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A Synergy Effect of Trisodium Phosphate and Ethanol on Inactivation of Murine Norovirus 1 on Lettuce and Bell Pepper

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The synergy effect of trisodium phosphate (TSP) and ethanol against murine norovirus 1 (MNV-1), as a surrogate for human noroviruses, on fresh produces was evaluated. More than 2% (w/v) of TSP effectively inactivated MNV-1. The single treatment of 1% TSP or 30% ethanol for 30 min was not effective on MNV-1; however, cotreatment showed inactivation of MNV-1 on stainless steel and the produces of lettuce and bell pepper under 15 min. The results suggest that cotreatment of TSP and ethanol at a low concentration and a short time of exposure might be useful for the reduction of norovirus in some produce.

Keywords: Murine norovirus, cotreatment, trisodium phosphate, ethanol, produce

Human noroviruses (NoVs) have been recognized as the most important agents causing acute gastroenteritis; human NoVs are highly infectious in people of all age groups and this may lead to widespread outbreaks [10]. NoVs are mainly transmitted through the fecal-oral route or ingestion of contaminated water and foods, particularly shellfish, fruits, and fresh vegetables [8]. Fresh produce can be easily contaminated with NoVs during transport and handling, and consumption of contaminated fruit or vegetables has been responsible for some of the reported outbreaks [12, 17]. Therefore, appropriate decontamination strategies are required to prevent outbreaks due to contamination occurring during the industrial process as well as raw conditions of ready-to-eat vegetables or fruits. These decontamination strategies should be easy, cost-effective, and rapid and should not cause harm to the environment [17].

Chemical disinfectants such as chlorine, alcohols, and organic acids have been widely used to inactivate NoVs. Chlorine, which is one of the most commonly used disinfectants, is known to be effective for inactivation of bacteria and viruses [1, 4]. However, a high concentration of chlorine and a long contact time are required to inactivate NoVs effectively, and chlorine can produce unfavorable sensory effects in food items [16]. Although ethanol has also been used as a disinfectant against vegetative bacteria and enveloped viruses, its activity against nonenveloped viruses such as feline calicivirus (FCV) is variable [6, 14]. Some studies have reported a strong effect of ethanol against FCV [19], whereas many others have shown that ethanol is ineffective against FCV [5, 6, 11, 21]. Hence, more effective decontamination methods are needed to inactivate human NoVs and prevent their associated gastroenteritis outbreaks. Trisodium phosphate (TSP) is nontoxic and is considered a "generally recognized as safe" chemical by the Food and Drug Administration [18]. Recent reports have evaluated TSP as a disinfectant against human NoV surrogates such as murine norovirus (MNV-1) and FCV. D'Souza and Su [6] reported that 5% TSP was effective (6 log reduction) against MNV-1 at both high and low initial MNV-1 titers, within a short contact time of 30 sec to 1 min. A 5% TSP concentration was more effective at reducing the MNV-1 plaque-forming unit (PFU) count than 10% sodium hypochlorite. Su and D'Souza [18] also showed that 5% TSP could cause a total reduction in titers of MNV-1 at 7 log reduction in titers of MNV-1.

However, D'Souza and Su [6] showed that TSP at 1% was not effective in inactivating MNV-1, with only 0.28 log reduction for MNV-1 being observed at high titers after 1 min exposure. Previous research studies have not examined the inactivation of MNV-1 by a combined treatment of low-concentration TSP with one of the other common disinfectants. Furthermore, there are few studies on the application of TSP for MNV-1 inactivation on fresh produce. Therefore, the objective of the present study was to evaluate the synergy effect of TSP and ethanol against MNV-1, as a surrogate for human NoVs, on fresh produce.

MNV-1, which is the most closely related NoV to humans and cultivated in the laboratory [20], was used at the titers of 7 log PFU/ml. The viral RNA was extracted by use of QIAamp Viral RNA Mini Kits (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA samples were used as the template for RT-PCR. RT-PCR was conducted with the One-Step RT-PCR PreMix Kit (iNtRON Biotechnology, Seoul, South Korea). Briefly, 10 µl of each viral template RNA was mixed with 1 µl of the primers [9]. The reverse transcription reaction took place at 45°C for 30 min, followed by RNA denaturation at 94°C for 5 min and then 40 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min), extension (72°C for 1 min), and final extension (72°C for 10 min). The RT-PCR products of 880 bp were confirmed by electrophoresis on 2% agarose gel. For inactivation of MNV-1 with ethanol (Sigma-Aldrich, St. Louis, MO, USA) and TSP (Ducksan Pure Chemical Co. Ltd., Ansan, Korea), 10 µl of MNV-1 was mixed with ethanol to a final 30%, 50%, and 70% for 1, 5, 15, and 30 min at 25°C. Inactivation effect of TSP against MNV-1 was tested at 1%, 2%, and 5% final concentrations (w/v) for the time intervals [6, 17]. At each time, 120 µl of DEPC water was added to the mixture and RT-PCR was conducted after viral RNA extraction from the total 140–150 µl mixture. To analyze the effect of the combined treatment, a solution of 1% TSP and 30% ethanol was used. First, 10 µl of MNV-1 was spotted on a No. 4 finish polished stainless steel disc (2 cm × 2 cm) and dried for 2 h in a sterile petri dish. The spotted disc was soaked in 10 ml of a combined solution of 1% TSP and 30% ethanol at 4°C for 0, 1, 5, 15, and 30 min. At each time point, the disc was rinsed three times with sterile water and washed with 7 ml of elution buffer (Tris-HCl 100 mM, glycine 50 mM, and 1% beef extract, pH 9.5) for 30 min by shaking at 300 rpm. The rinsed fluid was centrifuged at 10,000 ×*g* for 30 min, and the pH of the decanted supernatant was adjusted to 7.2 by 5 N HCl. The neutralized supernatant was filtered by a 0.2 µm syringe filter and supplemented with 10% polyethylene glycol (PEG) 8,000 (Sigma-Aldrich, USA) and 0.3 M NaCl. The mixture was incubated overnight at 4°C, after which viruses were concentrated by centrifugation at 10,000 ×*g* for 30 min at 4°C. The pellet was resuspended in 150 µl of DEPC water. The viral concentrate was used for viral RNA extraction and RT-PCR. All assays were carried out three times. The effect of combined TSP and ethanol treatment against MNV-1 was also studied on fresh produce in a manner identical to that described above, except for a different sample-processing procedure for lettuce and bell pepper [3, 7, 8, 13]. Lettuce and bell pepper were purchased from local grocery stores and cut into 1 cm × 2 cm pieces with a knife. Cut pieces of produce were washed with 70% ethanol for 30 min in a sterile beaker and rinsed three times with sterile water. Each piece of lettuce and bell pepper was dried in a sterile petri dish. Then 10 µl of MNV-1 was spotted on the lettuce and bell pepper, and dried at 4°C for 6 h in sterile petri dishes. The treatment of produce in a solution of TSP and ethanol, and the elution and concentration of MNV-1 for analysis, were conducted as described above.

The results of inactivation of MNV-1 by different

Table 1. Detection of PCR products of MNV-1 after ethanol or trisodium phosphate (TSP) treatment at different concentrations and times.

Time (min)	Ethanol (%)			TSP (%)		
	30	50	70	1	2	5
0	+	+	+	+	+	+
1	+	+	+	+	-	-
5	+	+	+	+	-	-
15	+	+	+	+	-	-
30	+	+	+	+	-	-

Symbol: +, Detection of PCR products for MNV-1; -, no detection.

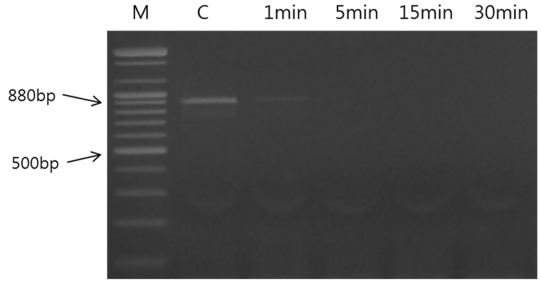

Fig 1. Detection of PCR products for MNV-1 collected on stainless steel after cotreatment of TSP (1%) and ethanol (30%) at the indicated times.

M, 100 bp DNA ladder; C, MNV-1 without cotreatment; 1 min–30 min, times for cotreatments.

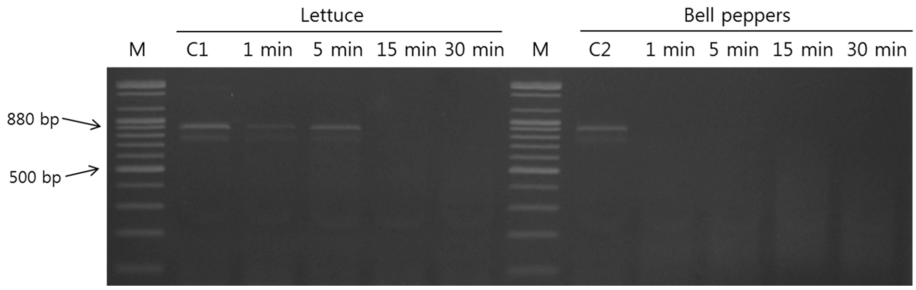

Fig. 2. Detection of PCR products for MNV-1 collected on lettuce and bell pepper after cotreatment of TSP (1%) and ethanol (30%) at the indicated times.

M, 100 bp DNA ladder; C1, MNV-1 without treatment on lettuce; C2, MNV-1 without treatment on bell pepper; 1 min–5 min, times of cotreatment.

concentrations of ethanol are shown in Table 1. RT-PCR products were present after 30 min exposure to 30% ethanol, and were even present after 30 min exposure to 50% and 70% ethanol. Alcohols, including ethanol, are widely used disinfectants [16], but our results revealed that ethanol did not effectively inactivate MNV-1. These results were in contrast to those of a previous study by Belliot *et al.* [2] that showed more than 4 log PFU/ml reduction of MNV after 30 sec and 1 min contact with ethanol. Tung *et al.* [19] reported that log reductions in MNV of 0.5, 2, and 3 were observed after a 30 sec exposure to 50%, 70%, and 90%, respectively. Although some studies are in contrast to our result, D'Souza and Su [6] showed a similar result to ours, demonstrating no efficacy of 70% ethanol against MNV-1.

MNV-1 was effectively inactivated after exposure to both 2% and 5% TSP (Table 1). RT-PCR products were still present after 30 min exposure to 1% TSP, but were not detected after 1 min exposure to 2% and 5% TSP. This result indicates that MNV-1 can be inactivated by 2% and 5% TSP with a short exposure of 1 min. Similar to our results, Su D'Souza [18] reported that MNV-1 was reduced from 7 log PFU/ml to undetectable levels after only 15 sec and 30 sec exposure to 5% TSP. The ineffective inactivation of MNV-1 with 1% TSP after 1 min exposure, seen in the present study, is consistent with a previous result that showed almost no reduction in MNV-1 after 1 min exposure to 1% TSP [6]. TSP at a concentration of 1% for exposure of 1, 5, 15, and 30 min was ineffective at inactivating MNV-1 compared with 2% and 5% TSP.

For more efficient inactivation of MNV-1, TSP was chosen as one component of a combined treatment. Ethanol was also chosen among the common disinfectants used in the present study as the other component of the combined treatment. A solution containing 1% TSP and 30% ethanol was evaluated, and a synergy effect of this combination against MNV-1 on stainless steel discs was confirmed (Fig. 1). A weak RT-PCR band was present on the agarose gel at 1 min exposure to the TSP/ethanol solution, and MNV-1 was reduced to an undetectable level at 5 min. Compared with our result from single-treatment trials, this result indicates that combined treatment can effectively inactivate MNV-1 within a short exposure of 5 min. It shows that MNV-1 can be inactivated using lower concentrations of TSP and ethanol at short time when used in combination. The effect of a combined treatment using 1% TSP and 30% ethanol against MNV-1 on fresh produce was evaluated (Fig. 2). A weak RT-PCR product was present on the agarose gel when MNV-1 spiked on lettuce was treated with the combined TSP/ethanol for 5 min, and MNV-1 on lettuce was reduced to undetectable level at less than 15 min. MNV-1 spiked on bell pepper was effectively inactivated at 1 min exposure. MNV-1 may be more rapidly inactivated on bell pepper than on lettuce because the surface of bell peppers may be smoother than that of lettuce.

In conclusion, this study reveals the combination of TSP and ethanol as a more effective decontamination method for MNV-1 inactivation. This result shows that the combined treatment with TSP and ethanol at low concentrations and for a short contact time might be useful for the reduction of NoVs in some fresh produce. Therefore, the combined agent of TSP and ethanol might be a simple, rapid, and cost-effective method to prevent gastroenteritis outbreaks due to NoV contamination.

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