistence on blueberries under all conditions tested. Furthermore, to ensure that inactivation treatments are effective, more than two viruses from different families must be tested simultaneously, such as rotavirus, calicivirus, and HAV. With a size similar to most foodborne viruses and easily produced, bacteriophages can also be used as surrogates for foodborne viruses (Baert, 2013). In the majority of conditions tested in our study, MNV-1 had low persistence in comparison with the other two viruses, yet MNV is still widely used as a surrogate and is often used alone in inactivation studies. There is thus some question about the effectiveness of the treatments in those studies on other, more persistent viruses. In some food matrices, such as oysters, Tulane virus may be more appropriate than MNV to represent the calicivirus family (Araud et al., 2016; Drouaz et al., 2015; Polo et al., 2018).

Using culture methods to evaluate virus viability is the gold standard but is not feasible for all viruses. Molecular methods widely used for virus detection combined with enzymatic treatment have recently been considered for evaluating the capacity of the capsid to protect the viral RNA after heat treatment (Topping et al., 2009). Incubation with RNase before nucleic acid extraction followed by RT-qPCR has been used to determine whether the heat-treatment conditions for feline calicivirus and norovirus exposed all RNA and inactivated the viruses (Topping et al., 2009). The presence of residual RNA was detected in our study, in accordance with Escudero et al. (2012), who found a decrease of 3–4 log in infectivity for MNV-1 during a 21-day incubation period on surfaces even though viral RNA was detected over 42 days of incubation. In this study, the combination of viability values (PFU/ml) and the respective genomic copies per milliliter (GC/ml) highlights the portion of viral particles that are intact but are not infectious. The membrane integrity of some of the noninfectious particles was not compromised, and the membranes were not permeable to RNA release even though necessary components for infectivity were missing. In that context, Baert et al. (2008) reported that 100 times more RNA than PFU values was detected for MNV-1 after heat exposure. In our study, for MNV-1 dried on stainless steel coupons, no viable particles were detected after 14 days even though close to 6.87 $\log_{10}$ of RNA was still detected. The disparity between those two values may be linked to the fact that, although the viral particles were not permeable to RNase, they were defective after treatment and were missing essential components for infection to take place. On the other hand, the difference in average values between genomic copies per milliliter and PFUs per milliliter in the starting material reveals the portion of noninfectious viruses that are still intact, since RNAs are not removed by RNase treatment. These represent defective particles that have lost their infective capacities, possibly during production steps.

In conclusion, these three enteric viruses survived for more than 3 days at ambient temperature when dried on stainless steel and for 21 days at 21 °C, 4 °C, and −20 °C in bottled water and on blueberries. The residual infectious viral particles may pose a risk of gastrointestinal illness. The results of our study show that even though all three viruses are enteric viruses, their persistence varied with temperature and the nature of the commodity. From the perspective of the food industry, the best indicator viruses for assessing the safety of a food process for viral inactivation or elimination may be different depending on the commodity.

Declarations of interest

None

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103257.

Supplementary Material 1. Persistence of infectious murine norovirus (MNV-1), bovine rotavirus (boRV) strain C486, and hepatitis A virus (HAV) incubated at 21 °C, 4 °C, or −20 °C. ■: diluted in water; ●: diluted in medium.

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