International Journal of Food Microbiology 233 (2016) 29–33

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Short communication

Viability of murine norovirus in salads and dressings and its inactivation using heat-denatured lysozyme

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ARTICLE INFO

Article history:
Received 29 January 2016
Received in revised form 29 May 2016
Accepted 5 June 2016
Available online 7 June 2016

Keywords:
Murine norovirus
Viability
Inactivation
Salads
Lysozyme

ABSTRACT

In recent years, a number of food poisoning outbreaks due to the contamination of norovirus in ready-to-eat (RTE) foods such as salads have been reported, and this issue is regarded as a global problem. The risk of contamination of fresh vegetables with norovirus has been previously reported, but the survivability of norovirus that contaminates salads remains unknown. In addition, there have been limited reports on the control of norovirus in food products by using inactivating agents. In this study, the viability of norovirus in various types of salads and dressings was examined using murine norovirus strain 1 (MNV-1) as a surrogate for the closely related human norovirus. In addition, the inactivation of MNV-1 in salads was examined using heat-denatured lysozyme, which had been reported to inactivate norovirus. MNV-1 was inoculated in 4 types of salads (coleslaw, thousand island salad, vinaigrette salad, egg salad) and 3 types of dressings (mayonnaise, thousand island dressing, vinaigrette dressing), stored at 4 °C for 5 days. The results revealed that in the vinaigrette dressing, the infectivity of MNV-1 decreased by 2.6 log PFU/mL in 5 days, whereas in the other dressings and salads, the infectivity of MNV-1 did not show any significant decrease. Next, 1% heat-denatured lysozyme was added to the 4 types of salads, and subsequently it was found that in 2 types of salads (thousand island salad, vinaigrette salad), the infectivity of MNV-1 decreased by >4.0 log PFU/g, whereas in coleslaw salad, a decrease of 3.0 log PFU/g was shown. However, in egg salads, the infectivity of MNV-1 did not show such decrease. These results suggest that norovirus can survive for 5 days in contaminated salads. Further, these findings also indicated that heat-denatured lysozyme had an inactivating effect on norovirus, even in salads. In the future, heat-denatured lysozyme can be used as a novel norovirus-inactivating agent, although it is essential to investigate the mechanism of inactivating effect of heat-denatured lysozyme against norovirus.

1. Introduction

Norovirus (NoV) is a single-stranded RNA virus belonging to the family *Caliciviridae*; it is responsible for nonbacterial acute gastroenteritis (Koopmans and Duizer, 2004). Symptoms such as vomiting, diarrhea, abdominal pain, and mild fever start to appear 24 to 48 h after infection (Koopmans and Duizer, 2004). The main routes of NoV transmission are through the ingestion of NoV-contaminated foods, as well as human-to-human transmission through the feces or vomitus of infected individuals (Matthews et al., 2012). NoV is highly infectious, and can cause onset of symptoms with as low as 10 copies or less (Teunis et al., 2008). Therefore, if food handlers are infected with NoV, large-scale food poisonings can occur. In fact, a number of large-scale norovirus outbreaks in schools and restaurants have been reported (Baker et al., 2011; Lin et al., 2015; Liu et al., 2015).

In recent years, a number of NoV outbreaks caused by salads have been reported (Herman et al., 2015; Schmid et al., 2007; Wadl et al., 2010). The reasons for NoV contamination in salads include the cultivation of fresh vegetables using water or soil that are contaminated with NoV (El-Senousy et al., 2013) and secondary contamination from food handlers (Grove et al., 2015; Maritschnik et al., 2013). As salads cannot be heated during food processing, inactivation of NoV by heating is not possible. For this reason, analyzing the viability of NoV in salads is extremely important to assess the risk of NoV outbreaks; however, no previous study has examined the viability of NoV in salads. In addition, it remains unknown how NoV is affected by various types of dressings used on salads. There is no study what components in salads and dressings affect the viability of NoV.

Therefore, our study examined the viability of NoV in salads and dressings by using murine norovirus strain 1 (MNV-1) as a surrogate for NoV. MNV-1 belongs to the family *Caliciviridae*, genus norovirus (Karst et al., 2003). It has been reported that MNV-1 was preferable for use as a surrogate virus (Cannon et al., 2006) because MNV-1 is

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http://dx.doi.org/10.1016/j.ijfoodmicro.2016.06.006
0168-1605/

more closely related to NoV than the other viruses (Wobus et al., 2006). Therefore we used MNV-1 as a surrogate for NoV.

In addition, we examined whether using lysozyme was effective in inactivating NoV as an alternative to heating. Lysozyme is present in the egg white and human tears; it is an enzyme that hydrolyzes the cell walls of Gram-positive bacteria composed of peptidoglycans (Aminlari et al., 2014). Most commonly, lysozyme acts on bacteria as an enzyme. However, in a previous study, we found that heat-denatured lysozyme inactivated NoV (Takahashi et al., 2015). In this study, we tested whether heat-denatured lysozyme also inactivated MNV-1 in salads.

2. Materials and methods

2.1. Preparation of dressings and salads

In this study, 4 types of salads (coleslaw, thousand island salad, vinaigrette salad, egg salad) and 3 types of dressings (mayonnaise, thousand island dressing, vinaigrette dressing) were used to examine the viability of MNV-1.

For the mayonnaise and thousand island dressing which contains shredded onion, items purchased from the retail stores were used. For the vinaigrette dressing, extra virgin olive oil and white vinegar purchased from the retail stores were mixed at a ratio of 3:1.

For the coleslaw, the core of a cabbage purchased from a retail store was removed with a sterilized knife, and the remaining parts of the cabbage were cut into squares measuring 5–10 cm each with a sterilized knife, which were then dipped in water at 55 °C for 10 s. Using a food processor, the pieces were chopped into tiny squares measuring 5 mm and were immersed in 2% salt water for 1 min. After the water was drained off from the cabbage, mayonnaise was added at an amount equivalent to 20% of the cabbage's weight, and it was stirred until the mayonnaise was evenly spread over the whole mixture.

For the thousand island salad, lettuce (*Lactuca sativa*) purchased from a retail store was cut into squares measuring 3–5 cm using a sterilized kitchen knife, and the pieces were dipped in tap water. After the water was drained off from the lettuce, thousand island dressing was added at an amount equivalent to 20% of the lettuce's weight, and it was stirred until the dressing was evenly spread over the whole mixture.

For the vinaigrette salad, a procedure similar to that performed on the thousand island salad was used: lettuce was cut into slices and dipped in tap water, and the water was drained off. Then, the vinaigrette dressing that we prepared as described above was added at an amount equivalent to 20% of the lettuce's weight; the mixture was stirred until the dressing was evenly spread throughout.

For the egg salad, 10 eggs purchased from a retail store were boiled in water at 100 °C for 20 min, and the shells were peeled. The eggs were chopped into 5 mm squares using an egg slicer, and mayonnaise was added at an amount equivalent to 20% of the eggs' weight; the mixture was stirred until the mayonnaise was evenly spread throughout.

The pH of the prepared salads and dressings were measured using a pH meter (HM-25R, DKK-TOA Corp., Tokyo, Japan) after stomacher treatment as described above.

2.2. Virus and cells

In this study, MNV-1 was used as a surrogate to the human NoV. Mouse macrophage cells (RAW264.7) were cultured at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum (FBS) and penicillin (100 U/mL)–streptomycin (100 μg/mL).

After the RAW264.7 cells became confluent, they were inoculated with MNV-1 at a multiplicity of infection (MOI) of 0.1 and were incubated for 3 days in 5% CO_2 at 37 °C. After confirmation of cytopathic effect, the cells were subjected to freezing and thawing 4 times, and centrifugation at 8000 × *g* for 20 min. The supernatant was used as MNV-1 solution and stored at −80 °C until use in experiments.

2.3. Viability of MNV-1 in various types of salads

One milliliter of MNV-1 solution at a concentration of approximately 6 log PFU/mL was inoculated for every 10 g of the salads that we had prepared (coleslaw, thousand island salad, vinaigrette salad, egg salad), and the salads were stored at 4 °C after stomacher treatment at 230 rpm for 30 s to mix MNV-1 solution and salads.

Samples were collected at day 0 (immediately after inoculation and stomacher treatment), day 1, day 2, day 3, day 4, and day 5. Ninety milliliters of phosphate-buffered saline (PBS) was added to each 10 g of salad, and stomacher treatment was performed at 230 rpm for 30 s. The treated samples were centrifuged at 8000 × *g* for 10 min, and the supernatants were passed through a filter with a pore size of 0.20 μm. The resulting samples were 10-fold serially diluted in DMEM, and were stored at −80 °C until they were used in plaque assay (described in Section 2.6).

2.4. Viability of MNV-1 in various types of dressing

Thirty grams of each dressing that we had purchased or prepared (mayonnaise, thousand island dressing, vinaigrette dressing) were inoculated with 3 mL of MNV-1 solution at a concentration of 6 log PFU/mL, and were stored at 4 °C after stomacher treatment at 230 rpm for 30 s to mix MNV-1 solution and dressings.

Samples were collected at day 0, day 1, day 2, day 3, day 4, and day 5 after storage. One gram of dressing was added to 9 mL of PBS, and the mixture was stirred. The subsequent centrifugation, filtration, and serial dilution were carried out using the same procedure as described above. The resulting samples were stored at −80 °C until they were used for plaque assay.

2.5. Inactivation of MNV-1 in salads added with heat-denatured lysozyme

Heat-denatured lysozyme was prepared as previously reported (Takahashi et al., 2015). Egg white lysozyme (Kewpie Corporation, Tokyo, Japan) was dissolved in distilled water at a concentration of 5% (w/v, pH 6.5 ± 0.2) and filtered using a 0.20 μm filter. The resulting filtrate was heated in an oil bath at 100 °C for 40 min and then was cooled on ice in order to prepare heat-denatured lysozyme.

Next, 40 mL of the prepared 5% heat-denatured lysozyme was added to 160 g of each of the 4 types of salads (coleslaw, thousand island salad, vinaigrette salad, egg salad) in order to obtain a final concentration of 1%. The prepared salads were weighed, and 10 g of each salad was inoculated with 1 mL of MNV-1 at a concentration of approximately 6 log PFU/mL, and was stored at 4 °C after stomacher treatment at 230 rpm for 30 s to mix MNV-1 solution and salads.

Samples were collected on day 0, day 1, day 2, day 3, day 4, and day 5 of storage. Next, 90 mL of PBS was added to 10 g of the salad, and subsequently subjected to stomacher treatment, centrifugation, filtration, and serial dilution steps according to the procedure described above. The resulting samples were stored at −80 °C until they were used for the plaque assay.

2.6. Plaque assay

The infectivity of MNV-1 was measured by plaque assay, using the method described by Gonzalez-Hernandez et al. (2012). RAW264.7 cells were seeded into 6-well plates (Falcon, Becton, Dickinson and Company, Franklin Lakes, NJ) to obtain a final concentration of 6 log cells/mL. Cells were then incubated for 18 h at 37 °C in 5% CO_2.

Next, 500 μL of each of the prepared samples were inoculated in plates containing RAW 264.7 cells, and the plates were shaken at room temperature for 1 h. Subsequently, the virus samples were layered