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Occurrence of *Escherichia coli,* Noroviruses, and F-Specific Coliphages in Fresh Market-Ready Produce

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ABSTRACT

Forty samples of fresh produce collected from retail food establishments were examined to determine the occurrence of *Escherichia coli,* F-specific coliphages, and noroviruses. An additional six samples were collected from a restaurant undergoing investigation for a norovirus outbreak. Nineteen (48%) of the retail samples and all outbreak samples were preprocessed (cut, shredded, chopped, or peeled) at or before the point of purchase. Reverse transcription–PCR, with the use of primers JV 12 and JV 13, failed to detect norovirus RNA in any of the samples. All six outbreak samples and 13 (33%) retail samples were positive for F-specific coliphages (odds ratio undefined, $P = 0.003$). Processed retail samples appeared more likely to contain F-specific coliphages than unprocessed samples (odds ratio 3.8; 95% confidence interval 0.8 to 20.0). Only two (5.0%) retail samples were positive for *E. coli*; outbreak samples were not tested for *E. coli.* The results of this preliminary survey suggest that F-specific coliphages could be useful conservative indicators of fecal contamination of produce and its associated virological risks. Large-scale surveys should be conducted to confirm these findings.

Fresh produce has been recognized as a vehicle for the transmission of foodborne illnesses *(5, 6, 15–17),* most recently highlighted by several outbreaks of hepatitis A virus infection associated with green onions *(2, 4, 7, 13).* This is partly because viruses tend to be resistant to sanitizing treatments that are effective against bacteria *(1, 3, 6).* Monitoring produce and produce production environments for pathogenic viruses could reduce the risk of disease transmission by helping to prevent the contamination of these commodities or by limiting the use of contaminated products *(1).* Because reliable methods for detecting noroviruses (NoVs) on produce or in the environment are not yet available, there is considerable interest in identifying an indicator that would reliably indicate the presence of pathogenic viruses on fresh produce and other types of ready-to-eat foods *(14, 15).*

The F-specific RNA phages have been proposed as candidate indicators for the virological safety of food and water because they resemble enteric viral pathogens, are stable in the environment, and are resistant to treatment processes in a way similar to enteric viral pathogens *(5, 11).* However, there is significant disagreement in the published literature regarding the occurrence of F-specific coliphages in the environment, and studies that directly compare their presence with the presence of enteric viral pathogens on fresh produce have not yet been reported *(8).*

In this preliminary study, we investigated the occurrence of NoVs, F-specific RNA coliphages, and *Escherichia coli* on a wide variety of fresh produce. Our aim was to determine whether there was a correlation between the presence of NoVs and their potential indicators in the sampled produce.

MATERIALS AND METHODS

Sampling. Forty samples of produce were obtained from retail outlets (retail samples) throughout Minnesota. Another six samples were collected at a restaurant during an investigation of a NoV outbreak (outbreak samples). Where possible, samples were collected in their original intact packaging, kept at 4°C, and prepared for microbiological testing within 6 h. The sampled items were single unprocessed commodities, single preprocessed commodities, or preprocessed salad mixes containing two or more fruits or vegetables. The term ''processed'' is used here to describe items that were cut, chopped, peeled, or shredded by a food worker before the item was sampled or purchased. None of the samples were ''processed'' in our lab. The following data were recorded for each sampled item: source (domestic or imported), preparation method (processed or unprocessed), and growth method (organic or fertilized). All samples were tested for *E. coli,* F-specific coliphage, and NoV, except for six samples from the NoV outbreak, which were tested for F-specific coliphage and NoV only.

***E. coli* elution and detection.** *E. coli* elution and detection were performed with slight modifications according to the U.S. Food and Drug Administration's protocol for examining produce *(10).* In brief, approximately 200 g of the sampled produce was weighed into a Ziploc bag (S. C. Johnson & Son, Inc., Racine, Wis.) containing 200 ml of buffered deionized water with 1% Tween 80 (Difco, Becton Dickinson, Sparks, Md.). The bag was

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shaken for 3 to 5 min on each side on a rotary shaker (Lab-Line Inc., Melrose Park, Ill.) to help loosen attached organisms. After shaking, 36 ml of the rinse was used in a three-tube most-probable-number (MPN) assay. First, 10 ml of undiluted rinse was transferred to each of three vials of double-strength lauryl sulfate tryptose broth (EM Industries, Gibbstown, N.J.). Next, 1 ml of undiluted rinse was placed in each of three vials of lauryl sulfate tryptose broth. Finally, 1 ml of a 1:10 dilution of the rinse was placed in each of three vials of lauryl sulfate tryptose broth. Durham tubes were placed in all vials prior to the addition of the rinse samples. The vials were incubated at 35°C for 48 h, after which, a loopful of suspension was transferred from vials showing turbidity and gas formation to vials containing 10 ml of *E. coli* broth (Difco) in vials along with the Durham tubes. After incubation at 45.5°C for 48 h, a loopful of suspension from positive *E. coli* broth tubes (showing turbidity and gas formation) was streaked on Levine's eosin–methylene blue agar (EM Industries), which was incubated at 35°C for 18 to 24 h. Suspicious *E. coli* colonies (dark centered and flat, with or without metallic sheen) were transferred to brain heart infusion slants, and final identification was performed with the automated Vitek 32 biochemical assay (bioMérieux, Inc., Durham, N.C.) according to the manufacturer's instructions. MPN calculations were based on the results of final identification.

Virus elution. Approximately 50 g of the sampled produce was placed in a sterile Ziploc plastic bag with 100 ml of sterile 3% beef extract solution (pH 8.5). The bag was massaged by hand for 5 min, and the eluate was centrifuged at 1,000 × *g* for 10 min at 4°C to pellet plant material and other debris. The supernatant (~60 to 100 ml depending on produce) was recovered.

Virus enrichment. A 10-ml aliquot of the eluate was enriched with the use of a modified version of the U.S. Environmental Protection Agency's two-step enrichment method to increase the likelihood of detecting F-specific coliphages *(19)*. In brief, we added 0.125 ml of a 4 M solution of $MgCl_2$, 0.05 ml of log phase *E. coli* Famp (ATCC 700891), 0.5 ml of 10× tryptic soy broth, and 0.1 ml of an ampicillin-streptomycin stock solution (containing 0.15 g of ampicillin and 0.15 g of streptomycin per 100 ml) to the 10-ml sample aliquot. This was mixed thoroughly for 2 min and incubated for 24 h at 37°C to allow enrichment of the sample. Strict aseptic techniques were used in the handling of all samples to minimize the likelihood of cross-contamination during enrichment.

Virus concentration. Fifty to 90 ml of the sample eluate (depending on produce) was concentrated to decrease sample volume. Briefly, polyethylene glycol (8% wt/vol) was added, and the sample was stored overnight at 4°C to allow precipitate formation. After centrifugation at 4,500 × *g* for 20 min, the supernatant fluid was discarded and the pellet was resuspended in 1 ml of phosphate-buffered saline (pH 7.2). This suspension was tested for the presence of F-specific RNA coliphages and NoV.

Coliphage detection. Both enriched and polyethylene glycol–concentrated samples were tested for F-specific coliphages. For enriched samples, five 10-μl aliquots were spotted on prepoured lawns of *E. coli* Famp, which were dried at room temperature for 30 min and then incubated for 24 h at 37°C. Clear zones of lysis on the *E. coli* Famp lawns indicated the presence of F-specific RNA coliphages. For concentrated samples, a 0.5-ml aliquot of the sample was mixed with 100 μl of an exponential culture of *E. coli* Famp and 3 ml of 0.75% tryptic soy agar. This mixture was poured on top of a solidified bottom agar layer (1.5% tryptic soy agar contained in a petri dish) and allowed to solidify. The plates were then inverted and incubated at 37°C and observed for plaque formation after 24 h.

A 10-μl suspension of a purified MS2 (ATCC 13706-B1) stock was spotted on bacterial lawn along with the samples to verify the sensitivity of the host for F-specific coliphages. Sterilized media (10× tryptic soy broth) was enriched as a negative enrichment control.

Method sensitivity. The methods for virus detection were validated by applying 20 μl of a suspension of either F-specific coliphage or feline calicivirus (as a NoV surrogate) on 200 g of lettuce, 200 g of spinach, or 400 g of peeled carrots. An average of 55% of feline calicivirus and 60% of MS2 was recovered by the above methods.

NoV detection. Viral nucleic acid was extracted from concentrated eluate with the QIAamp viral RNA minikit (Qiagen, Valencia, Calif.). In brief, 140 μl of sample was mixed with 560 μl of a lysis buffer, vortexed briefly, and incubated for 15 min at room temperature. Next, 560 μl of absolute ethanol was added, and the mixture was passed through a QIAamp spin column. The column was then washed twice with 500 μl of washing buffers (AWI-1 and AWI-2). RNA bound to the spin column cartridge was eluted in 60 μl of QIAamp AVE elution buffer and was stored at −20°C until use.

Detection of NoV was performed by reverse transcription–PCR (RT-PCR) with the use of primers JV 12 (5′-ATACCAC TATGATGCAGATTA-3′) and JV 13 (5′-TCATCATCACCATA GAAAGAG-3′), which have been shown to be capable of detecting a wide range of NoV genotypes *(23)*. To minimize the likelihood of cross-contamination, a single-tube RT-PCR method was adopted with the use of the Qiagen single-tube RT-PCR kit. Amplification was carried out in a reaction volume of 50 μl containing 10 μl of 5× RT buffer, 2 μl of dNTP mixture (10 mM of each dNTP), 2 μl of enzyme mix and 2 μl of Q solution, 1 μl of each primer (50 pmol each), 10 μl of RNA, and 23 μl of nuclease-free water to make the 50-μl reaction mixture. Reverse transcription was carried out at 50°C for 1 h followed by enzyme inactivation at 95°C for 15 min. PCR was carried out in a GeneAmp PCR system 9600 machine (Perkin Elmer, Boston, Mass.) for 40 cycles (denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 2 min) followed by a final extension at 72°C for 5 min. Electrophoretic separation of PCR products was performed for 1 h at 101 V on a 3% agarose gel in 1× TAE buffer, and products were stained with ethidium bromide. The amplicons were visualized under a UV transilluminator. A 1-kbp DNA ladder (Invitrogen, Carlsbad, Calif.) was used as a marker for estimating product length. Nucleic acid–free water was run as a negative control and confirmed by RT-PCR; a NoV-positive fecal sample was used as a positive control.

RESULTS AND DISCUSSION

Retail ($n = 40$) and outbreak samples ($n = 6$) of fresh produce were examined in this study (Table 1). All items were grown in or near the ground. Two thirds of the samples (67%) were grown in the United States and one third (33%) were imported. Nineteen of the 40 retail samples (48%) and all outbreak samples were preprocessed (cut, chopped, peeled, or shredded) prior to being sampled.

NoVs were not detected in any of the samples (Fig. 1). Only two (5%) unprocessed retail samples were positive for *E. coli* (MPN > 0.03 per g). However, all of the outbreak samples ($n = 6$) and 13 (33%) retail samples were positive for F-specific coliphage (odds ratio undefined, *P*

TABLE 1. *Frequency table of sampled items by outbreak, preparation method, and test results*

Item	Frequency	Association with outbreak	Processed samples[a]	Samples positive for[b]:		
					FRNA[c]	
				E. coli	Concentrated	Enriched
Artichoke	1	0	0	0	0	0
Brussels sprouts	1	0	0	0	0	0
Broccoli	1	0	1	0	0	1
Cabbage	1	0	0	0	0	0
Cactus leaves	1	0	1	0	0	0
Cantaloupe	1	0	1	0	0	0
Carrots	2	0	2	0	1	2
Cauliflower	2	0	2	0	0	1
Celery	2	0	0	0	0	0
Green beans	1	0	0	0	0	0
Green onions	1	0	0	0	1	1
Huauzontle leaves	1	0	0	1	0	0
Leeks	1	0	0	0	0	0
Lettuce	10	0	7	0	3	6
Melon	1	0	1	0	0	0
Mixed salad[d]	4	0	4	0	1	1
Onions	2	2	2	NT[e]	0	2
Peas	2	0	0	0	0	0
Peppers	2	2	2	NT	0	2
Radishes	1	0	0	0	0	0
Spinach	1	0	0	0	0	1
Strawberries	1	0	0	0	0	0
Tomatillos	1	0	0	0	0	0
Tomatoes	2	2	2	NT	0	2
Verdolaga leaves	1	0	0	1	0	0
Yellow squash	1	0	0	0	0	0
Zucchini	1	0	0	0	0	0
Total	46	6	25	2	6	19

[a] Cutting, peeling, shredding, etc.
[b] None of the samples were positive for norovirus as detected by RT-PCR of sample concentrates.
[c] F-specific RNA coliphages.
[d] Mixed salads contained two or more shredded, chopped, or peeled fruits or vegetables.
[e] Not tested for *E. coli.*

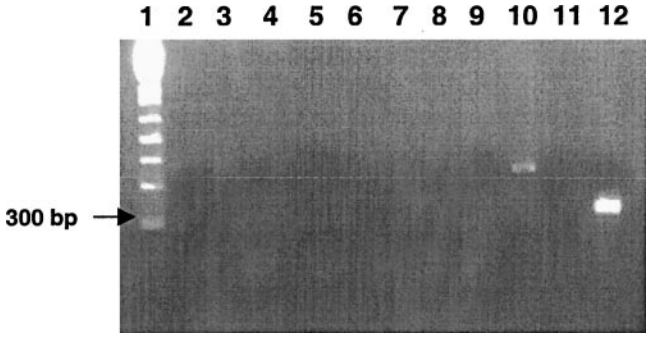

FIGURE 1. *Ethidium bromide–stained 3% agarose gel showing RT-PCR of nine produce samples for detection of norovirus with the use of broadly reactive primer sets JV 12 (5′-ATACCACTATGATGCAGATTA-3′) and JV 13 (5′-TCATCATCACCATAGAAAGAG-3′). Lane 1, 1-kbp DNA ladder; lanes 2–10, produce samples; lane 11, negative control (nucleic acid–free water); lane 12, positive control. The expected product size is 326 bp.*

= 0.003). Enrichment appeared to be more sensitive to coliphage detection because more than three times as many samples were positive after enrichment as were positive after eluate concentration without enrichment, probably because enrichment results in multiplication of the coliphages present in the rinse samples. Thus, even a few coliphages would increase in numbers and be detected easily. Concentration, on the other hand, does not result in phage multiplication and therefore could be less sensitive *(18).*

Among retail samples, processed samples appeared more likely to be contaminated with F-specific coliphages than unprocessed samples (odds ratio 3.8; 95% confidence interval 1.8 to 21.0). This finding emphasizes the potential usefulness of F-specific coliphages as indicators of enteric viruses in food. As demonstrated in this survey, F-specific coliphages can be more readily identified in an outbreak than NoVs. Thus, if it could be ascertained that there is a correlation between the presence of F-specific coliphages and the presence of enteric viral pathogens in food, they could play an important role in foodborne disease prevention.

F-specific coliphages possess certain characteristics that make them good candidate indicators of enteric viruses *(12, 13)*. For example, they are found only in the digestive tract of warm-blooded animals, certain serotypes are strongly associated with human waste, and they are relatively easy to detect *(1)*. The relative ease of detection of F-specific coliphages was highlighted in this study when they were detected in all six samples collected during a NoV outbreak, although NoVs could not be detected. Given the epidemiologic connection between the sampled items and illness among patrons, the failure to detect NoVs is likely because of the presence of low virus titers in the original samples.

Despite the positive attributes of F-specific coliphages as candidate indicators of enteric viruses, there are several questions about their suitability for this role. For example, unlike NoVs, these organisms can be found in the intestinal tract of both man and other animals; thus, their presence does not necessarily indicate human fecal contamination. Also, it has been reported that they occur in much lower numbers than bacterial indicators in environmental samples and are sensitive to heat *(10)*.

Detection of F-specific coliphages in all outbreak samples and in 47% of preprocessed and 19% percent of unprocessed retail samples suggests a possible correlation between increased handling of produce and the presence of F-specific coliphages. This type of association is plausible because F-specific coliphages are shed in human feces, and hand washing compliance among retail food workers is known to be relatively low *(20, 21)*. However, because of the small number of samples examined in this survey, we urge caution in making generalizations about the overall occurrence of F-specific coliphages on fresh produce.

Indicator organisms have played a major role in the prevention of food and waterborne diseases. Typically, the need for an indicator organism arises because the pathogen of interest is too difficult or costly to detect directly *(22)*. A lack of methods for routine detection of NoVs in nonshellfish foods or the environment justifies the use of F-specific coliphages as candidate indictors of these pathogens because coliphages are much easier to detect. However, inconsistent detection of F-specific coliphages in the environment and a lack of data regarding the correlation between the presence of enteric viral pathogens and F-specific coliphages have made their adoption as indicators controversial *(8)*. Regardless, F-specific coliphages have remained attractive candidate indicators of enteric viruses because they have been shown to be similar to enteric viruses in survival studies *(9, 10)*. We recommend that more comprehensive surveys of market-ready fresh produce be conducted to further investigate the occurrence of viral pathogens and their potential indicators on these commodities.

REFERENCES

1. Allwood, P. B., Y. S. Malik, C. W. Hedberg, and S. M. Goyal. 2003. Survival of F-specific RNA coliphage, feline calicivirus, and *Escherichia coli* in water: a comparative study. *Appl. Environ. Microbiol.* 69:5707–5710.
2. Beuchat, L. R., and J. H. Ryu. 1997. Produce handling and processing practices. *Emerg. Infect. Dis.* 3:459–465.
3. Burnett, S. L., and L. R. Beuchat. 2001. Human pathogens associated with raw produce and unpasteurized juices, and difficulties in decontamination. *J. Ind. Microbiol. Biotechnol.* 27:104–110.
4. Centers for Disease Control and Prevention. 2003. Hepatitis A outbreak associated with green onions at a restaurant—Monaca, Pennsylvania, 2003. *Morb. Mortal. Wkly. Rep.* 52:1155–1157.
5. Cliver, D. O. 1997. Virus transmission via food. *World Health Stat. Q.* 50:90–101.
6. Croci, L., D. De Medici, C. Scalfaro, A. Fiore, and L. Toti. 2002. The survival of hepatitis A virus in fresh produce. *Int. J. Food Microbiol.* 73:29–34.
7. Dentinger, C. M., W. A. Bower, O. V. Nainan, S. M. Cotter, G. Myers, L. M. Dubusky, S. Fowler, E. D. Salehi, and B. P. Bell. 2001. An outbreak of hepatitis A associated with green onions. *J. Infect. Dis.* 183:1273–1276.
8. Dore, W. J., K. Henshilwood, and D. N. Lees. 2000. Evaluation of F-specific RNA bacteriophage as a candidate human enteric virus indicator for bivalve molluscan shellfish. *Appl. Environ. Microbiol.* 66:1280–1285.
9. Endley, S., L. Lu, E. Vega, M. E. Hume, and S. D. Pillai. 2003. Male-specific coliphages as an additional fecal contamination indicator for screening fresh carrots. *J. Food Prot.* 66:88–93.
10. Feng, P., S. D. Weagant, and M. A. Grant. 1998. Enumeration of *Escherichia coli* and the coliform bacteria. *In* U.S. Food and Drug Administration (ed.), Bacteriological analytical manual online, 8th ed. Available at: http://www.cfsan.fda.gov/~comm/ift3–2c.html. Accessed 21 June 2003.
11. Grabow, W. O., M. B. Taylor, and J. C. de Villiers. 2001. New methods for the detection of viruses: call for review of drinking water quality guidelines. *Water Sci. Technol.* 43:1–8.
12. Havelaar, A., M. van Olphen, and Y. Drost. 1993. F-specific RNA bacteriophages are adequate model organisms for enteric viruses in fresh water. *Appl. Environ. Microbiol.* 59:2956–2962.
13. Hernroth, B. E., A. C. Conden-Hansson, A. S. Rehnstam-Holm, R. Girones, and A. K. Allard. 2002. Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: the first Scandinavian report. *Appl. Environ. Microbiol.* 68:4523–4533.
14. Jaykus, L. A. 1997. Epidemiology and detection as options for control of viral and parasitic foodborne disease. *Emerg. Infect. Dis.* 3:529–539.
15. Kurdziel, A. S., N. Wilkinson, S. Langton, and N. Cook. 2001. Survival of poliovirus on soft fruit and salad vegetables. *J. Food Prot.* 64:706–709.
16. Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5:607–625.
17. Parashar, U. D., and S. S. Monroe. 2001. ''Norwalk-like viruses'' as a cause of foodborne disease outbreaks. *Rev. Med. Virol.* 11:243–252.
18. Taku, A., B. R. Gulati, P. B. Allwood, K. Palazzi, C. W. Hedberg, and S. M. Goyal. 2002. Concentration and detection of caliciviruses from food contact surfaces. *J. Food Prot.* 65:999–1004.
19. U.S. Environmental Protection Agency. 2001. Method 1601: Male-specific (F+) and somatic coliphage in water by two-step enrichment procedure. Available at: http://www.epa.gov/microbes/1601ap01.pdf. Accessed 10 September 2003.
20. U.S. Food and Drug Administration. 1998. Guide to minimize microbial hazards for fresh fruits and vegetables. Center for Food Safety and Applied Nutrition, Washington, D.C.
21. U.S. Food and Drug Administration. 2000. Report of the FDA retail food program database of foodborne illness risk factors. Available at: http://www.cfsan.fda.gov/~acrobat/retrsk.pdf. Accessed 21 January 2004.
22. U.S. Food and Drug Administration. 2001. Analysis and evaluation of preventive control measures for the control and reduction/elimination of microbial hazards on fresh and fresh-cut produce. Available at: http://www.cfsan.fda.gov/~comm/ift3–2c.html. Accessed 5 June 2003.
23. Vinje, J., and M. P. Koopmans. 1996. Molecular detection and epidemiology of small round-structured viruses in outbreaks of gastroenteritis in the Netherlands. *J. Infect. Dis.* 174:610–615.