Differential Killing Activity of Cetylpyridinium Chloride with or without Bacto Neutralizing Buffer Quench against Firmly Adhered *Salmonella* Gaminara and *Shigella sonnei* on Cut Lettuce Stored at 4°C

MOEZNNIMANWATY OSMAN,† MARLENE E. JANES,‡ ROBERT STORY, RAMAKRISHNA NANNAPANENI, and MICHAEL G. JOHNSON*

Department of Food Science and Center for Food Safety, IFSE, 2650 North Young Avenue, University of Arkansas, Fayetteville, Arkansas 72704, USA

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**ABSTRACT**

Cetylpyridinium chloride (CPC) activity was quenched with Bacto neutralizing buffer on inoculated cut iceberg lettuce. This protocol permitted comparison of the numbers of *Salmonella* Gaminara- or *Shigella sonnei*-inoculated cells on lettuce that survived 1 min of CPC treatment. Cut lettuce was inoculated with about 6 log of *Salmonella* or 9 log of *Shigella* and stored in Whirl-Pak bags at 4°C for up to 4 days. Loosely adhered pathogen cells were washed off before CPC treatment. Firmly adhered cells of *Salmonella* Gaminara or *S. sonnei* on cut iceberg lettuce survived treatment with CPC even at the 0.4% CPC level if the CPC activity was quenched after 1 min by adding Bacto neutralizing buffer. The results confirm that there is extended killing activity of residual CPC against *Salmonella* Gaminara or *S. sonnei* if the residual CPC remaining in contact with the lettuce after the initial 1-min wash is not quenched. The CPC treatment was useful in reducing the numbers of these target pathogens on lettuce.

Consumers have been increasing their intake of fresh fruits and vegetables as a result of diet trends and interest in health. This increase has coincided with a marked increase in the number of produce-associated foodborne illnesses reported. The Centers for Disease Control and Prevention reported that between 1973 to 1987 and 1988 to 1992—excluding illness due to botulism, mushroom, and “salad”-associated illness (including salads that contain nonproduce items)—the number of produce-related outbreaks per year more than doubled (18, 22, 23). The Centers for Disease Control and Prevention estimated that fresh produce contributed at least 12% of the foodborne outbreak-associated cases in the 1990s (7).

*Salmonella* Gaminara, *Escherichia coli* O157:H7, and *Shigella* were selected by the U.S. Food and Drug Administration as focus organisms in their imported produce study as a result of the many outbreaks involving fresh and minimally processed produce in which these organisms were implicated (1). Of the 1,003 samples tested from 21 countries, 44 samples were found to be contaminated with pathogens (3). Of these 44 samples, 35 (80%) were contaminated with *Salmonella*, and 9 (20%) were contaminated with *Shigella* (3).

Cetylpyridinium chloride (CPC) is a water-soluble quaternary ammonium compound widely found in mouthwash, toothpaste, and throat lozenge products because of its ability to prevent dental plaques, gingivitis, and bacterial biofilm formation (1, 2, 8, 13, 17). Electron microscopy studies show that CPC kills bacterial cells by damaging their membranes, producing leakage of cellular materials (15). Studies have shown that CPC spray is effective in reducing *Salmonella* counts on poultry tissues (4, 5, 12, 25, 27, 28) and on beef surfaces (9). CPC treatments have also been found to be effective in reducing counts of *Salmonella* Typhimurium, *E. coli*, and *Listeria monocytogenes* cells on several different types of fresh produce, including broccoli, radishes, cauliflower (16, 24), and bean sprouts (16). Janes et al. (11) showed that CPC is effective in removing and killing *L. monocytogenes* cells inoculated onto cut lettuce.

Bacto neutralizing buffer (NB) is a water-soluble, tan-colored compound. NB is a modification of standard buffered, distilled water, and because of its ability to neutralize sanitizers, it can be used in the process of detection of persistent microbes after food processing equipment surfaces have been sanitized with chlorine and quaternary ammonium compounds. Additionally, the buffering ability of NB is provided by the monosodium phosphate (10).

This study was performed to investigate the efficacy of 1-min treatments of different concentrations of CPC in killing firmly attached *Salmonella* Gaminara and *S. sonnei* in-
oculated onto cut lettuce and held at 4°C for up to 4 days with and without a quench step immediately after CPC treatment. Loosely adhered cells are those that were detached easily by washing; firmly adhered cells are those that remained attached to the lettuce even after the wash step. Residual pathogen cells recovered in pooled rinses after CPC treatments without or with NB quench were enumerated to determine whether the detached pathogens cells could retain their viability in the washes when removed from the lettuce.

**MATERIALS AND METHODS**

**Lettuce sampling.** Whole heads of iceberg lettuce (*Lactuca sativa*) were obtained from a local supermarket. The core of each head and the outer leaves were removed and discarded. The remaining leaves were placed on a plate and covered with sterile cellophane wrap. The lettuce was then placed in a refrigerator at 4°C for 24 h. After 1 h, the lettuce was allowed to dry in a colander for approximately 30 min. The leaves were cut into approximately 2.5-cm squares. Twenty-five-g samples of lettuce were macerated in separate sterile 1,600-ml-size Whirl-Pak bags (Nasco, Fort Atkinson, Wis.) before inoculation with pathogen cells.

**Inoculation of target pathogen cells.** Two different bacterial cultures were used in this study, *Salmonella* Gaminara (F2712), from an orange juice-associate outbreak (6), and *Shigella sonnei* (F6129), from a 1998 parsley-associated outbreak (26). This *Shigella* strain is resistant to various antimicrobial agents, including tetracycline, trimethoprim-sulfamethoxazole, ampicillin, sulfisoxazole, and streptomycin. Stock cultures were stored in brain heart infusion broth (Difco, Becton Dickinson, Sparks, Md.) with 20% glycerol (wt/wt) at −80°C.

For each experiment, 10 µl of a suspension of each strain was inoculated into 10 ml of brain heart infusion broth and incubated for 18 h at 37°C to achieve titers of about 10⁸ CFU/ml; these cell suspensions were then decimally diluted in phosphate-buffered saline (PBS). Cultures of *S. sonnei* were grown on constant agitation (200 rpm) to avoid cell clumping.

One milliliter of the starting inoculum containing 10⁸ CFU/ml of *Salmonella* Gaminara or 10⁷ CFU/ml *Shigella sonnei* was inoculated onto each 25 g amount of cut lettuce and allowed to have 1 min of contact time in each Whirl-Pak bag. In order to emulate an actual contamination scenario, which has a low probability of being uniformly spread, the cut lettuce, samples were inoculated by dripping the inoculum over the lettuce inside the Whirl-Pak bags. Inoculated lettuce samples that were assigned for treatments on day 2 and 4 were kept in separate Whirl-Pak bags in a refrigerator at 4°C (with the 1 ml inoculum left in contact with the lettuce in each bag). After 1 min at day 0 (or at day 2 or day 4), the inoculated cut lettuce in each Whirl-Pak bag was washed twice in 400 ml of sterile deionized (DI) water for 1 min each to remove the loosely attached microbes (20). These wash water washes were decanted and discarded. Each cut lettuce sample was then transferred into a sterile bag with sterile forceps. Two hundred milliliters of CPC wash was poured into the bag, and the lettuce was washed by manually shaking the bags vigorously. After 1 min, the wash solution was poured off and collected. This step was repeated for both NB and water wash. Two hundred twenty-five milliliters of PBS was poured into the bag before being stomached for 2 min. Liquid suspension from the bag (1 ml) was then plated on appropriate media, and the colonies were enumerated. Pooled wash solutions were mixed and then centrifuged (14,000 × g at RT for 20 min) before plating. For treatment with CPC alone, 25 ml of PBS was poured into the bag after decanting the CPC wash solution. After the stomaching step, liquid suspension collected from the stomacher bag was centrifuged (14,000 × g at RT) for 5 min before plating (1 ml) on the appropriate media for enumeration. The decanted wash solution was also centrifuged (14,000 × g at RT for 20 min) before plating. Different volumes of PBS were used to increase the possibility of obtaining survivors from the CPC treatment alone.

**CPC treatments and quenching.** Three different types of solutions were used in this study: CPC (Sigma, St. Louis, Mo.), Bacto neutralizing buffer dehydrated (NB) (Difco), and sterile DI water. Four different concentrations of CPC—0.05%, 0.1%, 0.2%, and 0.4% wt/vol—were prepared by adding the appropriate amount of dry CPC into sterile DI water. NB was prepared according to the manufacturer’s instructions (10). Both DI water and NB were autoclaved before use.

Two hundred milliliters of each solution was used for washing each 25-g lettuce sample for 1 min. The washing steps were done by manually shaking the bags vigorously. After 1 min, the wash solution was poured off and pooled (when the treatment protocol called for use of more than one wash solution). After the respective washing treatments, the cut lettuce was macerated in 225 ml (for all CPC treatments with NB and water wash) or 25 ml (for all CPC treatments without NB and water wash) of PBS in a homogenizer (Tekmar Co., Cincinnati, Ohio) for 2 min.

**Enumeration of target pathogen cells.** The homogenates or the pooled wash solutions were either serially diluted in PBS (if needed) or centrifuged to concentrate the cells before samples of dilutions were surface-plated onto appropriate enumeration media. Various volumes of the pooled wash solutions containing CPC, NB, and water wash were centrifuged at 14,000 × g at RT for 20 min before plating in order to maximize the possibility of obtaining survivors in the pooled wash. The CPC wash solutions alone underwent the same treatment. Various sections of each experiment were staggered to minimize to 20 to 30 min the lag times between the steps of washing and plating or between macerating and plating of cells. *Salmonella* Gaminara was plated on xylose lysine deoxycholate agar medium (Difco), and colonies typical of *Salmonella* were counted after incubation at 37°C for 24 h. *Shigella sonnei* was plated on MacConkey with tetracycline (MAT) (26) and colonies typical of *Shigella* were counted after incubation at 37°C for 24 h. Dry tetracycline was added to autoclaved MacConkey agar to yield a final concentration of 20 µg/mL. Experiments with both cultures were repeated twice. The minimum levels of detection were 2 CFU/g for nonquenched lettuce, 10 CFU/g for NB-quenched lettuce, and 10 or 1 CFU/ml for pooled wash solutions without or with centrifugation before plating.

**Statistical analysis.** Each treatment constituted duplicate assays from one 25-g lettuce sample by evaluating two rinse subsamples within each experiment; all experiments were repeated twice. Data collected from the various plates (CFU per gram or CFU per milliliter) were converted to units of log CFU per gram or CFU per milliliter before analysis. All quantitative data were converted to units of log CFU per gram or CFU per milliliter before analysis. All quantitative data were converted to units of log CFU per gram or CFU per milliliter before analysis.
analyzed by JMP IN (SAS Institute, Cary, N.C.) by Student’s *t* test after one-way analysis of variance, with the level of significance of α<sub>0.05</sub>.

RESULTS

Quenching effects of NB on CPC. A preliminary control experiment was performed to determine the quenching effects of Bacto NB on CPC (data not shown). Two different washes were used, a CPC wash and a 1:1 mixture of CPC and NB. It was found that the CPC wash (at 0.05% concentration) was able to reduce both loosely and firmly attached *Salmonella* Gaminara on cut lettuce by 4.4 log and *S. sonnei* by 5.8 log, whereas the 1:1 mixture of 0.1% CPC and 2× concentration NB wash caused no death of cells of either *Salmonella* Gaminara or *S. sonnei*. These results indicate that NB is not toxic to *Salmonella* Gaminara or *S. sonnei*, but also that NB is able to completely neutralize the killing ability of 0.05% CPC. Another preliminary control experiment was performed to show that up to 0.4% of CPC concentration caused no significant reductions of either pathogen on lettuce if the CPC was neutralized after 1 min with NB, indicating the NB quench treatment used was adequate to neutralize the killing action of up to 0.4% (wt/vol) of CPC, the maximum concentration used in this study (data not shown).

Reductions of firmly adhered *Salmonella* Gaminara and *S. sonnei* on cut lettuce treated with CPC without a NB quench and water-wash step. For lettuce treated with CPC without a quench step and water-wash step, thereby leaving residual CPC on the lettuce, on day 0, 0.1% CPC treatment reduced *Salmonella* Gaminara counts from 4.8 log CFU/g lettuce to nondetectable levels (Fig. 1A). After 2 days at 4°C, the 0.1% CPC and 0.2% CPC treatments gave about 4 log CFU/g reduction (about 99.99% kill), whereas a level of 0.4% CPC treatment with no quench was required to reduce survivors to nondetectable levels. Inoculated lettuce stored for 4 days at 4°C required at least 0.2% CPC to give nondetectable levels of *Salmonella* Gaminara (Fig. 1A). Figure 2A shows the results for different concentrations of CPC wash against firmly adhered *S. sonnei* on lettuce without the NB quench and water wash. Compared with the *Salmonella* results, *Shigella* on lettuce seemed to be more susceptible to residual CPC, showing about a 4-log reduction of CFU per gram of lettuce with the 0.05% CPC at all three sampling days. The 0.1% CPC treatment without quench reduced counts of *Shigella* to nondetectable levels at days 0, 2, and 4. For both *Salmonella* and *Shigella* cells, even when the CPC washes from inoculated lettuce (without NB and water) were concentrated by centrifugation (minimum level of detection, 1 CFU/ml), no detectable levels of survivors of either pathogen were observed in any of the CPC wash solutions used from 0.05% to 0.4% (data not shown).

Survival of firmly adhered *Salmonella* Gaminara and *S. sonnei* on cut lettuce treated with CPC with NB quench and water-wash step. There were no significant reductions (less than 1 log CFU/g lettuce reduction) of firmly adhered *Salmonella* Gaminara observed on the cut lettuce with 1-min treatment of CPC, even when 0.4% of CPC was used, for all three sample times (Fig. 1B).

Results for 1-min treatments of different concentrations of CPC wash followed by an NB quench and then a water wash on firmly attached *S. sonnei* on lettuce are shown in Figure 2B. Similar to the *Salmonella* data, for all three sampling times, the 1-min treatment with up to 0.4% CPC yielded less than 1 log reduction of CFU/g lettuce for firmly attached *S. sonnei* on cut lettuce, compared with untreated controls.

Survival of *Salmonella* Gaminara and *S. sonnei* cells in pooled rinses containing CPC, NB, and water. A sterile DI water wash pretreatment was given to all inoculated
lower than the control treatment. There is, however, a 1.3-log kill for the 0.4% CPC pooled wash. At day 4, these CPC levels produced no killing of *Salmonella* Gaminara cells in the washes if quenched in 1 min with NB, and the pooled 0.4% CPC wash killed only 0.6 log CFU/ml compared with the control. Results for survival of *S. sonnei* in pooled washes from inoculated lettuce are summarized in Figure 2C. On day 0, the 0.05 to 0.2% CPC treatments killed only 1.1 log CFU/ml, compared with the control, whereas the 0.4% CPC killed 2 log CFU/ml. After 2 or 4 days at 4°C, the pooled 0.05 to 0.2% washes killed less than 0.5 log CFU/ml of *S. sonnei*, whereas 0.4% CPC-NB-water–pooled wash solutions, compared with the control, killed just 0.9 and 1.8 log CFU/ml, respectively.

**DISCUSSION**

Firmly attached *Salmonella* and *Shigella* cells were the major target cells in this study. On the basis of a method described by Takeuchi and Frank (20), about 4.8 to 5.3 log of firmly adhered *Salmonella* Gaminara and about 4.1 to 4.8 log of firmly adhered *S. sonnei* cells remained on the control samples of cut lettuce after the two successive initial washes with sterile DI water. The model assumption we made was that the cells enumerated in the lettuce count are cells that remained attached on the lettuce after the CPC treatment and NB plus water wash. These cells only became loose as a result of the maceration action on the lettuce by the stomacher and became suspended in the PBS buffer, allowing the released cells to be enumerated. The cells that became dislodged as a result of the CPC treatment, the NB-neutralizing step, and the water-wash step were assumed to be present in the pooled wash column, and solutions and were enumerated separately from the lettuce samples.

The NB solution used was sufficient to completely neutralize the killing action of the CPC solutions used in this study (up to 0.4% concentration [wt/vol]). It was also demonstrated in a preliminary experiment that a CPC wash alone yielded a higher log reduction, compared with a water wash alone. The difference in the log reduction demonstrates the killing action of CPC taking effect and was not simply the result of the washing action contributed by the water wash alone (data not shown). When the cut lettuce was treated with CPC wash alone with no subsequent quenching with NB and washing with water, CPC appear to be effective in killing or removing firmly attached *Salmonella* and *Shigella* cells from the cut lettuce. CPC solutions caused foaming in bags when mixed with the lettuce. Although the CPC was decanted after lettuce treatment, a large amount of residual CPC probably remained on the lettuce. This residual CPC and the longer contact time (20 to 30 min between treatment and sample dilution for enumeration) likely account for the substantial reductions of the *Salmonella* or *Shigella* inocula on lettuce samples without subsequent NB quench or water wash.

In addition to testing the efficacy of incorporating a CPC wash step before packaging the cut lettuce in a food-processing environment, this study also encompassed the possibility of using CPC as a household wash. This household wash approach was designed with the assumption that...
either the cut lettuce was contaminated during packaging and held refrigerated up to 4 days before being consumed, or, if the cut lettuce was not completely consumed upon opening the package, it became contaminated when it was first opened and then kept refrigerated for up to 4 days.

For firmly adhered Salmonella Gaminara, a 0.05% CPC concentration without quench was inadequate to reduce the cell counts to nondetectable levels over 4 days. Although the CPC treatments produced far lower counts than the controls at all days, a minimum of 0.4 or 0.2% CPC were needed at days 2 or 4, respectively, to reduce counts of Salmonella Gaminara to nondetectable levels on lettuce held at 4°C. Conversely, for firmly attached S. sonnei cells on lettuce held over 4 days at 4°C, a CPC treatment of just 0.1% with no NB quench was adequate to reduce counts to nondetectable levels.

When the inoculated cut lettuce samples were treated with CPC washes for just 1 min and were followed by quenching with NB and then washing with water, the killing effect of the CPC on pathogens on the lettuce or dislodged in the pooled washes was greatly diminished. The results are summarized in Figures 1B and 2B for lettuce counts and in Figures 1C and 2C for the pooled washes.

Firmly adhered cells of Salmonella Gaminara or S. sonnei on cut lettuce were completely resistant to a 1-min treatment of CPC, even one containing up to 0.4% CPC wt/vol. Cells that are firmly attached on the lettuce, as a whole, are more resistant to the CPC wash treatment. The lettuce may provide a microenvironment that shields and therefore protects the cells from harmful chemicals. When the cells became dislodged and suspended (as a result of the washing action), they are more susceptible to attack by chemicals such as CPC, as clearly observed for treatments day 0. Allowing the inocula to adapt for 2 or 4 days on the lettuce at 4°C without many nutrients, however, may have encouraged the cells to go into resting or stationary phase, or it may have triggered a starvation-survival mode that caused the cells to become more resistant to the killing action of CPC, regardless of whether the cells were still attached to the lettuce or after becoming dislodged into the wash solutions. Furthermore, allowing the cells to attach to the lettuce may have caused them to develop some sort of attachment mechanism—for example, formation of a biofilm—which may have aided to protect or strengthen them from the killing action of the CPC after they had become dislodged from the lettuce and fallen into the pooled wash solutions as a result of the washing action.

Collectively, our results are apparently contradictory to those previously reported by other researchers who found CPC was able to remove or kill up to 4 to 6 log CFU/g of several different selected pathogens inoculated onto freshcut vegetables (11, 24, 16). However, two major differences in our protocol compared with those used by other laboratories (11, 24, 16) were that we used only firmly attached cells (Figs. 1B and 2B) (20, 21), whereas the other groups did not use a water prewash, so their sample contained both loosely and firmly attached cells before CPC treatments; and they did not use a quench step with NB. It is possible that Lukasik et al. (14) were able to achieve a 2-log reduction of Salmonella Montevideo attached on strawberry fruit because they quenched only with (0.05%) sodium thiosulfate after CPC treatment, whereas the NB we used has not only sodium thiosulfate but also aryl sulfate, which is specifically able to neutralize quaternary ammonium compounds such as CPC.

Firmly attached cells of our test strain of Salmonella and Shigella were apparently much more resistant to CPC inactivation, similar to the resistance to 200 ppm chlorine observed for firmly attached E. coli O157:H7 cells on the surfaces of lettuce by Takeuchi and Frank (20). Sharma and Beuchat (19) showed that a 0.1% solution of CPC would kill suspensions of cells of E. coli O157:H7 that were preexposed to 0.05% peptone, exposed to 0.1% CPC in liquid suspensions, and then quenched in a Dey-Engley NB, yielding viable cell reductions of 1 to 3 log. However, in this last case, there apparently was no solid food substrate provided to protect the cells from the killing action of the CPC.

In summary, to our knowledge, this is the first report in which the exposure times for CPC treatments of pathogens on surfaces of vegetables were defined more accurately by use of a quench step with a chemical agent, namely NB, that contains aryl sulfate—a chemical known to be able to neutralize CPC, a quaternary ammonium compound. With such a quench step, the reductions caused by CPC of the two gram-negative pathogens tested were considerably less than those previously reported. This result may also be due to the use of firmly attached cells that were more protected from the killing action of the CPC. Further work will be needed to determine how much more contact time than 1 min is needed in order for CPC to kill more firmly adhered cells of Salmonella or Shigella on lettuce.

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