Short Communication

Simultaneous recovery of bacteria and viruses from contaminated water and spinach by a filtration method

Julie Brassard *, Évelyne Guévremont, Marie-Josée Gagné, Lisyanne Lamoureux

Agriculture and Agri-Food Canada, Food Research and Development Centre, 3600 Casavant Boulevard West, Saint-Hyacinthe, Québec, Canada, J2S 8E3

ABSTRACT

Water and leafy vegetables eaten fresh are increasingly reported as being involved in food-borne illness cases. The pathogenic agents responsible for these infections are mainly bacteria and viruses and are present in very small quantities on the contaminated food matrices. Laboratory techniques used to isolate or detect the contaminating agent differ enormously according to the type of microorganisms, generating time and economical losses. The purpose of this study was to optimize a single method which allows at the same time the recovery and concentration of these two main types of pathogenic organisms. Water and spinach samples were artificially contaminated with the feline calicivirus (FCV), rotavirus, hepatitis A virus (HAV), *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter jejuni* and *Salmonella* Typhimurium. The principle behind the recovery technique is based on the use of a positively charged membrane which adsorbs both viruses and bacteria present in the water or in the rinse from the vegetables. Using conventional microbiology, PCR and RT-PCR, this filtration technique allowed a detection level superior to 10^2 CFU/g for *S.* Typhimurium, *E. coli*, *L. monocytogenes* and *C. jejuni* and to 10^1 PFU/g for FCV, HAV and rotavirus. This combined method can also be applied to other bacterial and viral species for the identification of the responsible agent for food-borne illnesses.

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1. Introduction

Fresh produce consumed raw or minimally processed, such as fruits and vegetables, provide an ideal route for the transmission of certain enteric pathogenic bacteria and viruses including Norovirus, hepatitis A virus, rotavirus, enteroviruses, *Salmonella* spp, *Bacillus cereus*, *Staphylococcus aureus*, *Shigella*, *Escherichia coli*, *Campylobacter jejuni*, and *Listeria monocytogenes* (Islam et al., 2004; Newell et al., 2010). The perceptions that consuming fresh produce is safe and that most food-borne illness outbreaks are caused by food primarily of animal origin are generally widespread in the population (Sivapalasingam et al., 2003). However, fresh fruits and vegetables are increasingly recognized as a source of food-borne outbreaks in the US and throughout the world (Lynch et al., 2009). Food-borne infectious intestinal and hepatic illnesses are serious public health concerns and different factors can influence this situation. Among them, market globalization for fresh produce, long-distance transport of commodities, changes in consumers' eating habits (such as consumption of raw food) and intensification of crop production using reclaimed water for irrigation (Hamilton et al., 2006; Newell et al., 2010).

Fresh fruits and vegetables can potentially be contaminated directly in the field through contact with water. In horticultural production, water is commonly used for irrigation, fertilizer or pesticide applications, during post harvest transport (ice) and for washing. In order to determine the origin of the microbial contamination, to evaluate the impact of the irrigation water and afterward, to develop effective prevention and control strategies, the use of sensitive recovery methods for microorganisms present in vegetable and water is necessary. Presently, the techniques used to isolate or detect the contaminating agents differ enormously according to the type of microorganisms and matrices, generating time and economical losses. Some pathogenic bacteria are largely characterized and already monitored in the food industry by surveillance programs and accredited isolation methods. For food-borne viral diseases, no systematic surveillance has been developed and only few methods for the recovery and detection of viral pathogens in food and water are recognized by the authorities. Currently, no universal method exists to allow the recovery of bacterial and viral particles at the same time from the same food or water sample. The combination of a single recovery technique for pathogenic viruses and bacteria in fresh produce along with the surveillance programs already in place could be very helpful for measuring the incidence of food-borne illnesses and for reducing the cost and the time of laboratory analysis. It is thus important, for a better control of potential microbial contamination of

fresh produce via irrigation water at the farm, to obtain new knowledge on the microbiological quality of the irrigation water and of fresh vegetables pre and post harvest.

The purpose of this study was to optimize a single method which simultaneously recovers and concentrates bacterial and viral pathogenic microorganisms from the same food sample. Water and spinach were artificially contaminated with the feline calicivirus (FCV) as a sample process control, rotavirus, hepatitis A virus, *E. coli*, *L. monocytogenes*, *C. jejuni* and *Salmonella Typhimurium* and tested through a filtration technique.

2. Materials and methods

2.1. Bacterial and viral strains and standardization of the inoculum

The following bacterial strains were used: *S. Typhimurium* (ATCC 14328), *L. monocytogenes* (ATCC 7644), *E. coli* OLC811 (kindly provided by Burton Blais, CFIA, Ottawa, where the original strain O157:H7 was modified to be resistant for nalidixic acid and verotoxin negative), and *C. jejuni* (ATCC 33291). For each experiment, *C. jejuni* was grown overnight in 25 ml of Bolton broth with supplements (SR0084, Oxoid Nepean, ON) at 42 °C in microaerophilic atmosphere. *S. Typhimurium*, *L. monocytogenes* and *E. coli* were grown overnight in 25 ml of TSB at 37 °C. Ten-fold dilutions, ranging from 1×10^3 to 1×10^7 colony-forming units (CFU)/ml, of each bacterial cell suspension were made in peptone water (0.1%) determined by OD at 600 nm values analyses.

Rotavirus strain Wa (kindly provided by P. Payment, Institut National de Recherches Scientifique (INRS), Montréal, QC, Canada), hepatitis A virus (HAV) strain HM-175 (ATCC# VR-1402) and the feline calicivirus (FCV) strain F9 (ATCC VR-782), propagated in FRhK-4, MA-104 and CrFK cells respectively as previously described (Ansari et al., 1988; Bidawid et al., 2003; Mbithi et al., 1990), were also used. Viral suspensions of each species with final concentrations of 1×10^3 PFU/ml to 1×10^5 PFU/ml, based on the viral production titer, were prepared in phosphate saline buffer. Finally, four cell suspensions containing each bacterial and viral strains were prepared with the following standardized concentrations: 1×10^5 CFU/ml and 1×10^4 PFU/ml (Mix 1) and 1×10^4 CFU/ml and 1×10^3 PFU/ml (Mix 2) for water samples; 1×10^6 CFU/ml and 1×10^4 PFU/ml (Mix 3) and 1×10^5 CFU/ml and 1×10^3 PFU/ml (Mix 4) for spinach samples.

2.2. Recovery of microorganisms from spinach and water samples

The procedure for testing viruses and bacteria elution and concentration method is summarized in Fig. 1. Spinach samples (25 g) were placed in sterile polypropylene container and inoculated with 250 µl of the different bacterial and viral suspensions. The leaves were dried for 1 h in the safety cabinet at room temperature. Spinach were transferred into a stomacher bag and 225 ml of washing buffer (glycine 0.05 M, NaCl 0.14 M, pH 7.5) were added and gently mixed for 90 min at room temperature in order to detach the microorganisms from the vegetables. The washing buffer was passed through a Zetaplus 60 S filter (Peacock, LaSalle, Qc, Canada). Adsorbed viruses and bacteria were eluted by incubating the filter at room temperature with agitation for 30 min with 10 ml of TPBG buffer (2.9% Tryptose Phosphate Broth and 6% glycine, pH 9.0). The eluate was recovered and pH was adjusted to 7.0–7.4 with HCl 1 N. At this step, plate counts on selective media were carried out to isolate the four tested bacterial species (see Section 2.3). Viruses in elution buffer underwent a supplementary concentration step in an Amicon centrifugal unit ($5000 \times g$, 10 min) before nucleic acid extraction.

For water sample analysis, the OPFLP-04 standard method for the recovery and concentration of viruses present in artificially and naturally contaminated water from Health Canada's compendium of analytical methods was used with some modifications (Brassard et al.,
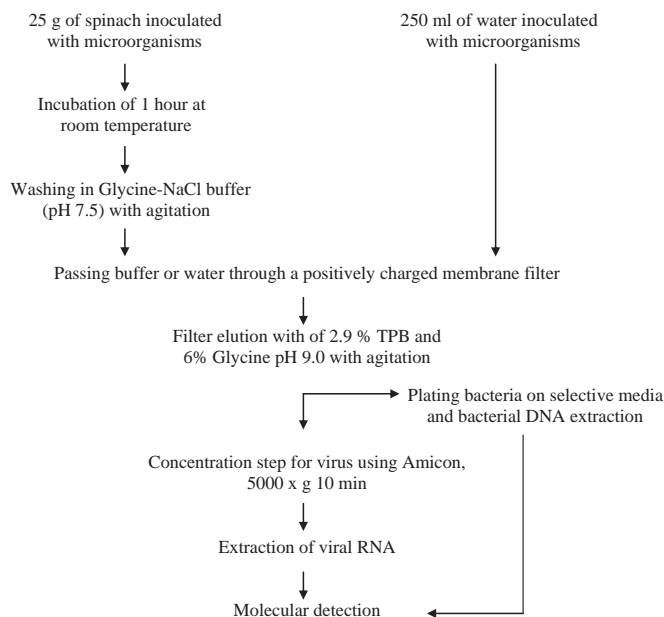

Fig. 1. Diagram for recovery, concentration and detection of viruses and bacteria from inoculated spinach and water samples.

2005, 2007). Briefly, 250 ml of water was spiked with 250 µl of cell suspensions and passed through a Zetaplus 60 S filter (Peacock). The filter was retrieved and placed in 10 ml of TPBG for elution. Eluate was used for dilution series and plated on selective media for bacterial detection and was also concentrated on an Amicon centrifugal unit for viral particles recovery. Each experimental assay included non-inoculated spinach or water sample as negative control and was performed in triplicate.

2.3. Bacteriological analysis

Ten-fold dilutions of eluate were plated on selective media for the growth of the different bacteria. Hektoen and MacConkey Sorbitol media supplemented with 25 µg/ml of nalidixic acid were used and incubated at 37 °C for 24 h for the detection of *S. Typhimurium* and *E. coli*, respectively. Palcam media supplemented with selective supplement (SR0150E, Oxoid) and incubated at 37 °C for 48 h was used for *L. monocytogenes* detection. Karmali agar was used to enumerate *C. jejuni* and plates were incubated at 42 °C for 48 h under microaerophilic atmosphere. Plate counts were carried out after the incubation period and were used to determine the detection limit of the recovery technique in classical microbiology.

2.4. DNA/RNA extraction and molecular detection

Viral RNA was extracted using the QIAamp RNA Viral Mini Kit (Qiagen, Mississauga, ON) as recommended by the manufacturer. For rotavirus, an additional step was performed before extraction. Virus concentrate was incubated at 37 °C with 1% SDS and 100 µg/ml of proteinase K for 1 h. The FCV, HAV and rotavirus from water and spinach samples were detected after the recovery and concentration steps using a conventional RT-PCR assay in 25 µl of reaction mixture with 2 µl of extracted RNA and according to the procedures previously described (Brassard et al., 2005; Guevremont et al., 2006; Mattison et al., 2009; Papafragkou et al., 2008).

The QIAamp DNA mini kit (Qiagen) was used as recommended by the manufacturer for bacterial DNA extraction from a 200 µl volume of the filter eluate. PCR amplification was performed as described by Inglis et al. (Inglis et al., 2003) for *C. jejuni*. *S. Typhimurium*, *L. monocytogenes* and *E. coli* were detected by multiplex conventional

Table 1
Detection limits by classical and molecular (PCR and multiplex PCR) microbiology for water and spinach samples artificially inoculated with different concentrations of bacterial strains following the combined recovery method.

Type of matrix and detection method	Bacterial spiking concentration (CFU/ml or CFU/g)											
	E. coli			C. jejuni			S. Typhimurium			L. monocytogenes		
	10^4	10^3	10^2	10^4	10^3	10^2	10^4	10^3	10^2	10^4	10^3	10^2
Water												
Classical	3/3	3/3	3/3	3/3	3/3	2/3	3/3	3/3	2/3	3/3	3/3	2/3
Molecular	3/3	1/3	1/3	3/3	3/3	1/3	3/3	3/3	0/3	3/3	3/3	0/3
Spinach												
Classical	3/3	Na	Na	3/3	3/3	3/3	3/3	3/3	1/3	3/3	3/3	2/3
Molecular	3/3	1/3	0/3	3/3	3/3	3/3	3/3	2/3	0/3	3/3	3/3	1/3

Na: Not applicable.

PCR as previously described (Kawasaki et al., 2005). RT-PCR and PCR amplicons were analysed on 2% w/v agarose gels stained with SYBRsafe.

3. Results and discussion

This work describes the use of a universal recovery method for food-borne bacteria and viruses from contaminated leafy vegetables and water samples. It is based on the use of positively charged membrane, which allows the adsorption of microorganisms from filtered samples of water or vegetable rinses. These filters are mainly used for the concentration of food-borne viruses in water, although a few authors have also indicated the possibility of capturing bacteria when using this kind of filters (Polaczyk et al., 2007; Watt et al., 2002). The method presented in this paper was an adaptation to bacterial species (S. Typhimurium, E. coli, C. jejuni, and L. monocytogenes) of a standardized method for recovery of viruses in water samples developed in our laboratory (Brassard et al., 2005; Brassard et al., 2007). For water sample analysis, the detection limit for each bacterial strain was determined using ten-fold dilutions followed by filtration and two types of analysis: classical microbiology and molecular biology (Table 1). These tests demonstrated the method's effectiveness in recovering various bacterial strains alone from contaminated water samples with a detection limit around 10^2 CFU/g. It also showed the ability of the filter to retain the studied bacteria and not only viruses. The data are expressed as the number of positive assay over three replicates. A positive assay corresponds to the presence of typical colonies on agar at the end of the process. This method has also been tested for the simultaneous recovery from water samples of the

different bacterial and viral species targeted. Two mixtures were prepared with different concentrations of viruses and bacteria based on the detection limits previously obtained (Table 1) in order to evaluate possible interference in the presence of multiple microorganisms. The detection limits of the different bacterial and viral species are comparable whether they are alone or with other microorganisms (Table 2) (Brassard et al., 2005). An extensive number of microbiological methods for identifying those pathogens in various food matrices are already available. However, very few methods enable the recovery than one type of microorganisms from the same matrix. This technique therefore enables the effective recovery of more types of microorganisms that may be present in water from a single sample, which considerably reduces the time and expense of laboratory analyses.

Using the same laboratory equipment and almost the same protocol (Fig. 1), it was possible to adapt the method to more complex food matrices such as leafy vegetables. The challenge lay in choosing a washing buffer that would make it possible to efficiently recover bacteria and viruses while maintaining their natural negative charge, so that their electrostatic interactions with the cationic filter would not be lost. It was suggested that salts, like $MgCl_2$ or NaCl and protein-enriched eluants may disrupt electrostatic interactions and hydrophobic interactions. Application of physical forces like shaking may dissociate and release attached organisms from leafy vegetable matrices (Fino and Kniel, 2008; Shields and Farrah, 1983). In this study, the use of a pH-neutral, glycine- and NaCl-based washing buffer on spinach resulted in detection limits similar to those obtained for contaminated water with bacteria (Table 1). Detection limits obtained for each individual virus on artificially contaminated spinach are generally 1 log greater than those

Table 2
Detection by classical and molecular microbiology of bacterial and viral strains prepared in different concentration mix and artificially inoculated in water and spinach samples following the combined recovery method.

Micro-organisms	Type of matrix and detection methods							
	Water				Spinach			
	Mix 1[a]		Mix 2[b]		Mix 3[c]		Mix 4[d]	
	Classical	Molecular	Classical	Molecular	Classical	Molecular	Classical	Molecular
Bacterial strains								
S. Typhimurium	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3
E. coli	3/3	3/3	3/3	3/3	3/3	3/3	2/3	1/3
L. monocytogenes	3/3	3/3	1/3	1/3	3/3	3/3	3/3	3/3
C. jejuni	3/3	3/3	2/3	2/3	3/3	3/3	3/3	3/3
Viral strains								
Feline calicivirus	Na	3/3	Na	2/3	Na	0/3	Na	0/3
Hepatitis A virus	Na	3/3	Na	3/3	Na	3/3	Na	1/3
Rotavirus	Na	3/3	Na	3/3	Na	3/3	Na	3/3

Na: Not applicable.
[a] Mix 1, final concentration of bacteria and viruses in water 10^3 CFU/ml and 10^2 PFU/ml, respectively.
[b] Mix 2, final concentration of bacteria and viruses in water 10^2 CFU/ml and 10^1 PFU/ml, respectively.
[c] Mix 3, final concentration of bacteria and viruses on spinaches 10^4 CFU/g and 10^2 PFU/g, respectively.
[d] Mix 4, final concentration of bacteria and viruses on spinaches 10^3 CFU/g and 10^1 PFU/g, respectively.

for bacteria, that is, 1×10^1 PFU/g for rotavirus and HAV and 1×10^2 PFU/g for FCV. Since an enrichment step is not possible for viruses, an additional concentration step with ultrafiltration device allows an increasing of the detection limit by molecular biology.

Bacteria-virus mixtures were also prepared and used to artificially inoculate spinach samples. Again, no interference was observed and detection limits are equivalent to those obtained in tests on bacteria and viruses separately (Table 2). They are also equivalent to other detection limits reported in the literature for tests using either bacteria or viruses only (Cheong et al., 2009; Elizaquivel and Aznar, 2008; Fumian et al., 2009; Ibekwe et al., 2004; Morales-Rayas et al., 2010). However, the recovery of FCV, used here as a sample process control (Mattison et al., 2009), was not constant in various assays on spinach while being stable in water. Recently, FCV was described as being not as stable as some other enteroviruses in the environment, in foods and might be influenced by RT-PCR inhibitors from the matrix (Cannon et al., 2006; Su et al., 2010). A sample process control should allow a measure of comparison between extractions and processing methods for testing food. In spinach samples, it seems difficult to have proper repeatability using this virus in comparison with the other tested microorganisms. This result raises questions about using FCV as an internal control with certain foodstuffs in which matrix components or pH level can limit virus recovery and detection.

During food poisoning outbreak, research laboratories must often investigate for the source of infection, pathogenic bacteria or viruses, starting sometimes with a very small quantity of suspected food (for example, the remains of a meal). It is therefore important to optimize methods that avoid the use of a large quantity of material and tests duplication. The used of the electropositive charged membrane is a promising and polyvalent alternative for simultaneous recovery of small quantities of bacteria and viruses from water and fresh vegetables and could be very useful in the future for tracking sources of microbial contamination and evaluating their impact on the safety of fresh produce such as leafy vegetables. This combined method could be applied to other bacterial and viral species. Also, the cost and the time associated with laboratory manipulations for the search and identification of the agent responsible of food-borne illnesses could be possibly reduced with the use of this technique.

Acknowledgement

References

Ansari, S.A., Sattar, S.A., Springthorpe, V.S., Wells, G.A., Tostowaryk, W., 1988. Rotavirus survival on human hands and transfer of infectious virus to animate and nonporous inanimate surfaces. Journal of Clinical Microbiology 26, 1513–1518.

Bidawid, S., Malik, N., Adegbunrin, O., Sattar, S.A., Farber, J.M., 2003. A feline kidney cell line-based plaque assay for feline calicivirus, a surrogate for Norwalk virus. Journal of Virological Methods 107, 163–167.

Brassard, J., Seyer, K., Houde, A., Simard, C., Trottier, Y.L., 2005. Concentration and detection of hepatitis A virus and rotavirus in spring water samples by reverse transcription-PCR. Journal of Virological Methods 123, 163–169.

Brassard J., Simard C., Müller P., Houde A. and Trottier Y.L., 2007. Concentration of hepatitis A virus and rotavirus in spring or mineral bottled water samples and their detection by the reverse-transcription polymerase chain reaction. http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume5/opflp_04-eng.php. accessed on October 1, 2008. ed.

Cannon, J.L., Papafragkou, E., Park, G.W., Osborne, J., Jaykus, L.A., Vinje, J., 2006. Surrogates for the study of norovirus stability and inactivation in the environment:

a comparison of murine norovirus and feline calicivirus. Journal of Food Protection 69, 2761–2765.

Cheong, S., Lee, C., Choi, W.C., Lee, C.H., Kim, S.J., 2009. Concentration method for the detection of enteric viruses from large volumes of foods. Journal of Food Protection 72, 2001–2005.

Elizaquivel, P., Aznar, R., 2008. Comparison of four commercial DNA extraction kits for PCR detection of Listeria monocytogenes, Salmonella, Escherichia coli O157:H7, and Staphylococcus aureus in fresh, minimally processed vegetables. Journal of Food Protection 71, 2110–2114.

Fino, V.R., Kniel, K.E., 2008. Comparative recovery of foodborne viruses from fresh produce. Foodborne Pathogens and Diseases 5, 819–825.

Fumian, T.M., Leite, J.P., Marin, V.A., Miagostovich, M.P., 2009. A rapid procedure for detecting noroviruses from cheese and fresh lettuce. Journal of Virological Methods 155, 39–43.

Guevremont, E., Brassard, J., Houde, A., Simard, C., Trottier, Y.L., 2006. Development of an extraction and concentration procedure and comparison of RT-PCR primer systems for the detection of hepatitis A virus and norovirus GII in green onions. Journal of Virological Methods 134, 130–135.

Hamilton, A.J., Stagnitti, F., Premier, R., Boland, A.M., Hale, G., 2006. Quantitative microbial risk assessment models for consumption of raw vegetables irrigated with reclaimed water. Applied and Environmental Microbiology 72, 3284–3290.

Ibekwe, A.M., Watt, P.M., Shouse, P.J., Grieve, C.M., 2004. Fate of Escherichia coli O157: H7 in irrigation water on soils and plants as validated by culture method and real-time PCR. Canadian Journal of Microbiology 50, 1007–1014.

Inglis, G.D., Kalischuk, L.D., Busz, H.W., 2003. A survey of Campylobacter species shed in faeces of beef cattle using polymerase chain reaction. Canadian Journal of Microbiology 49, 655–661.

Islam, M., Morgan, J., Doyle, M.P., Phatak, S.C., Millner, P., Jiang, X., 2004. Persistence of Salmonella enterica serovar typhimurium on lettuce and parsley and in soils on which they were grown in fields treated with contaminated manure composts or irrigation water. Foodborne Pathogens and Diseases 1, 27–35.

Kawasaki, S., Horikoshi, N., Okada, Y., Takeshita, K., Sameshima, T., Kawamoto, S., 2005. Multiplex PCR for simultaneous detection of Salmonella spp., Listeria monocytogenes, and Escherichia coli O157:H7 in meat samples. Journal of Food Protection 68, 551–556.

Lynch, M.F., Tauxe, R.V., Hedberg, C.W., 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. Epidemiology and Infection 137, 307–315.

Mattison, K., Brassard, J., Gagne, M.J., Ward, P., Houde, A., Lessard, L., Simard, C., Shukla, A., Pagotto, F., Jones, T.H., Trottier, Y.L., 2009. The feline calicivirus as a sample process control for the detection of food and waterborne RNA viruses. International Journal of Food Microbiology 132, 73–77.

Mbithi, J.N., Springthorpe, V.S., Sattar, S.A., 1990. Chemical disinfection of hepatitis A virus on environmental surfaces. Applied and Environmental Microbiology 56, 3601–3604.

Morales-Rayas, R., Wolffs, P.F., Griffiths, M.W., 2010. Simultaneous separation and detection of hepatitis A virus and norovirus in produce. International Journal of Food Microbiology 139, 48–55.

Newell, D.G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., Opsteegh, M., Langelaar, M., Threfall, J., Scheutz, F., van der Giessen, J., Kruse, H., 2010. Food-borne diseases — the challenges of 20 years ago still persist while new ones continue to emerge. International Journal of Food Microbiology 139 (Suppl 1), S3–S15.

Papafragkou, E., Plante, M., Mattison, K., Bidawid, S., Karthikeyan, K., Farber, J.M., Jaykus, L.A., 2008. Rapid and sensitive detection of hepatitis A virus in representative food matrices. Journal of Virological Methods 147, 177–187.

Polaczyk, A.L., Roberts, J.M., Hill, V.R., 2007. Evaluation of 1MDS electropositive microfilters for simultaneous recovery of multiple microbe classes from tap water. Journal of Microbiological Methods 68, 260–266.

Shields, P.A., Farrah, S.R., 1983. Influence of salts on electrostatic interactions between poliovirus and membrane filters. Applied and Environmental Microbiology 45, 526–531.

Sivapalasingam, S., Barrett, E., Kimura, A., Van Duyne, S., De Witt, W., Ying, M., Frisch, A., Phan, Q., Gould, E., Shillam, P., Reddy, V., Cooper, T., Hoekstra, M., Higgins, C., Sanders, J.P., Tauxe, R.V., Slutsker, L., 2003. A multistate outbreak of Salmonella enterica Serotype Newport infection linked to mango consumption: impact of water-dip disinfestation technology. Clinical Infectious Diseases 37, 1585–1590.

Su, X., Howell, A.B., D'Souza, D.H., 2010. The effect of cranberry juice and cranberry proanthocyanidins on the infectivity of human enteric viral surrogates. Food Microbiology 27, 535–540.

Watt, P.M., Johnson, D.C., Gerba, C.P., 2002. Improved method for concentration of Giardia, Cryptosporidium, and poliovirus from water. Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances and Environmental Engineering 37, 321–330.