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Short communication

Viability and heat resistance of murine norovirus on bread

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ABSTRACT

Contaminated bread was the cause of a large-scale outbreak of *norovirus* disease in Japan in 2014. Contamination of seafood and uncooked food products by *norovirus* has been reported several times in the past; however the outbreak resulting from the contamination of bread products was unusual. A few reports on the presence of *norovirus* on bread products are available; however there have been no studies on the viability and heat resistance of *norovirus* on breads, which were investigated in this study. ce:italic>/ce:italic> strain 1 (MNV-1), a surrogate for human *norovirus*, was inoculated directly on 3 types of bread, but the infectivity of MNV-1 on bread samples was almost unchanged after 5 days at 20 °C. MNV-1 was inoculated on white bread that was subsequently heated in a toaster for a maximum of 2 min. The results showed that MNV-1 remained viable if the heating period was insufficient to inactivate. In addition, bread dough contaminated with MNV-1 was baked in the oven. Our results indicated that MNV-1 may remain viable on breads if the heating duration or temperature is insufficient.

1. Introduction

Norovirus is a primary cause of acute gastroenteritis (Koopmans and Duizer, 2004) and numerous cases of mass *norovirus* gastroenteritis have been reported globally. *Norovirus* outbreaks have occurred in areas where people tend to gather, such as in hospitals, schools, and restaurants (Kroneman et al., 2008; Vivancos et al., 2010; Xu et al., 2013). This is also the case in Japan; *norovirus* contamination of food products was involved in 31% of foodborne diseases in 2014 (Ministry of Health, Labour and Welfare, 2014), having a significant economic impact and human disease burden. Apart from *norovirus* gastroenteritis via oral route by ingestion of contaminated food or beverages, *norovirus* can also spread to others via feces or vomits of infected people (Koopmans and Duizer, 2004). *Norovirus* is extremely infectious, with less than ten viral particles required to cause onset of symptoms (Teunis et al., 2008).

There have also been several cases of *norovirus* detected in aqueous environments, such as seawater (Hellmér et al., 2014; Katayama et al., 2008), and bivalve mollusk species caught as seafood have also been reported as a primary source of *norovirus* gastroenteritis (Koopmans and Duizer, 2004). In addition, *norovirus* has also been detected in ready-to-eat (RTE) food products (Malek et al., 2009). In the case of RTE food products in particular, it is necessary to exercise caution since consumers often eat them without heating (Todd et al., 2007). Reports focusing specifically on foodservice workers who transferred the virus to food products are available (D'Souza et al., 2006; Maritschnik et al., 2013; Rönnqvist et al., 2014; Verhaelen et al., 2013). In the case of a mass outbreak of *norovirus* in Japan in 2014, bread contaminated with *norovirus* which transferred from a worker at the manufacturing plant infected 1,271 people including elementary school children (National Institute of Infectious Diseases, 2014).

Currently, several reports on *norovirus*-contaminated bread products are available. de Wit et al. (2007) reported that the cause of a 2007 outbreak in Holland was a worker in the bread manufacturing plant. Additionally, efforts to quantify *norovirus* present in bread using real-time PCR to improve recovery methods have also been reported (Arita et al., 2008). However, a study of *norovirus* viability in bread has not yet been conducted.

To this end, an investigation of the viability of ce:italic>/ce:italic> strain 1 (MNV-1) as a surrogate for human *norovirus* on bread products was conducted. As there is currently no established cultivation method for human *norovirus*, a common procedure employing cultivatable MNV-1 was used to measure the level of infectivity. MNV-1 also belongs to the family *Caliciviridae*, genus *norovirus* (Karst et al., 2003), and being more closely related to human *norovirus* in comparison to other viruses (Wobus et al., 2006), it has been reported as preferable for use as a surrogate virus (Cannon et al., 2006; Hirneisen and Kniel, 2013a). In this study we used MNV-1 as a surrogate virus and assessed the viability and heat resistance of the virus inoculated on breads as well as the transfer of MNV-1 from gloved hands to the breads.

2. Materials and methods

2.1. Bread samples

In this study, we used 3 types of breads, purchased from a retail store, namely butter-rich roll, French bread, and white bread.

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Characteristics of bread samples are measured by pH spear (WD-35,634–40, OAKTON, VIC, AUS) and water activity meter (AQUALAB CX-3, Decagon Devices, WA, USA) (Table 1).

2.2. Virus and cells

In this study, ce:italic>/ce:italic> (MNV-1) was used as a surrogate for human *norovirus*.

Murine macrophage cells (RAW 264.7) cultured in Dulbecco's modified Eagle's medium (DMEM) which contains 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin were infected with MNV-1 at 0.1 multiplicity of infection (MOI) and cultured for 3 days at 37 °C in 5% CO_2. After development of cytopathic effect, the cells were frozen and thawed 4 times and centrifuged at 8,000 ×*g* for 20 min for the virus particles to be purified. Infectivity was measured by plaque assay as described below (Section 2.7) and the extracted virus and cell cultures solution was stored at −80 °C until use as MNV-1 solution.

2.3. Viability of MNV-1 on bread

The surface of bread samples (10 g) of each type (butter-rich roll, French bread, and white bread) were inoculated with 100 μL MNV-1 solution at 7.3 log PFU/mL (6.3 log PFU) onto crusts and inside the breads, then dried at room temperature for 30 min.

Breads, incubated at 20 °C, were sampled at 0, 1, 3, 5 days post-inoculation. To 10 g of the bread samples, 90 mL of sterile phosphate buffered saline (PBS) was added and homogenized at 230 rpm for 30 s with a stomacher. Then, the mixture was centrifuged at 8,000 ×*g* for 10 min and filtrated with CA filter (0.20 μm pore size, Advantec, Tokyo, Japan). The samples were stored at −80 °C before plaque assay.

2.4. Transfer of MNV-1 from gloved hands to bread

A hundred microliters of MNV-1 solution at a concentration of 6.6 log PFU/mL (5.6 log PFU) were inoculated onto the palm of a gloves (Diamond Grip Plus, Microflex, NV, USA). Following dried at room temperature for 1 h or without drying, 10 g bread (butter-rich roll, French bread, and white bread) was grabbed with the inoculated gloved-hand for 5 s. Grabbed breads were diluted with 90 mL of PBS and homogenized as mentioned above. After the filtration, the samples were stored at −80 °C before plaque assay.

2.5. Inactivation of MNV-1 on the white bread surface by toasting

A hundred microliters of MNV-1 solution at a concentration of 7.3 log PFU/mL (6.3 log PFU) were inoculated onto the white bread and dried at room temperature for 30 min. After air-drying, the bread was heated using an 800-W household toaster (DOT-240; Dohshisha, Osaka, Japan). The breads were sampled at 30, 60, 90, and 120 s, and the homogenization, centrifugation, filtration, and the storage were done with the methods described above. Temperature of bread surface was measured by thermal data acquisition system (E830, Takara Thermistor Co., Iwate, Japan).

Table 1
Property of the breads used in this study.

		pH	Aw*
Butter-rich roll	Crust	4.71 ± 0.028	0.907 ± 0.001
	Inside		0.917 ± 0.001
French bread	Crust	5.02 ± 0.048	0.877 ± 0.013
	Inside		0.936 ± 0.009
White bread	Crust	4.58 ± 0.045	0.928 ± 0.005
	Inside		0.967 ± 0.001

Values are expressed as mean ± SEM ($n = 3$).
* Aw: water activity.

2.6. Inactivation of MNV-1 in bread dough by baking

Bread dough was prepared by mixing ingredients in Table 2 using a bread machine. The dough was leavened at room temperature for 30 min, and divided into 25 g each. Then, 100 μL MNV-1 at 6.8 log PFU/mL (5.8 log PFU) was inoculated onto the flattened dough, which was then rolled up into a ball.

Each dough was baked in an oven at 130 °C, 160 °C, and 190 °C until the weight of the bread dough decreased by 10%; 12 min for 130 °C, and 10 min for 160 °C and 190 °C. The dough baked at 130 °C was sampled at 2, 5, 8, 10, and 12 min, and the dough baked at 160 °C and 190 °C were sampled at 2, 5, 8, and 10 min. Then 225 mL of PBS was added to each of the dough samples. The homogenization, centrifugation, filtration, and the storage were done with the methods described above. Temperature of bread dough was also measured by thermal data acquisition system as mentioned above.

2.7. Plaque assay

We determined viral infectivity by plaque assay (Gonzalez-Hernandez et al., 2012). RAW 264.7 cell was seeded into a 6-well plate (Falcon, Life Science, MA, USA) at 1×10^6 cells/mL. The plate was incubated at 37 °C in 5% CO_2 for 18 h.

Viral samples were diluted in 10% FBS-DMEM, and 500 μL aliquots were inoculated into a RAW 264.7 plate. Then the plate was incubated at room temperature with shaking for 1 h. After incubation, viral samples were layered with 2 mL of 1.5% SeaPlaque Agarose (Lonza, Valais, Switzerland) + 10% FBS-DMEM. The plates were incubated at 37 °C in 5% CO_2 for 48 h.

Finally, 2 mL of 0.03% Neutral Red-PBS was added to the plates and incubated at 37 °C in 5% CO_2 for 1 h. The plaques were then counted.

2.8. Statistical processing

All experiments were performed in triplicate, and results are shown as mean ± standard error. Significant differences were tested by Duncan's method using MS Excel.

3. Results

3.1. Viability of MNV-1 on bread

Viability of MNV-1 directly inoculated on the surface of French bread, butter-rich roll, and white bread indicated that the infectivity of MNV-1 declined slightly after 5-day incubation, with no difference between the different bread types ($p > 0.01$) (Fig. 1). MNV-1 in the interior of the butter-rich roll exhibited greater viability than on the crust after a 5-day period ($p < 0.01$) (Fig. 1a), but in the cases of the French bread (Fig. 1b) and white bread (Fig. 1c), no statistically significant difference in viable MNV-1 titer was observed between the crust and interior regions of the bread ($p > 0.01$).

3.2. Transfer of MNV-1 from gloved hands to bread

Latex gloves contaminated with 5.6 log PFU of MNV-1 then dried up for 1 h or without drying were used to handle bread to evaluate transfer

Table 2
Ingredients recipe for making dough.

Ingredient	
Wheat flour	250 g
Water	170 mL
Dried yeast	3 g
Sugar	30 g
Salt	5 g
Butter	15 g