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Fate of *Shigella sonnei* on Parsley and Methods of Disinfection

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ABSTRACT

Outbreaks of shigellosis associated with chopped parsley used as a garnish for foods occurred in four states in the United States and in two Canadian provinces in 1998. This prompted a study to determine survival and growth characteristics of *Shigella sonnei* inoculated onto raw parsley. Two inoculum levels (~10^3 and 10^6 CFU/g) were applied to parsley leaves, portions of which were then chopped. Inoculated whole and chopped parsley leaves were held at 4°C or 21°C for up to 14 days. Initial populations of the organism on chopped parsley receiving high or low levels of inoculum increased by approximately 3 $\log_{10}$ CFU/g, within 1 day at 21°C. Populations of *S. sonnei* on inoculated chopped or whole parsley leaves held at 4°C decreased by 2.5 to 3.0 $\log_{10}$ CFU/g during a 14-day storage period. The pathogen multiplied, without a lag phase, on inoculated (2.72 $\log_{10}$ CFU/g) chopped parsley held at 21°C, exceeding 6 $\log_{10}$ CFU/g within 24 h. Treatment of inoculated whole parsley leaves with vinegar containing 5.2% (vol/vol) acetic acid or 200 ppm free chlorine for 5 min at 21°C reduced the population of *S. sonnei* by more than 6 $\log_{10}$ CFU/g, whereas treatment with vinegar containing 7.6% acetic acid or 250 ppm free chlorine reduced initial populations of 7.07 and 7.26 $\log_{10}$ CFU/g, respectively, to undetectable levels (<0.6 $\log_{10}$ CFU/g). These studies revealed that *S. sonnei* can grow rapidly on chopped parsley held at ambient temperature and remain viable for at least 14 days at 4°C. Treatment of contaminated parsley with vinegar or chlorinated water offers a simple method to reduce markedly or eliminate the pathogen in food-service or home settings.

Shigellosis accounted for only 3.1% (25 of 796 outbreaks) of foodborne illness outbreaks caused by bacterial pathogens in the United States during 1988 through 1992; however, the percentage of cases was much higher (14.4%, 4,788 of 33,206 cases) *(2).* This discrepancy was attributable to a large outbreak of *Shigella sonnei* infections involving 3,175 cases in Michigan in 1988. Although *Shigella* is not considered to be a major diarrheal pathogen in the United States, it is a major cause of diarrheal disease in tropical developing countries, where it has been estimated to cause at least 500,000 deaths each year in young children *(24).*

Shigella species have the potential to cause large outbreaks because of their low infectious dose (~10 cells) and because both food and water can serve as vehicles of transmission *(16, 19, 25).* Contaminated human feces are the major sources of shigellae associated with food and water *(24).* Foods implicated in shigellosis outbreaks usually have been subjected to hand processing or preparation, received limited heat treatment, or have been served raw. Raw vegetables are among the common vehicles of shigellosis *(2, 7, 8, 12, 14, 18).*

In August 1998, outbreaks of shigellosis occurred in Minnesota, California, Massachusetts, Florida, and Canada *(6).* Epidemiologic investigations revealed that chopped, uncooked curly parsley was the vehicle of infection. In all outbreaks, *S. sonnei* was isolated from patients' stools. The ability of this pathogen to survive or grow on chopped parsley is not known. The purposes of this study were (i) to determine, on whole and chopped parsley leaves, survival and growth characteristics of three *S. sonnei* isolates from the 1998 outbreaks and (ii) to identify practical food-service and home-use treatments such as chlorinated water or vinegar to disinfect parsley.

MATERIALS AND METHODS

Bacterial strains. Three isolates of *S. sonnei* (F6129, 10304-98, and 10305-98) associated with the 1998 outbreaks were used to inoculate parsley. All three strains were resistant to multiple antimicrobial agents, including trimethoprim/sulfamethoxazole, tetracycline, ampicillin, sulfisoxazole, and streptomycin. Each isolate was grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) under constant agitation (150 rpm) for 18 h at 37°C, then held at 4°C as stock cultures for up to 1 week, or maintained frozen after adding 50% glycerol at −30°C for long-term storage.

Preparation of inoculum and inoculation of parsley. Raw curly parsley (*Petroselinum crispum*) leaves purchased from a local supermarket were washed three times with sterile water. Parsley was placed in a laminar flow hood for 1 h at 21°C to remove surface water before inoculating with a suspension composed of approximately equal numbers of cells of the three strains of *S. sonnei.* Each *S. sonnei* strain was grown individually in 10 ml of tryptic soy broth at 37°C for 16 to 18 h; a loopful of culture was then inoculated into 50 ml of tryptic soy broth and incubated at 37°C with agitation (150 rpm) for 24 h. Cell suspensions were sedimented by centrifugation at 3,100 × *g* for 10 min at 21°C, washed three times with 0.05 M potassium phosphate-buffered saline, pH 7.2, and resuspended in 0.05 M potassium phosphate-

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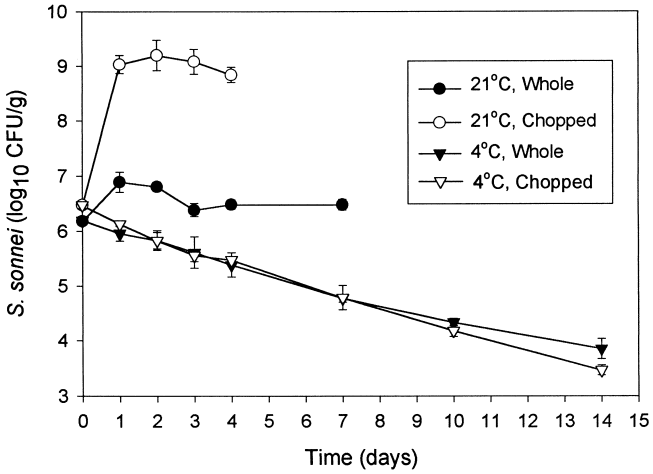

FIGURE 1. *Survival and growth of* S. sonnei *on whole or chopped parsley, initially containing 10^6 to 10^7* S. sonnei*/g.*

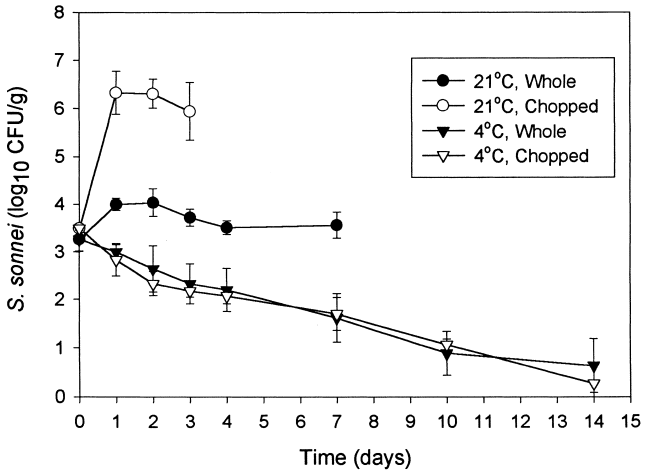

FIGURE 2. *Survival and growth of* S. sonnei *on whole or chopped parsley, initially containing 10^3 to 10^4* S. sonnei*/g.*

buffered saline, pH 7.2 to obtain a cell suspension with an optical density at 630 nm of 0.50 ($\sim 5 \times 10^8$ to 8×10^8 CFU/ml). Equal volumes of each strain suspension were mixed together and diluted in 0.05 M potassium phosphate-buffered saline, pH 7.2 to obtain cell suspensions of approximately 10^7CFU/ml (high level) and 10^4 CFU/ml (low level). Parsley leaves (1,200 g) were submerged in the respective cell suspensions for 1 min while under constant agitation (50 rpm). The inoculated parsley was removed from the cell suspension, shaken to remove excess inoculum, and surface dried at 21°C in a laminar flow hood for 1 h. This procedure provided parsley with two levels of inoculum, i.e., approximately 10^6 and 10^3 CFU/g. A portion of inoculated parsley was chopped into 0.5-cm pieces with a sharp knife. Both whole and chopped leaves (300 g in 9-liter plastic pans) were held at 4°C and 21°C. To achieve a higher inoculum (10^7 CFU/g) on parsley used in disinfection experiments, a cell suspension containing 10^8 CFU/ml was used as an inoculum.

Enumeration of *S. sonnei* on parsley. Inoculated parsley (whole and chopped leaves) was held in darkness in covered plastic containers at 4°C or 21°C and sampled in duplicate at 0, 1, 2, 4, 7, 10, and 14 days for enumeration of *S. sonnei.* In another experiment, inoculated chopped parsley was held at 21°C and sampled in duplicate at 0, 2, 4, 6, 8, 10, 12, 16, 20, and 24 h for enumeration of *S. sonnei.* Parsley (10 g) was removed from storage containers after being mixed with stainless steel tongs and was added to 90 ml of sterile 0.1% peptone water in a sterile stomacher bag. The mixture was pummeled for 2 min at normal speed in a stomacher (model 400, Seward Medical, London, UK). MacConkey agar (Oxoid Ltd., Hampshire, UK) containing tetracycline (20 μg/ml; MAT) was used to select for *Shigella* in the presence of other microflora on parsley. The peptone wash water was then serially diluted in 0.1% peptone water and surface plated on MAT; quadruplicate 0.25-ml portions and duplicate 0.1-ml portions of undiluted peptone wash were also plated on MAT. Plates were incubated at 37°C for 18 h, and colonies typical of *Shigella* were enumerated. Three presumptive *S. sonnei* colonies were picked randomly, inoculated onto triple sugar iron slants (Carr Scarborough Microbiologicals Inc., Decatur, Ga.), incubated at 37°C for 18 h, and confirmed by an agglutination test with *Shigella* poly O group D antiserum (Centers for Disease Control and Prevention, Atlanta, Ga.). All experiments were performed in duplicate.

Treatment with vinegar and chlorine. White vinegar (H. J. Heinz Co., Pittsburgh, Pa.) containing 5.2 and 7.6% (vol/vol) acetic acid was used. Vinegar containing 5.2% acetic acid was diluted with deionized water to contain 0.5%, 1.0%, and 2.6% acetic acid; these diluted vinegar solutions as well as undiluted vinegar containing 5.2% and 7.6% acetic acid were used to treat whole parsley leaves inoculated with *S. sonnei.* Water containing 0, 1, 5, 50, 100, 150, 200, and 250 ppm free chlorine prepared with sodium hypochlorite (Sigma Chemical Co., St. Louis, Mo.) was also tested for efficacy in killing *S. sonnei* on parsley.

Inoculated parsley (10 g) was combined with 40 ml of treatment solution in a sealable plastic bag and constantly agitated (50 rpm) for 5 min at 21°C. Undiluted treatment solutions (quadruplicate 0.25-ml or duplicate 0.1-ml portions) and duplicate 0.1-ml portions of solutions serially diluted in Dey–Engley neutralizing broth (Difco) were surface plated on MAT. The pH values of a mixture of 9 ml of Dey–Engley broth and 1 ml of vinegar wash solution containing 0.5% or 7.6% acetic acid were 6.7 and 4.1, respectively. The parsley and treatment solution mixture was then combined with 40 ml of double-strength tryptic soy broth containing 20 μg of tetracycline/ml and incubated 18 h at 37°C. The enrichment mixture was serially diluted, surface plated on MAT, and incubated 18 h at 37°C; plates were examined for presumptive *S. sonnei* colonies and randomly selected colonies were subjected to confirmation tests. Experiments were done in triplicate.

RESULTS AND DISCUSSION

Survival and growth of *S. sonnei* on parsley. Parsley inoculated with high levels (6.19 and 6.48 $\log_{10}$ CFU/g of whole and chopped parsley, respectively) and low levels (3.23 and 3.49 $\log_{10}$ CFU/g of whole and chopped parsley, respectively) of *S. sonnei* was monitored for changes in populations during storage at 4°C and 21°C for up to 14 days. Studies were terminated when visual examination revealed that parsley was inedible, i.e., the tissue began to liquefy and develop an off odor. Survival and growth patterns of *S. sonnei* on parsley with both levels of inocula were similar. The pathogen grew to 9.20 and 6.32 $\log_{10}$ CFU/g on chopped parsley initially containing 6.48 $\log_{10}$ CFU/g (Fig. 1) and 3.49 $\log_{10}$ CFU/g (Fig. 2), respectively, within 2 days at 21°C. An increase of less than 1 $\log_{10}$

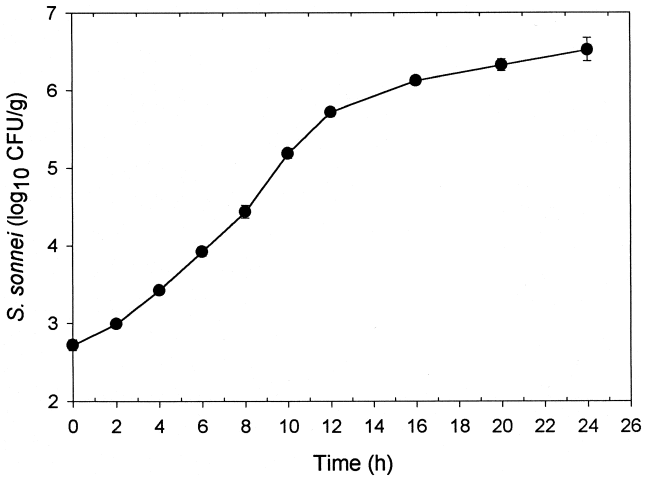

FIGURE 3. *Growth of* S. sonnei *on chopped parsley held at 21°C for 24 h.*

TABLE 1. *Populations of* S. sonnei *on whole parsley leaves treated with vinegar or chlorine for 5 min at 21°C*

Treatment	Concentration	*S. sonnei* ($\log_{10}$ CFU/g)[a]
Water (deionized)	0	7.07 ± 0.28
Vinegar (% acetic acid, vol/vol)	0.5	6.09 ± 0.36
	1.0	5.72 ± 0.08
	2.6	3.76 ± 0.21
	5.2	<0.60[b]
	7.6	<0.60[c]
Water (deionized)	0	7.28 ± 0.29
(tap)	0	6.68 ± 0.29
Free chlorine (ppm)	1	6.79 ± 0.20
	5	6.02 ± 0.13
	50	4.95 ± 0.50
	100	2.94 ± 0.60
	150	<0.60[b]
	200	<0.60[b]
	250	<0.60[b]

[a] Mean values from three replicates.
[b] Not detected on MAT, but detected by enrichment.
[c] Not detected on MAT or by enrichment.

occurred on whole parsley leaves within 1 day at 21°C, followed by a decline in population after 2 days. The increase in population of *S. sonnei* on whole parsley leaves was less than 1 $\log_{10}$ during storage for 7 days at 21°C, regardless of the initial level of inoculum. Escartin et al. *(11)* reported that *Shigella* grew on cubes of papaya (pH 5.97) held at 25°C to 27°C. Increases of 4 to 5 $\log_{10}$ CFU/ml of a 1% to 20% suspension of papaya occurred within 24 h at 22°C.

S. sonnei grew rapidly on chopped parsley held at 21°C for 24 h (Fig. 3), increasing without a lag phase, from 2.72 $\log_{10}$ CFU/g to 6.53 $\log_{10}$ CFU/g. The growth curve of *S. sonnei* on chopped parsley resembles that which occurs in a nutritious liquid medium, indicating that the tissue fluid provides a good medium for growth. Rapid growth is attributed to increased availability of nutrients, resulting from the release of tissue fluid from broken cells. This was unexpected, because parsley reportedly contains antimicrobial furocoumarins, compounds that have been reported to inhibit *Escherichia coli* O157:H7, *Listeria monocytogenes*, and spoilage microorganisms *(17).* A possible explanation for the lack of inhibitory activity against *S. sonnei* is that furocoumarins require photoactivation to be effective. Implications of the ability of *S. sonnei* to grow rapidly on chopped parsley are significant in food-service or home settings.

Unlike the dramatic increase in populations of *S. sonnei* on chopped parsley incubated at 21°C, populations of the organism on both whole and chopped parsley leaves held at 4°C declined throughout the 14-day storage period. However, the pathogen survived these storage conditions, regardless of initial populations. Studies reported by Rafii and Lunsford *(21)* on the survival of *Shigella flexneri* in vegetables and commercially prepared salads held at 4°C yielded similar results. In their study, populations decreased during incubation, with the pathogen surviving 12 to 26 days depending on the type of vegetable.

Satchell et al. *(23)* studied the growth of *S. sonnei* in shredded cabbage packaged under vacuum, modified atmosphere, and aerobic conditions and held at room temperature (24 ± 2°C) or under refrigeration (0 to 6°C). Results were similar to those observed on parsley in our study. Under all three packaging conditions, *S. sonnei* reached highest populations on cabbage, i.e., 3 to 4 $\log_{10}$ CFU/g increase, within 1 or 2 days at room temperature, then declined. Populations of *S. sonnei* on cabbage packaged under vacuum or in a modified atmosphere and held refrigerated were stable for at least 7 days. Populations decreased continuously on cabbage stored aerobically throughout the incubation period at 0 to 6°C. Islam et al. *(13)* studied survival and growth of *S. flexneri* in rice, mashed potato, milk, lentil soup, fish, beef, mashed brinjal, and cucumber, and determined that maximal growth at 25°C occurred within 1 or 2 days; then populations decreased. Hence, it is apparent that *Shigella* can survive on refrigerated raw vegetables for periods of time exceeding expected shelf life.

Treatment with vinegar and chlorinated water. Vinegar, or more specifically acetic acid, has been used to reduce populations of foodborne pathogens in several foods *(9, 15, 20).* In our study, treatment of whole parsley leaves at 21°C for 5 min with vinegar containing 5.2% acetic acid reduced the population of *S. sonnei* more than 6 $\log_{10}$ CFU/g, whereas treatment with 7.6% acetic acid reduced the initial inoculum (7.07 $\log_{10}$ CFU/g) to an undetectable level (<0.6 $\log_{10}$ CFU/g) (Table 1). Vinegar containing ≤2.6% acetic acid reduced the population of *S. sonnei* by <3.3 $\log_{10}$ CFU/g. However, parsley treated with vinegar containing ≥2.6% acetic acid noticeably discolored and had a strong vinegar odor. A simple wash with tap water for 1 min restored color and diminished the odor.

Other researchers have evaluated acetic acid and vinegar for their lethality against foodborne pathogens. Kara-

pinar and Gonul *(15)* used 1 to 5% (vol/vol) acetic acid or 30 to 50% of vinegar containing 4.9% acetic acid to treat parsley inoculated with *Yersinia enterocolitica.* Treatment with 2 to 5% acetic acid or 40 to 50% vinegar for at least 15 min reduced initial inocula of 8.6×10^7 and 1.8×10^7 CFU/g, respectively, to an undetectable level (<1 CFU/g). Sodium chloride enhances the lethal effect of vinegar against *E. coli* O157:H7 *(10, 26).*

Chlorinated water is used commercially to sanitize whole and freshcut produce. However, treatment of produce with up to 300 ppm free chlorine is not exceptionally effective *(3–5, 22, 27, 28).* In our study, treatment of whole parsley leaves with 150 ppm free chlorine reduced the population of *S. sonnei* by more than 6 $\log_{10}$ CFU/g (Table 1), and treatment with 250 ppm free chlorine reduced the initial population of 7.28 $\log_{10}$ CFU/g to an undetectable level (<0.6 $\log_{10}$ CFU/g). This magnitude of inactivation is much greater than that observed for inactivation of other foodborne pathogens on other vegetables. The greater lethality of chlorine against *S. sonnei* on parsley compared with other foodborne pathogens on other vegetables may be a result of exceptional sensitivity of *S. sonnei* to chlorine and perhaps also to the large surface/weight ratio of parsley leaves compared to many other vegetables.

Beuchat et al. *(4)* suggested that the lack of effectiveness of chlorine in killing pathogenic bacteria on lettuce, tomatoes, and apples may be due to inaccessability of chlorine to cells lodged in microenvironments. Adams et al. *(1)* hypothesized that microbial cells could be protected from sanitizers because of hydrophobic pockets on the surface of salad vegetables. These researchers demonstrated that the antimicrobial efficacy of hypochlorite solution could be increased by surface-wetting lettuce leaves with a surfactant. In addition to the type of vegetable and target microorganism, other factors affecting the efficacy of chlorine in decontaminating vegetables include time of exposure, temperature, free chlorine concentration, and pH *(5, 28).*

Our studies revealed that *S. sonnei* inoculated onto chopped parsley can grow rapidly at ambient temperature. Washing parsley with tap water or storing at refrigeration temperature does not eliminate *S. sonnei.* However, washing parsley with vinegar (containing ≥5.2% acetic acid) or chlorinated water (containing >150 ppm free chlorine) will greatly reduce, if not eliminate, *S. sonnei.*

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