FOOD BIOLOGICAL CONTAMINANTS

Survival and Detection of *Shigella flexneri* in Vegetables and Commercially Prepared Salads

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The normal bacterial microflora of 4 commercially prepared salads (coleslaw, crab salad, carrot salad, and potato salad) and 3 vegetables (green pepper, onion, and cabbage) were evaluated. Twenty-eight species of bacteria, including potential pathogens, were isolated. The foods were artificially inoculated with an avirulent mutant strain of *Shigella flexneri* 5 (pHS1059) to develop a method for the rapid detection of *Shigella* spp. Bacteria were separated from insoluble and particulate salad ingredients by filtration through shark skin filter paper and by low speed centrifugation. *S. flexneri* survived at 4°C in all salads for at least 11 days and up to 20 days in crab salad. The polymerase chain reaction (PCR), using primers for amplification of a 118-base pair DNA fragment from the virulence-associated *spa* region, present in all *Shigella* spp., was used to detect *S. flexneri* in filtrates from salads inoculated with *S. flexneri* 5 (pHS1059). DNA was amplified from all of the artificially contaminated salads and vegetables except green pepper. After 3–5 days of storage, the PCR also amplified *S. flexneri* DNA from salads that had been enriched with nutrients to increase the number of bacteria. Green peppers contained a PCR inhibitory substance that was attenuated by dilution and enrichment before the PCR. No amplification of DNA was observed in foods to which *S. flexneri* had not been added.

The genus *Shigella* is composed of 4 species of Gram-negative facultative anaerobes; *S. dysenteriae, S. boydii, S. sonnei,* and *S. flexneri* (1), which cause shigellosis (bacillary dysentery) in humans and other primates. *Shigella* spp. invade the epithelial cells of the gastrointestinal tract, causing acute inflammation and, in severe cases, ulceration (1–5). The infections are frequently mild and self-limiting (1, 5). However, because the minimum infecting dose is small (as few as 200 organisms produce disease in some individuals), the contamination of food and water may be significant even when only small numbers of bacterial cells are present (1).

Most shigellosis outbreaks are caused by *S. flexneri* and *S. sonnei* (5–11). The route of transmission is the consumption of food or water contaminated with fecal bacteria (5–11). Foods that commonly transmit *Shigella* either received minimal or no heat treatment before consumption or were delivered fresh to the consumer (6–11).

We previously showed that *S. flexneri* can survive on vegetables at room and refrigeration temperatures (12). Commercially prepared salad vegetables are readily available in supermarkets and restaurants, both as fresh produce and mixed with other ingredients in prepared salads. Prepared salads may contain fresh produce that was contaminated during growth, harvesting, and processing or during preparation, packaging, distribution, and display. These foods, which do not require cooking before consumption, could be the source of contamination in geographically widespread shigellosis outbreaks (7, 9–11). Rapid detection of *Shigella* spp. before or after an outbreak is needed to identify the source of the outbreak and to reduce further risk to humans. An effective rapid detection method is the polymerase chain reaction (PCR) which uses primers that amplify a segment of DNA unique to all 4 *Shigella* spp. PCR has been used to detect *Shigella* spp. in various vegetables (12, 13).

In this study, 4 types of commercially prepared salads were studied for their ability to support the survival of *S. flexneri,* and a method was developed for the rapid detection of *Shigella* spp. in salads. Because these salads contain soluble and particulate ingredients, an elution-and-filtration method was devised to separate bacteria for PCR amplification. An avirulent mutant of *S. flexneri* 5 (pHS1059) (14–19) (to ensure the safety of laboratory personnel) was used to inoculate or artificially contaminate prepared salads. This procedure allowed us to determine survival time and to test the rapid PCR detection method.

Strain 5 (pHS1059) of *S. flexneri* has almost the same genetic makeup as virulent wild-type strains, lacking only the products of the *spa* locus (15) required for

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presentation of *S. flexneri* invasion plasmid antigens (12, 19) on the surface of the cell (14, 17). Using this strain and primers for amplification of 118 base pairs of ORF-3 from the virulence-associated *spa* region, a PCR method could detect *S. flexneri* rapidly and differentiate it from bacteria of other genera. This primer also amplifies 118 base pairs of the *spa* region of *S. boydii, S. dysenteriae*, and *S. sonnei* (12).

Experimental

Bacteria and Media

S. flexneri serotype 5 (pHS1059) (14) was provided by T.L. Hale (Walter Reed Army Institute of Research, Washington, DC). Luria-Bertani (LB) medium (17) was used for bacterial growth and for determination of the normal bacterial flora in the prepared salads. Xylose-lysine-desoxycholate (XLD) agar (Difco Laboratories, Detroit, MI) was used to differentiate *Shigella* spp. from other bacteria.

Isolation, Identification and Enumeration of Normal Bacterial Flora from Prepared Salads and Vegetables

Prepared salads were obtained, either packaged or from a salad bar, in local supermarkets. Each salad was mixed with an equal volume of sterile water for 15 min on a rotary shaker (150 rpm) and then centrifuged for 3 min at 360 × *g*. The supernatants were decanted and filtered through either shark skin or No. 595 paper filters (medium porosity, 8–12 μm, Schleicher & Schuell, Keene, NH). The filtrates were serially diluted, and 100 μL of each dilution was spread on duplicate LB and XLD plates. The plates were incubated for 24 h at 37°C, and then counted. Morphologically different bacterial colonies were streaked on sterile LB plates for single colony isolation; isolated bacteria were then grown on blood agar plates. The bacteria were identified with either the AMS (Automatic Biology Microstation) system (Biology, Inc., Hayward, CA) or the API System (bioMerieux Vitek, Hazelwood, MO).

For detection of normal bacterial flora of vegetables, green pepper, cabbage, and onion were cut into small pieces (6–10 mm) with a sterile knife or scissors and placed in sterile beakers. The vegetables were then shaken with an equal volume of sterile water for 15 min on a rotary shaker (150 rpm). The liquid was removed with a sterile Pasteur pipette and serially diluted. Each dilution (100 μL) was plated on duplicate LB plates, which were incubated for 24 h at 37°C.

Evaluation of Survival of S. flexneri in Salads and Vegetables

Prepared salads were transferred to conical 50 mL polypropylene centrifuge tubes (Fisher Scientific, Pittsburgh, PA) and inoculated and mixed with *S. flexneri* 5 (pHS1059) by adding 2.49×10^6 to 9.79×10^6 colony forming units (cfu) in water/g salad, and then refrigerated. At day zero and at daily intervals until the salads were visibly spoiled, ca 5 g of each inoculated salad was measured into a sterile 50 mL centrifuge tube, and 5 mL sterile water was added. The mixture was shaken on a rotary shaker (150 rpm) for 15 min and centrifuged for 3 min at 360 rpm. The supernatant was withdrawn with a sterile pipette and filtered through shark skin or No. 595 paper filters (medium flow with particle retention of 8–12 μm). The fluid was serially diluted, 100 μL was plated on duplicate LB and XLD plates, and the plates were incubated for 24 h at 37°C. The number of colonies of *S. flexneri* 5 (pHS1059) surviving in the salads was recorded.

To evaluate the survival of *S. flexneri* on green pepper, cabbage, and onion, the vegetables were placed in 50 mL polypropylene centrifuge tubes and inoculated by adding 5.5×10^6 cfu *S. flexneri* 5 (pHS1059) in water/g vegetable. The vegetables were mixed thoroughly and refrigerated. *Shigella* colonies were counted at day zero and at periodic intervals until the appearance of the vegetable had deteriorated or until no *Shigella* could be detected.

For counting, a portion of the vegetables was shaken with an equal volume of water for 15 min on a rotary shaker (150 rpm). The fluid was removed with a sterile Pasteur pipette, serially diluted, and plated on duplicate LB plates. The plates were incubated at 37°C for 24 h.

DNA Extraction

Colonies of *S. flexneri* 5 (pHS1059) were grown on LB plates, collected in 10 mM Tris-HCl with 1 mM EDTA (pH 8.0), and centrifuged for 30 min at 2680 × *g*. The bacterial pellet was washed, using 10 mM Tris-HCl with 1 mM EDTA (pH 8.0), and centrifuged at 2680 × *g* for 10 min. The bacteria were incubated at room temperature with lysozyme buffer (5 μg lysozyme/mL, Sigma Chemical Co., St. Louis, MO) for 10 min. Proteinase K (1 μg/mL; Sigma) and 0.1% sodium dodecyl sulfate (Sigma) were added to the bacterial suspension, which was incubated at 65°C for 5 min. The DNA was extracted with phenol-chloroform-isoamyl alcohol (Gibco BRL, Gaithersburg, MD) and precipitated with isopropanol (J.T. Baker, Phillipsburg, NJ). The precipitate was washed with 70% ethanol, dried, and dissolved in 10 mM Tris-HCl with 1 mM EDTA (20).

Rapid Detection Using PCR for Amplification of S. flexneri DNA

Either bacterial DNA or 68 μL bacterial lysate was used as the PCR template. Each 100 μL PCR reaction mixture contained 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 1.5 mM $MgCl_2$; 0.001% gelatin; 200 μM each of dATP, dCTP, dGTP, and dTTP; 0.45 μmol of each primer: 5'CGAGATGTGGAGGCAT3' and

5'AGCGATCTTACGTCTTG3' [selected from Gen Bank, accession no. D13663 (18) and synthesized by National Bioscience (Plymouth, MN)]; and 5 units of Ampli-Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). Each sample was overlaid with 20 μL sterile mineral oil, heated for 1 min at 94°C, and amplified in a thermal cycler (Perkin-Elmer Cetus model 480) for 30 cycles of 94°C denaturation for 1 min, 45°C annealing for 1.5 min, and 72°C extension for 2 min, with a final extension of 5 min. The reaction products were separated by electrophoresis in 3% agarose (Perkin-Elmer Cetus), stained in ethidium bromide solution, destained, and photographed.

Detection of S. flexneri 5 (pHS1059) in Salads and Vegetables by PCR

Prepared salads were inoculated with 2.49×10^6 to 9.79×10^6 cfu *S. flexneri 5* (pHS1059)/g salad, and mixed. After addition of water to the salad and shaking, the liquid was drained from a portion of the salad, and filtered through shark skin filter paper or No. 595 filter paper. No filtration was performed when eluates from individual vegetables were used. The bacteria were collected by centrifugation at $16\,755 \times g$ for 30 min and washed with 1 mL sterile distilled water. A 100 μL amount of 0.1% Triton X-100 solution (Fisher Scientific, Fair Lawn, NJ) was added to the bacterial pellet and mixed. Bacterial lysates were prepared by boiling the bacteria for 5 min in 0.1% Triton X-100, and 68 μL of the resulting lysate was added to the PCR reaction mixture for amplification.

To enrich for detection of the low number of *S. flexneri* cells that survived in the salad after refrigeration, bacteria were collected from a portion of the salad that had been refrigerated for 3–5 days. The fluid was removed with a sterile Pasteur pipette and filtered through shark skin filter paper. A 1 mL amount of each filtrate was inoculated into 10 mL LB broth, which was incubated at 37°C for 6–16 h. Bacteria were collected by centrifugation ($9000 \times g$) for 30 min, washed with sterile distilled water, and suspended in 1 mL distilled water. From each suspension, 100 μL was used for PCR amplification. This method was also used to amplify bacteria from green pepper, so that materials in green pepper that inhibited PCR amplification were diluted. For green pepper, both 100 and 500 μL amounts of the final bacterial suspension were used for PCR amplification. The bacteria from 100 or 500 μL suspensions were pelleted and suspended in 100 μL 0.1% Triton X-100 solution, before boiling, to lyse the bacteria for PCR amplification.

Results

Normal Microflora of Salads

Twenty-eight different species of bacteria were isolated from coleslaw, crab salad, carrot salad, cabbage, and potato salad (Table 1). Some species were found in more than one type of salad. The total populations of aerobic bacteria ranged from 6.0×10^1 to 1.75×10^5 cfu/g salad.

Table 1. Bacteria isolated from commercially prepared salads

Coleslaw	Crab salad
Bacillus spp. strain D	*Bacillus* spp. strain E
Staphylococcus epidermidis	*Staphylococcus auricularis*
	Streptococcus equinus
	Rathayibacter tritici
	Sphingomonas paucimobilis
Carrot salad	**Potato salad**
Bacillus spp. strain A	*Bacillus* spp. strain C
Bacillus spp. strain B	*Enterobacter cloacae*
Corynebacterium spp.	*Klebsiella oxytoca*
Staphylococcus auricularis	*Staphylococcus auricularis*
Staphylococcus epidermidis	*Staphylococcus hominis*
Staphylococcus spp.	*Staphylococcus warneri*
Staphylococcus warneri	*Streptococcus equinus*
Streptococcus constellatus	*Streptococcus* spp.
Streptococcus intermedius	*Clavibacter michiganense*
Streptococcus uberis	*Klebsiella pneumoniae*
	Stenotrophomonas maltophilia

Cabbage leaf surfaces were contaminated with 160 bacteria/g. No bacteria were isolated from green pepper or the interior tissues of onion at the dilutions tested either because of the low numbers of bacteria in eluates from these vegetables or the inability of these bacteria to grow under our experimental conditions.

Survival of S. flexneri on Salads and Vegetables

The number of *S. flexneri* cells that survived in distilled water at 4°C declined gradually until the final count at 26 days (Table 2). The bacterial count on day zero in water was 2.80×10^8 cfu/mL. After 26 days, the count was 9.20×10^7 cfu/mL (32.8% survival).

The survival of *S. flexneri* 5 (pHS1059) in salads and vegetables at 4°C was recorded daily until spoilage occurred or until no count could be determined. *S. flexneri* survived in all salads for at least 11 days (Figure 1) and survived on vegetables for at least 12 days at 4°C (Table 2).

The number of *S. flexneri* cells/g coleslaw was 1.18×10^5 at the time of inoculation. The number decreased at 4°C, but 2.16×10^4 bacteria/g coleslaw (18.3%) were still detected on day 13, when spoilage was evident and no further counts were performed.

For the crab salad, the count of *S. flexneri* before storage was 1.09×10^6 cfu/g salad. By day 8, the number of bacteria had decreased to 2.10×10^5 cfu/g. It stayed approximately the same until the final count on

Table 2. Survival of *S. flexneri* in water (cfu/mL) and on vegetables (cfu/g) stored at 4°C

Day	Water	Green pepper	Onion	Cabbage
0	2.80×10^8	5.25×10^6	5.10×10^6	3.45×10^6
6	ND[a]	8.80×10^5	3.20×10^5	1.85×10^4
12	1.49×10^8	2.20×10^4	2.10×10^5	9.50×10^3
14	1.10×10^8	ND	ND	9.60×10^3
19	9.90×10^7	ND	ND	7.60×10^3
26	9.20×10^7	ND	ND	1.13×10^3

[a] ND, not done.

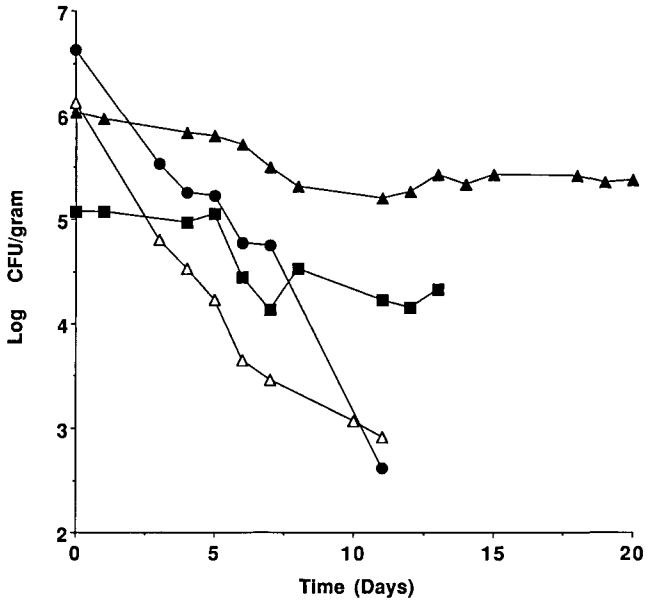

Figure 1. Survival of *S. flexneri* 5 (pHS1059) in commercially prepared salads at 4°C. Log of cfu/g of crab salad (▲), carrot salad (●), potato salad (△) and coleslaw (■).

day 20, when the level was 2.40×10^5 cfu/g salad (22.0% survival). The counts were terminated after day 20 because of spoilage of the crab salad.

The count of *S. flexneri* in the carrot salad at inoculation was 4.30×10^6 cfu/g. By day 3, the count had decreased to 3.52×10^5 cfu/g (8.2%). From day 3 to day 11, the number of bacteria slowly decreased; the count on day 11 was only 4.20×10^2 cfu/g (0.01% survival).

The count of *S. flexneri* in potato salad just after inoculation was 1.32×10^6 cfu/g. On day 3, the number had decreased to 6.40×10^4 cfu/g (4.8% survival). It continued to decrease to 8.50×10^2 cfu/g (0.06% survival) at the end of the experiment on day 11.

The survival of *S. flexneri* 5 (pHS1059) on inoculated vegetables stored at 4°C was also determined at different intervals until the appearance of the vegetables had deteriorated or until no bacteria could be detected. *S. flexneri* survived on all of the vegetables (green pepper, cabbage, and onion) for at least 12 days at 4°C; however, the number of cells decreased. The numbers of *S. flexneri* cells/g detected after inoculation of pepper, onion, and cabbage were 5.25×10^6, 5.10×10^6, and 3.45×10^6 cfu/g, respectively. The numbers had declined to 2.20×10^4, 2.10×10^5, and 9.50×10^3 cfu/g, respectively, after 12 days at 4°C. The counts of *S. flexneri* on onion and green pepper were discontinued after 12 days because of deterioration.

However, *S. flexneri* survived on cabbage and was detected even after 26 days at 4°C (Table 2).

Amplification of S. flexneri with PCR

The sequence of ORF-3 from *S. flexneri* (19) was selected for designing the primers used for DNA amplification. This segment has no significant homology with other DNA sequences in the SWISS-PROT R22 database. The primers exhibited homology only with the *S. flexneri* sequence in GenBank. These primers amplified a 118-base pair DNA region from the *spa* locus of *S. flexneri* DNA (Figure 2, lane 2).

After artificial inoculation of coleslaw, potato salad, crab salad, and carrot salad with *S. flexneri,* the bacteria were collected from the salads, filtered through shark skin filter paper, and used for DNA amplification. This process retained particulate materials from the salad while the bacteria went through the filters. These bacteria were used for PCR amplification. The soluble materials that passed through the filters did not inhibit the PCR reaction. The primers amplified a fragment of 118 base pairs from salads with *S. flexneri* (Figure 2) and from the onion and cabbage eluates (Figure 3). Green pepper contained one or more substances that inhibited amplification of the 118-base pair fragment (Figure 3) when it was added to the mixture containing *S. flexneri* DNA as a template. The primers did not amplify DNA from other bacteria found in salads or vegetables. No amplified DNA fragments were observed in salads not inoculated with *S. flexneri* (Figures 2 and 3).

For the detection of *S. flexneri* from salads stored at 4°C for 3–5 days and from green pepper, the bacteria were enriched in LB broth. This step resulted in the dilution of substances that interfered with the amplification (Figure 4) and increased the number of bacteria.

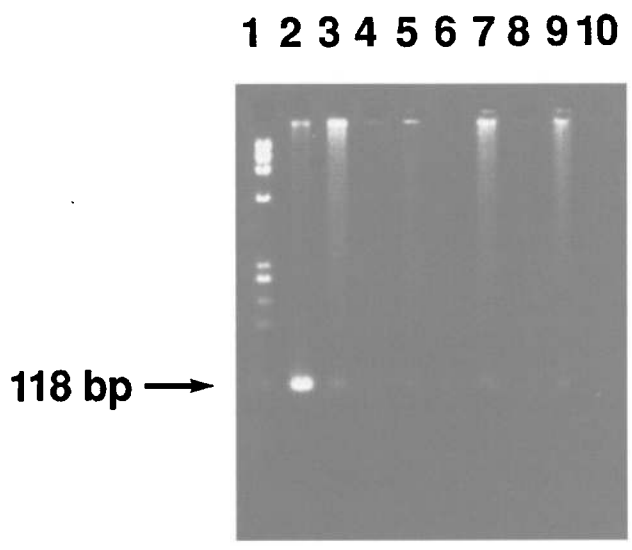

Figure 2. Agarose gel electrophoresis of PCR-amplified DNA (118-base pairs) after elution of bacteria from salads, before and after inoculation with *S. flexneri* 5 (pHS1059), using ORF-3 primers. Lane 1: ϕx174 DNA digested with *Hae* III (Gibco BRL) used as a molecular weight marker. Lane 2: DNA amplified from *S. flexneri* 5 (pHS1059). Lane 3: *S. flexneri* 5 (pHS1059) eluted from potato salad. Lane 4: Eluate from noninoculated potato salad. Lane 5: *S. flexneri* 5 (pHS1059) eluted from carrot salad. Lane 6: Eluate from noninoculated carrot salad. Lane 7: *S. flexneri* 5 (pHS1059) eluted from crab salad. Lane 8: Eluate from uninoculated crab salad. Lane 9: *S. flexneri* 5 (pHS1059) eluted from coleslaw. Lane 10: Eluate from noninoculated coleslaw.

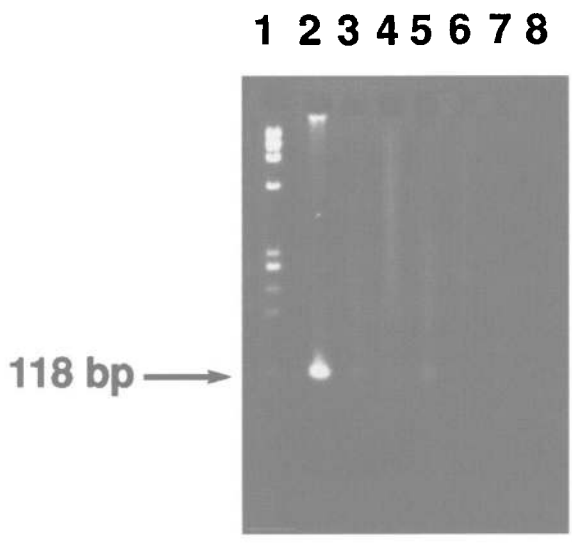

Figure 3. Agarose gel electrophoresis of PCR-amplified DNA (118-base pairs) after elution of bacteria from vegetables, before and after inoculation with *S. flexneri* 5 (pHS1059). Lane 1: ϕx174 DNA digested with *Hae* III (Gibco BRL), used as a molecular weight marker. Lane 2: DNA amplified from *S. flexneri* 5 (pHS1059). Lane 3: *S. flexneri* 5 (pHS1059) eluted from cabbage. Lane 4: Eluate from noninoculated cabbage. Lane 5: *S. flexneri* 5 (pHS1059) eluted from onion. Lane 6: Eluate from noninoculated onion. Lane 7: *S. flexneri* 5 (pHS1059) eluted from green pepper, which contained materials inhibitory to DNA amplification. Lane 8: Eluate from noninoculated green pepper.

Discussion

All salads, including crab salad, contained different vegetables in small or large quantities. The bacteria that were isolated varied from salad to salad. No pathogenic bacteria were isolated from any of the salads. However, potentially pathogenic bacteria (*Klebsiella pneumoniae* and *K. oxytoca*) that could be threats to immunocompromised individuals were isolated. *Shigella* species were not detected from any of the salads, which were purchased from 2 different sources. Commercially prepared salads may include bacteria from the vegetables, which could have been contaminated prior to harvesting or during shipping, handling, or processing, and bacteria introduced during salad preparation, packaging, display, or distribution. Garg et al. (21) noted that washing and sanitizing procedures were inefficient in removing bacteria from processed vegetables.

S. flexneri 5 (pHS1059), which has all of the characteristics of pathogenic strains except invasiveness (15, 16), survived in commercially prepared salads for at least 11 days, or up to 20 days in crab salad. This indicated that *S. flexneri* was not rapidly killed by the normal microflora or the low pH of the salads (the pH of carrot salad was 2.7–2.9, potato salad 3.3–4.4, coleslaw 4.1–4.2, and crab salad 4.4–4.5). Pathogenic strains of *Shigella* spp. surviving on foods have been responsible for several outbreaks of shigellosis. After an initial decline in the number of viable *S. flexneri* cells in crab salad, the count stabilized, but in carrot and potato salads the number of *Shigella* cells decreased to zero. Crab salad supported the longest survival of *S. flexneri* and appeared usable in the refrigerator longer than other salads.

S. flexneri also survived on green pepper, onion, and cabbage, which are used as salad ingredients. We previously showed that *S. flexneri* survives on carrot, celery, lettuce, and broccoli (12). We found no increase in the number of *S. flexneri* cells at 4°C, either in salads or on vegetables, to indicate growth at this temperature. The fluctuation observed in the bacterial count per gram of these salads and vegetables may reflect unequal distribution of bacteria during mixing.

The method used to detect *S. flexneri* appeared to separate bacteria from larger food particles and to remove smaller particulate matter, allowing amplification of DNA from the bacteria. The PCR primers used in this study were genus-specific, amplifying a 118-base

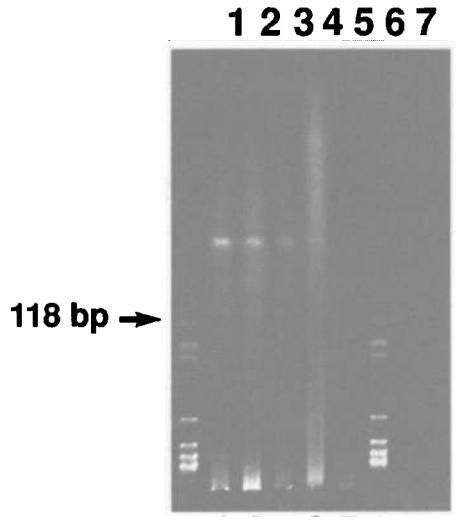

Figure 4. Agarose gel electrophoresis of PCR-amplified DNA (118-base pairs) after elution and enrichment in LB medium of *S. flexneri* 5 (pHS1059) from green pepper and salads refrigerated for 3–5 days after inoculation, using ORF-3 primers for amplification. Lanes 1 and 7: ϕx 174 DNA digested with *Hae* III (Gibco BRL), used as a molecular weight marker. Lane 2: DNA amplified from *S. flexneri* 5 (pHS1059). Lanes 3 and 4: *S. flexneri* 5 (pHS1059) eluted from green pepper and enriched before amplification (500 μL in lane 3, and 100 μL in lane 4; note the inhibition to DNA amplification at 500 μL). Lanes 5 and 6: Eluates from potato salad (lane 5) and crab salad (lane 6), enriched after inoculation with *S. flexneri* 5 (pHS1059) and storage at 4°C.

pair DNA fragment from all 4 *Shigella* spp. (12; data not shown).

One or more substances from green pepper inhibited amplification of this fragment; however, during enrichment, dilution of the inhibitor eliminated this problem. At the same time, the dilution increased the number of bacteria and therefore the quantity of DNA template for the PCR. Other ingredients of the salads did not inhibit PCR amplification, although they may have made PCR conditions less than optimal by affecting the salt concentration or pH, or by adding inhibitory substances. Partial or complete inhibition of PCR amplification associated with components of food matrixes has been reported (22, 23). There was no amplification of DNA from the microflora of salads that had not been inoculated with *S. flexneri*.

The observation that *S. flexneri* and other bacterial contaminants (some of which were potential pathogens) survived in salads for 11–20 days under refrigeration conditions indicates the need for increased attention to the microbiological safety of salads that are commercially packaged or available from salad bars.

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