Chlorine inactivation of coliphage MS2 on strawberries by industrial-scale water washing units

Michael J. Casteel, Charles E. Schmidt and Mark D. Sobsey

ABSTRACT

Fruits and vegetables (produce) intended for minimal processing are often rinsed or washed in water. Chlorine and other sanitizers are used during washing to inactivate produce spoilage microbes, but such procedures may also inactivate pathogens epidemiologically linked to produce, such as hepatitis A virus (HAV). However, no information exists on the efficacy of chlorinated wash water to inactivate HAV and other viruses on produce in actual practice, because of obvious safety concerns. In contrast, coliphage MS2 (a bacterial virus) is commonly used as a surrogate for some pathogenic viruses and may be safely used in field studies. In the present investigation, strawberries seeded with MS2 were passed through industrial-scale water washing units operated with or without added sodium hypochlorite. MS2 on strawberries was inactivated by 68%, 92% and 96% at free chlorine (FC) concentrations of ≤ 2, 20 and 200 ppm in wash water, respectively. MS2 was detected in wash water containing ≤ 2 ppm FC in one trial, but was not detected in water containing 20 or 200 ppm FC. The presence and absence of MS2 in wash water containing various levels of FC highlight the importance of controlling sanitizer levels to prevent viral cross contamination of strawberries.

Key words | chlorine, coliphage MS2, disinfection, strawberries, virus, water

Michael J. Casteel (corresponding author)
Microbial Intelligence Group, LLC,
PO Box 1292,
San Bruno, California 94066,
USA
Tel.: +1 650-9864-3453
E-mail: *mc@microbial-intelligence-group.us*

Charles E. Schmidt
Agrana Fruit US, Inc.,
6850 Southpointe Parkway,
Brecksville, OH 44141,
USA

Mark D. Sobsey
Department of Environmental Sciences and Engineering,
University of North Carolina at Chapel Hill,
Chapel Hill, NC 27599,
USA

INTRODUCTION

Minimally processed fruits and vegetables (produce) may be washed, cooled or transported in water using industrial washing units, sprayers, dump tanks, flumes and other equipment. During such procedures, microbes present on produce surfaces and in water may be transferred to surrounding product, thereby resulting in cross-contamination. Hence, sanitizers are recommended for use in produce washing systems whenever possible (Beuchat 1996). Chemical sanitizers used by the produce industry to reduce microbes on produce and in wash water include, but are not limited to chlorine, chlorine dioxide, ozone and peroxyacetic acid; however, chlorination is the most widely utilized method, and is often the standard to which other chemical sanitizers are compared. In general, chlorine concentrations, contact times and microbial reductions for produce and wash water are ≤ 200 ppm, ≤ 2 minutes and $\leq 2 \log_{10}$, respectively (Beuchat 1998). Most work in the area of produce sanitization has focused on various combinations of parameters to inactivate pathogenic bacteria and produce spoilage microbes (e.g. yeasts and moulds), and standard protocols to assess sanitizer effectiveness have been proposed (Beuchat *et al.* 2001*a*,*b*).

While many reported outbreaks and cases of produce-borne disease are attributed to pathogenic bacteria such as *Salmonella* and *Escherichia coli* O157:H7, fecal viruses in crop growing areas and on produce are also a public health concern. Acute infectious hepatitis (caused by hepatitis A virus; HAV) and gastroenteritis (caused by noroviruses) have been epidemiologically linked to the consumption of raw and minimally processed strawberries, raspberries, blueberries, green onions, lettuce and other produce (Cliver *et al.* 2006), and it is estimated that these and other enteric

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viruses are responsible for approximately two-thirds of all food-borne illness with known aetiology (Mead *et al.* 1999). Historically, the coliform bacteria and *E. coli* have been used to assess disinfection effectiveness and to determine the overall sanitary quality of environmental media, including food. However, there is now appreciable evidence showing that vegetative bacteria are poor predictors of the occurrence and survival of viruses and parasites following exposure to disinfectants and environmental conditions (Feachem *et al.* 1983; Sobsey 1989; Payment *et al.* 1991), and the need to match appropriate microbial surrogates with specific pathogens for determining the efficacy of produce safety procedures is increasingly recognized (Busta *et al.* 2003).

Coliphages, which are bacterial viruses infecting *E. coli*, have been proposed for use as surrogates for the disinfection efficacy of viruses such as HAV (Sobsey *et al.* 1988; IAWPRC 1991). A number of previous studies have shown that male-specific, ribonucleic acid (RNA) containing (i.e. F + RNA) coliphages such as MS2 are adequate surrogates for predicting chlorine (Sobsey *et al.* 1988; Sobsey 1989), ozone and hydrogen peroxide (Hall & Sobsey 1993) inactivation of HAV in water. Studies examining or using various coliphages in shellfish have been reported for decades (Canzonier 1971; Vaughn & Metcalf 1975), and there have been several published studies on the detection, survival and occurrence of coliphages in various other food types (Kennedy *et al.* 1984, 1986*a*,*b*; Kennedy & Bitton 1987; Hsu *et al.* 2002). Coliphages have also been used to screen produce for fecal contamination (Allwood *et al.* 2004*b*), to examine virus uptake by plant root systems (Ward & Mahler 1982), and to determine virus survival on produce following exposure to various environmental conditions and disinfection treatments (Mariam & Cliver 2000*a*,*b*; Chaidez *et al.* 2003; Allwood *et al.* 2004*a*; Dawson *et al.* 2005; Casteel *et al.* 2008). However, there is no information available on the ability of chlorine and other sanitizers to inactivate viruses, including coliphages, on produce and in water from actual postharvest processing operations. To address these issues, experiments were performed to determine the ability of various concentrations of chlorine to inactivate MS2 on strawberries passed through industrial-scale washing units. Levels of ambient microbes (aerobic bacteria, yeasts and moulds) on strawberries and their inactivation by chlorinated water were also determined. An elution technique was employed to recover all of the microbes from strawberries, and water samples from washing units were assayed for the presence or absence of MS2 using a sensitive enrichment method.

METHODS

Sources of strawberries and description of washing/sanitation processes

Strawberries used in the present study were delivered to commercial processing facilities in California. Fresh strawberries were selected, seeded with virus (see below) and passed through industrial-scale continuous produce washing systems (Figure 1). Tap water (380 l) was added directly to the main compartment of the washing units, which used simple mechanisms to gently agitate and wash the strawberries. Tap water in the main compartment of the washers was used without additional added chlorine (i.e. the residual free chlorine level was $\leq$2 ppm) or was injected with sodium hypochlorite (NaOCl) to achieve a final concentration of

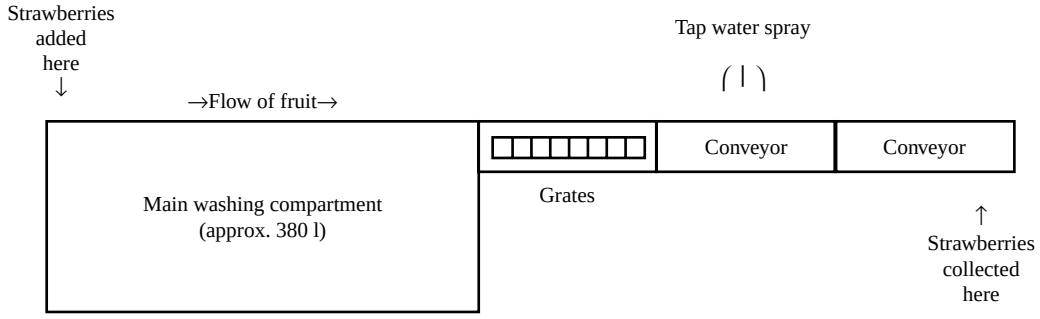

Figure 1 | Schematic of strawberry washing unit.

approximately 20 or 200 ppm free chlorine. As shown in Figure 1, strawberries were added to the main compartment of a washing unit, travelled over perforated grates, were sprayed with tap water (no additional chlorine added) and collected for further processing. With the exception of pH and free chlorine levels, other water quality parameters were not measured. In this study, exposure of strawberries to various dosages of free chlorine in water in the main compartments of washers was approximately 12 seconds at pH 6.5. Because the flow of fruit was controlled for the volume of wash water, there was no loss of free chlorine at dosages $\geqq$ 20 ppm due to oxidant demand. The washers were sanitized with 200 ppm free chlorine followed by a tap water rinse between trial replications.

Preparation of virus stock used in experiments

Coliphage MS2 (ATCC 15597-B1) used in these experiments was grown and assayed using the double agar layer technique (Adams 1959), producing confluent or discrete (plaque) lysis, respectively, of an overnight culture of *E. coli* C3000 (ATCC 15597) in molten tryptic soy agar (TSA). Suspensions of MS2, *E. coli* C3000 host, and top agar were overlaid on TSA plates and incubated overnight at 37°C. Virus stocks were purified and monodispersed as previously described (Shin & Sobsey 2003), and areas of confluent lysis (plaques) in TSA overlays were enumerated and expressed as $\log_{10}$ plaque-forming units (PFU).

Procedure for seeding viruses on strawberries

Strawberries were seeded with 10-μl volumes of stock MS2 for a concentration of approximately 7 $\log_{10}$ PFU per strawberry, and this virus-containing suspension was allowed to dry on the surface of the fruit as previously described (Casteel *et al.* 2008). Fifteen fresh, medium-sized strawberries without visible bruising, rot or decay were selected for these experiments, and five of the strawberries were seeded with MS2. Weights of representative strawberries ($n = 45$) in this study ranged from 14 to 41 g (mean ± SD = 23 ± 5.8 g). The ten unseeded strawberries were included with the five seeded strawberries to compose one sample batch of 15 strawberries. One sample batch of strawberries was passed through a washing unit for each free chlorine dose.

Experimental design and analysis of samples

Separate trials were performed in which batches of strawberries seeded with MS2 were sent through washers using municipal tap water containing no added chlorine or approximately 20 or 200 ppm free chlorine. For each trial, a single sample batch of strawberries was run through a washing unit alone, in the absence of other fruit. Control strawberries (without MS2) were passaged first. Free chlorine and pH levels of the wash water were measured using test strips (Hach Co., Loveland, Colorado). After passage through a washing unit, strawberries were collected in sterile polypropylene containers and subjected to an elution procedure, which consisted of shaking (100 rpm for 60 min) in 500 ml of 3% beef extract/0.1% Tween-80, pH 8 (Casteel *et al.* 2008). Grab samples of water (1,000 ml) from washing units were aseptically collected and mixed with sodium thiosulfate (*Standard Methods* 1995) to neutralize residual free chlorine.

Water and strawberry eluate samples were sent in coolers containing ice packs by overnight delivery to the University of North Carolina at Chapel Hill. Descriptions of the samples are shown in Table 1. Strawberry eluate volumes of 100 ml were assayed for MS2 using the single agar layer technique (USEPA 2001*b*), and eluate volumes of $\leq$ 1 ml were assayed using the double agar layer technique (Adams 1959). Coliphages in water samples were processed using an enrichment procedure (USEPA 2001*a*), and analysis of strawberry eluates and water for MS2 used the bacterial

Table 1 | Sample descriptions

Strawberries* seeded with coliphage MS2 (control)
Strawberries* without coliphage MS2, analysed for background levels of F + and somatic coliphages (control)
Strawberries* seeded with coliphage MS2, passed through washers using municipal (tap) drinking water ($\leq$ 2 ppm free chlorine)
Strawberries* seeded with coliphage MS2, passed through washers using water containing 20 ppm free chlorine
Strawberries* seeded with coliphage MS2, passed through washers using water containing 200 ppm free chlorine
1 litre volumes of water† ($\leq$ 2, 20, and 200 ppm free chlorine)

*Analysed for MS2 and other microbes.
†Analysed for MS2 only.

Table 2 | Log_{10} PFU of coliphage MS2 per ml of strawberry eluate and mean % inactivation after exposure to ≤2, 20 or 200 ppm free chlorine

Sample	Log_{10} PFU MS2 per ml eluate Trial 1	Log_{10} PFU MS2 per ml eluate Trial 2	Mean ± SD (log_{10} PFU MS2 per ml eluate)	% inactivation
Control*	4.3	4.2	4.3 ± 0.07	–
≤2 ppm free chlorine	3.9	3.6	3.8 ± 0.21	68
20 ppm free chlorine	3.2	3.2	3.2 ± 0	92

*Strawberries not passed through washer.
SD = standard deviation.

host *E. coli* HS [pFampR] (*E. coli* F_{amp}). The bacterial hosts *Salmonella typhimurium* WG49 and *E. coli* CN13 were used to assay control strawberries (not seeded with MS2) for background levels of F + and somatic coliphages, respectively. Portions of the strawberry eluate were also sent to a commercial laboratory for the analysis of aerobic bacteria and total yeasts and moulds (TYM), in which 1-ml portions of the eluates were assayed on 3M™ Petrifilm™ Count Plates. Results for MS2 and ambient microbes recovered from strawberries are reported as the mean (± one standard deviation) log_{10} number of PFU or colony-forming units (CFU) per ml of strawberry eluate. Microbial reductions are reported as a percentage reduction, calculated by subtracting the log_{10} number of microbes per ml of eluate recovered from strawberries exposed to chlorine-containing water from the number of microbes per ml of eluate recovered from control strawberries.

RESULTS AND DISCUSSION

Inactivation levels of MS2, aerobic bacteria and TYM on strawberries passed through industrial-scale washing units are shown in Tables 2, 3 and 4, respectively. Neither male specific (F +) nor somatic coliphages were detected on control strawberries (data not shown). In contrast, mean levels of aerobic bacteria and TYM on control strawberries were 6.7 and 6.5 log_{10} CFU per ml strawberry eluate, respectively, which corresponds to approximately 6.9 and 6.7 log_{10} of these microbes per gram of strawberry. The levels of ambient microbes on the control strawberries differed by about an order of magnitude between trials one and two; levels of aerobic bacteria and TYM were 1.2 and 0.8 log_{10} units higher, respectively, in trial two compared with trial one. While there was appreciable variation in levels of ambient microbes on strawberries between trials, the levels and observed variation are similar to previous findings (Nguyen-the & Carlin 1994).

There were measurable reductions of MS2 on strawberries under all conditions tested. As shown in Table 2, MS2 on strawberries passed through washing units was inactivated by an average of 68%, 92% and 96% at free chlorine concentrations of ≤2 (tap water), 20 and 200 ppm, respectively. Unlike ambient microbes, MS2 recovery from control strawberries in trials 1 and 2 differed by only 0.1 log_{10} unit, and the extent of its inactivation was similar in both trials. Although MS2 was inactivated by an average of 68% (0.5 log_{10}) on strawberries after washing in tap water

Table 3 | Log_{10} CFU of aerobic bacteria per ml of strawberry eluate and mean % inactivation after exposure to ≤2, 20 or 200 ppm free chlorine

Sample	Log_{10} CFU aerobic bacteria per ml eluate Trial 1	Log_{10} CFU aerobic bacteria per ml eluate Trial 2	Mean ± SD (log_{10} CFU aerobic bacteria per ml eluate)	% inactivation
Control*	6.1	7.3	6.7 ± 0.85	–
≤2 ppm free chlorine	6.0	5.8	5.9 ± 0.14	84
20 ppm free chlorine	5.4	4.4	4.9 ± 0.71	98
200 ppm free chlorine	5.4	4.3	4.9 ± 0.78	98

*Strawberries not passed through washer.
SD = standard deviation.

Table 4 | Log_{10} CFU of total yeasts and moulds (TYM) per ml of strawberry eluate and mean % inactivation after exposure to ≤2, 20 or 200 ppm free chlorine

Sample	Log_{10} CFU TYM per ml eluate Trial 1	Log_{10} CFU TYM per ml eluate Trial 2	Mean ± SD (log_{10} CFU TYM per ml eluate)	% inactivation
Control*	6.1	6.9	6.5 ± 0.57	–
≤2 ppm free chlorine	6.0	6.7	6.4 ± 0.50	21
20 ppm free chlorine	4.5	5.8	5.2 ± 0.92	95
200 ppm free chlorine	5.2	5.2	5.2 ± 0	95

*Strawberries not passed through washer.
SD = standard deviation.

without additional chlorine, the virus was detected in a water sample taken from the main washing compartment in the first trial. However, MS2 was not detected (i.e. was <1 PFU l^{-1}) in water samples taken from the washer's main compartment in the second trial (Table 5).

Like MS2, aerobic bacteria and TYM on strawberries were inactivated more extensively with 20 or 200 ppm free chlorine compared with tap water. As shown in Tables 3 and 4, however, there are appreciable differences in the inactivation of ambient microbes on strawberries when the individual data from trials 1 and 2 are compared. For example, the inactivation of TYM in trial 1 was greater at 20 ppm free chlorine compared with 200 ppm, and the inactivation of aerobic bacteria by 20 ppm free chlorine was 80% in the first trial and almost 99.9% in the second trial. Compared with MS2, aerobic bacteria were less extensively inactivated during the first trial but were more extensively inactivated than MS2 during the second trial. The inactivation levels of TYM were similar to the inactivation of MS2 in both trials.

Although variable, the levels of inactivation of ambient microbes by 200 ppm free chlorine are within the ranges of inactivation reported in fruit sanitization studies (Stapleton 1986). However, problems exist in using ambient microbes for determination of sanitizer and washing effectiveness. In the present study, levels of aerobic bacteria and TYM on strawberries differed by approximately one order of magnitude during separate trials; ambient microbes are also acclimated to the ecology of the produce, may propagate following disinfection treatment (O'Connor & Mitchell 1991), or may exist in various physiological states, affecting their ability to be cultured. In contrast, the present study used a well-characterized laboratory strain of the F + RNA coliphage group (MS2) to assess sanitizer effectiveness. Coliphage MS2 is available from the American Type Culture Collection (see Methods), and the use of it and other laboratory propagated viruses in disinfection studies is preferred, as environmental virus isolates may have varying degrees of resistance (Payment *et al.* 1985). The levels of inactivation of coliphage MS2 on strawberries observed in this study are similar to results obtained in bench-scale experiments in which strawberries and other produce items seeded with MS2 and HAV were washed in water containing 20 and 200 ppm free chlorine (Casteel *et al.* 2008).

Table 5 | Presence (+) or absence (−) of MS2 in strawberry wash water containing ≤2, 20 or 200 ppm free chlorine

Water sample	MS2 present (+) or absent (−) Trial 1	MS2 present (+) or absent (−) Trial 1
≤2 ppm free chlorine	+	−
20 ppm free chlorine	−	−
200 ppm free chlorine	−	−

CONCLUSIONS

The use of chlorine at typical levels used in postharvest washing procedures resulted in appreciable inactivation (92–96%) of coliphage MS2 on strawberries. When strawberries were washed in tap water only, virus inactivation levels were about 0.5 log_{10} (68%) compared with 1.4 log_{10} (96%) when washed in water containing 200 ppm chlorine. The washing units evaluated in this study are representative of those used by strawberry processors

to clean fruit before it is sliced or combined with other material for inclusion as food ingredients, and the field results reported here are similar to those obtained in bench-scale studies. The absence of MS2 in the chlorinated wash water and the presence of virus in the main wash compartment water in one of the trials demonstrate the importance of maintaining and controlling chlorine levels to prevent cross-contamination of produce, and to inactivate waterborne microbes that could infiltrate produce through stem scars and other openings of strawberries. However, additional studies are needed to verify the results presented here, and disinfection practices are best used in combination with other procedures (i.e. a multiple barrier approach) to reduce the risk of transmission of fecal viruses on fruits and vegetables.

Coliphage MS2 is a convenient model to study virus inactivation on chlorinated strawberries under actual processing conditions. The use of coliphage MS2 and perhaps other F + RNA coliphages is beneficial over the direct analysis of fecal viruses such as HAV for a number of reasons. Compared with fecal viruses, results for F + RNA coliphages can be quickly and easily obtained, and the assays involved are less expensive than other types of virological assay. Coliphages are nonpathogenic to humans and can be used to evaluate virus survival following exposure to disinfection procedures, and for studying virus survival on crops exposed to varying temperature, humidity or other environmental conditions. However, bacteriophages are in fact different viruses compared with HAV and other human enteric viruses. Hence, future bench-scale studies should examine various combinations of produce type, sanitizer and contact time to inactivate culturable human enteric viruses, cytopathic variants of HAV (e.g. HAV HM175) and bacteriophages in the presence and absence of soil and fecal material. Future studies should also quantitatively determine both seeded and ambient levels of F+ and other RNA coliphages in processing water and on strawberries and other produce exposed to various water washing processes. The differences observed in the levels of inactivation of microbes between trials in the present study may be partially explained by variables such as organic load on strawberries and differences in water quality; hence, future studies should also consider such factors.

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