Research Note

PCR Identification of *Salmonella*: Potential Contamination Sources from Production and Postharvest Handling of Cantaloupes

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ABSTRACT

*Salmonella* is one of the most frequently reported etiological agents in outbreaks of foodborne diseases associated with the consumption of cantaloupes. Sensitive and reliable methods for detecting and identifying foodborne microorganisms are needed. The PCR can be used to amplify specific DNA fragments and thus to detect and identify pathogenic bacteria. In this study, a PCR method was used to evaluate the incidence of *Salmonella* at cantaloupe production, harvest, and packaging steps, and the results were compared with those of the standard method for detection of *Salmonella in foods* (Mexican NOM-114-SSA1-1994). *Salmonella* was detected by both standard and PCR methods in 23.5% of the irrigation water samples but only by the PCR method in 9.1% of the groundwater samples, 4.8% of the chlorinated water samples, 16.7% of samples from the hands of packing workers, 20.6% of samples from the packed cantaloupes, and 25.7% of samples from the in-field cantaloupes. With the standard method, *Salmonella* was found in 8.3% of the crop soil samples. Statistical analysis indicated a significant difference in sensitivity (P < 0.05) between the two methods; the PCR method was 4.3 times more sensitive than the standard method. *Salmonella* was found at seven of the eight points evaluated during the production and postharvest handling of cantaloupe melons.

The increase in the incidence of *Salmonella* on fresh fruits and vegetables in the United States is correlated with more cases of foodborne illnesses (5, 19). Products such as cantaloupe have been implicated less often than other fruits, although some years ago salmonellosis outbreaks were associated with the consumption of contaminated melons (4, 6). Two reported outbreaks of salmonellosis have been related epidemiologically to Mexican cantaloupes. In 1997, the Californian Department of Health Services detected 24 patients infected with *Salmonella* serogroup Saphra presumably from consumption of cantaloupe from Mexico (11). More recently, during 2000 through 2002, three multistate outbreaks of *Salmonella* Poona infections were associated with consumption of Mexican cantaloupes. In response, the U.S. Food and Drug Administration conducted an on-farm investigation in Mexico and concluded that good agricultural practices and good manufacturing practices were not in place to minimize microbial contamination in the growing, harvesting, packaging, and cooling of cantaloupes. Possible sources of contamination included irrigation water contaminated with sewage, contaminated water used in processing produce, poor hygienic practices of workers, and inadequate cleaning and sanitizing of equipment in contact with cantaloupes (6). Unfortunately, only a few outbreaks investigations of fresh product have resulted in clear identification of the point of contamination (4, 11, 12, 20), underscoring the need for rapid and accurate methods of identifying pathogens.

Detection of *Salmonella* by standard methods, such as the Mexican standard NOM-114-SSA1-1994, is labor intensive and time-consuming, requiring 3 to 4 days for negative results and up to 7 days for a confirmed positive result. This method involves more than one enrichment step, selective plating media, and biochemical and serological tests (1, 14). Even so, sensitivity may be poor for samples with low levels of contamination (7). Food safety controls require rapid, sensitive, and specific detection methods for pathogenic bacteria. The PCR technique is one of the most promising of the rapid microbiological methods for the detection and identification of bacteria in a wide variety of samples. This method is based on amplification of a specific target DNA sequence and analysis of the amplification product (8, 16). PCR methods are currently widely used in clinical, research, environmental, and food microbiology, but the complex composition of food matrices can hinder the PCR and lower the sensitivity of the assay (8, 13, 22). Because the bacterial concentration on contaminated fresh food products is usually low, a relatively long preenrichment step usually is necessary to give an acceptable level of sensitivity before PCR can be used (2, 7, 15).
The purpose of this study was to identify potential sources of *Salmonella* contamination during production, harvest, and packaging of cantaloupe melons grown on some farms in Sonora, México, and to compare the PCR method with the standard method for detection of *Salmonella* on foods.

**MATERIALS AND METHODS**

**Farm selection and sampling plan.** Samples were collected on five commercial farms during September 2003 and from May to July 2004. At each farm, samples were collected from melons in the field, melons at the packing house, irrigation water, groundwater, chlorinated water, hands of personnel harvesting and packing cantaloupe, and crop soil. A total of 190 samples were obtained, of which 35 were collected from the field (during harvest) and 34 were from the packing plant. Eleven samples were taken from groundwater (after filtration), 17 were taken from irrigation water, both from drippers or furrows according to the method used at each farm, and 21 were taken from chlorinated water. Personnel samples were obtained in buffered phosphate water from 48 workers (24 field workers and 24 packing house workers). The 24 crop soil samples were taken from the same place in the field as the cantaloupe samples.

Samples from crop soil and in-field and packinghouse cantaloupes were collected in sterile plastic bags, water samples were collected in 500-ml sterile screw-cap bottles, and samples from workers’ hands were collected by rubbing the right hand in a sterile plastic bag with 100 ml of sterile buffered phosphate water. All samples were aseptically collected, placed on ice, transported to the laboratory (about 1 h), stored at 4°C, and processed within 24 h.

**Bacteriological method of reference.** All the samples in this study were tested for *Salmonella* contamination using standardized methods of PCR and the Mexican official method (NOM-114-SSA1-1994). This method for *Salmonella* identification in foods (14) is similar to the *Bacteriological Analytical Manual* protocol for detection of *Salmonella* (1).

**Control strain.** At the beginning of the study, a stock culture of *Salmonella* Typhimurium ATCC 14028 was prepared by adding sterile glycerol (Sigma, St. Louis, Missouri) at a final concentration of 16% to a 24-h culture of the bacteria grown in lactose broth (Difco, Becton Dickinson, Sparks, MD). The culture was dispensed into microtubes, mixed with glycerol, and stored at -55°C. For each experiment, the contents of one microtube were thawed and used as a positive control.

**Sample processing and conventional microbiological testing.** Lactose broth (225 ml) was added to each 25-g or 25-ml sample (skin and pulp of cantaloupe were taken) as a preenrichment step for *Salmonella* analysis. After incubation of preenrichment samples at 37°C for 24 ± 2 h, 1 ml was transferred into a tube containing 10 ml of tetrathionate broth (Difco, Becton Dickinson), and 1 ml was transferred into a tube containing 10 ml of Selenite cystine broth (Difco, Becton Dickinson). Both enrichment tubes were incubated at 37°C for 24 ± 2 h, and then 3-mm loops of each culture were streaked onto brilliant green agar (Difco, Becton Dickinson), Hektoen enteric agar (Difco, Becton Dickinson), and xylose-lysine-desoxycholate agar (Difco, Becton Dickinson) and incubated overnight at 37°C. Plates were observed for typical *Salmonella* colonies. At least two typical colonies from each selective agar plate were screened by inoculation of triple sugar iron agar (Difco, Becton Dickinson), lysine iron agar (Difco, Becton Dickinson), and urea broth (Difco, Becton Dickinson) incubated at 37°C for 24 ± 2 h. Isolates with a positive result in the screening test were confirmed as *Salmonella* by growth on Simmons citrate agar (Merck, Darmstadt, Germany), SIM medium (Difco, Becton Dickinson), MR-VP medium (Difco, Becton Dickinson), and mannitol broth (Difco, Becton Dickinson), all starting from a positive triple sugar iron result.

**PCR preparation.** The same preenrichment samples were used for the PCR assay and for the standard method. The control strain was also preenriched to activate cells. DNA template was extracted from all preenrichment samples and the control strain by a modification of the lysis method of Marmur (10). A 10-ml aliquot of preenrichment sample was subjected to low-speed centrifugation (1,500 × g for 10 min), the supernatant was removed, and the pellet was resuspended in 10 ml of sterile 50 mM EDTA (Sigma) followed by centrifugation (1,500 × g for 5 min). The pellet was washed twice with 1 ml of sterile physiological saline solution (0.85%) and once with 1 ml of distilled water. Then, the samples were centrifuged (3,600 × g for 5 min). The supernatant fluid was removed, the pellet was resuspended in 600 μl of buffered solution (0.15 M NaCl, 0.1 M EDTA, pH 8.0), and 30 μl of lysozyme (10 mg/ml; Sigma) was added. The cell suspension was incubated at 37°C for 1 h and then centrifuged at 3,600 × g for 10 min. The resulting pellet was resuspended in 600 μl of buffered Tris solution (20 mM, pH 8.0; Sigma), 60 μl of 1% sodium dodecyl sulfate (Sigma), and 60 μl of 20 mg/ml protease from *Streptomyces griseus* (Sigma) and then incubated for 30 min at 37°C and then for 20 min at 65°C. The solution was centrifuged (8,000 × g for 10 min), and the supernatant was washed with 600 μl of saturated phenol, phenol-chloroform-isomyl alcohol (25:24:1), and chloroform-isomyl alcohol (24:1) (Sigma). The aqueous phase was recovered by centrifugation at 8,000 × g for 20 min at room temperature and transferred to a new microtube. Enough 5 M NaCl to obtain a 1:10 ratio and 0.6 volumes of ice-cold isopropanol (Sigma) were added, and the samples were vortexed and left at -20°C for at least 20 min. A pellet was obtained by centrifugation at 8,000 × g for 30 min, washed with one volume of cold 70% ethanol, and centrifuged again at 8,000 × g for 10 min. The ethanol was discarded, and the pellet was dried at 65°C for 10 min and then resuspended in 50 μl of buffered Tris-EDTA solution (pH 8.0; Sigma). The DNA solution was treated with 1 μl of RNase (1 μg/ml; Sigma) at 37°C for 1 h.

**Amplification of the ori*C gene.** Two oligonucleotide PCR primers that amplify a 163-bp fragment of the *ori*C gene encoding the origin of DNA replication of *Salmonella* were used for the PCR. The sequences of the primers were as follows: forward primer Sal1, 5’-TTA TTA GGA TCG CGC CAG GC-3’; and reverse primer Sal2, 5’-AAA GAA TAA CCG TTG TTC AC-3’ (8, 21).

The PCR mixture (50 μl) consisted of 5 μl of purified chromosomal DNA (approximately 10 ng/μl), 2 μl of 25 pmol/μl primer Sal1, 2 μl of 25 pmol/μl primer Sal2 (Bioselec, Mexico City, Mexico), 1 μl of 25 mM MgCl2, 1 μl of 2.5 mM (each) dCTP, dGTP, dATP, and dTTP (Promega, Madison, Wis.), 1 U of Taq DNA polymerase (Promega), and 26 μl of denitized water. The mixture in each reaction tube was covered with 35 μl of mineral oil. Amplification was conducted on a Thermo-cycler 480 (Perkin-Elmer Instruments, Norwalk, Conn.) for 36 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. Amplification products (15 μl) were subjected to electrophoresis (C.B.S. Scientific Company, Inc., Del Mar, Calif.) at 75 V on a 2% agarose gel (Sigma). Fine-
TABLE 1. Frequency of detection of Salmonella from cantaloupes, irrigation water, groundwater, chlorinated water, soil, and hands of personnel by PCR and standard methods

<table>
<thead>
<tr>
<th>Sample source</th>
<th>No. of positive samples/total no. of samples analyzed (%)</th>
<th>PCR method</th>
<th>Standard method (NOM-114-SSA1-1994)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irrigation water</td>
<td>4/17 (23.5)</td>
<td>4/17 (23.5)</td>
<td></td>
</tr>
<tr>
<td>Groundwater</td>
<td>1/11 (9.1)</td>
<td>0/11</td>
<td></td>
</tr>
<tr>
<td>Chlorinated water</td>
<td>1/21 (4.8)</td>
<td>0/21</td>
<td></td>
</tr>
<tr>
<td>Crop soil</td>
<td>0/24</td>
<td>2/24 (8.3)</td>
<td></td>
</tr>
<tr>
<td>In-field cantaloupes</td>
<td>9/35 (25.7)</td>
<td>0/35</td>
<td></td>
</tr>
<tr>
<td>In-field workers’ hands</td>
<td>0/24</td>
<td>0/24</td>
<td></td>
</tr>
<tr>
<td>Packed cantaloupes</td>
<td>7/34 (20.6)</td>
<td>0/34</td>
<td></td>
</tr>
<tr>
<td>Packing workers’ hands</td>
<td>4/24 (16.7)</td>
<td>0/24</td>
<td></td>
</tr>
</tbody>
</table>

Characterization of oriC gene amplification. The presence of a DNA fragment of 163 bp was taken as a positive result for Salmonella. To confirm the presence of the oriC Salmonella gene, one of each type of sample positive for the oriC fragment was sequenced. The isolated PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, Calif.) and sequenced at the Genomic Analysis and Technology Core facility at the University of Arizona (Tucson, Az.). The same Sal1 and Sal2 primers were utilized for sequencing (8, 21). The Clustal W program (17) was used to compare the sequences obtained with the sequence of the oriC gene of Salmonella Typhimurium in GenBank (accession no. J01808).

Data analysis. Data were analyzed with the chi-square test from the Number Cruncher Statistical Systems software (9) to compare the sensitivity of the standard method with that of the PCR method.

RESULTS AND DISCUSSION

Detection of Salmonella. From the 190 samples collected from cantaloupes, water, soil, and personnel, 6 samples (3.2%) were positive for the standard method and 26 samples (13.7%) were positive for viable Salmonella by the PCR method. Data analysis indicated a significant difference ($P < 0.05$) in sensitivity between the two methods; the PCR method was 4.3 times more sensitive than the standard method. The combination of preenrichment and the PCR technique enhanced the sensitivity of the assay. Preenrichment increases the number of microorganisms, and the PCR method was able to detect stressed cells that could not be detected by standard methods (7).

The frequency of detection of Salmonella from cantaloupes, water, soil, and personnel by both methods is given in Table 1. The incidence of Salmonella in water samples was as follows. Salmonella was found in 4 (23.5%) of 17 irrigation water samples. This number reflects the contamination at two farms that used groundwater as a water source; this water is transferred to the field by an open irrigation canal. Samples collected from this canal were positive for Salmonella by both the PCR and standard methods. The use of contaminated irrigation water and neglect of good agricultural practices in the field could favor the presence of Salmonella on the in-field cantaloupes. Only 1 (9.1%) of 11 groundwater samples produced the 163-bp amplified fragment of the Salmonella oriC gene. However, these data reflect the contamination at only one farm that used a groundwater source located near a corral of cattle. Salmonella was found by PCR in 1 (4.8%) of 21 chlorinated wash water samples.

Salmonella was detected with the PCR method in 4 (16.7%) of 24 samples from the surface of the hands of workers packing cantaloupes, indicating possible exposure to fecal sources and poor hygienic practices. However, Salmonella was never found on the surface of the hands of field workers with either the PCR or standard methods. Salmonella was detected in 2 (8.3%) of 24 soil samples with the standard method but not with the PCR method. The usefulness of the PCR method for detection Salmonella in soil may have been limited by the presence of substances that inhibit the activity of the enzymes used in the PCR technique. The composition of some inorganic fertilizers present in soil samples and on the surface of hands of field workers may have inhibited the Taq DNA polymerase, resulting in no amplification of the Salmonella oriC DNA fragment (8).

Salmonella was detected in 9 (25.7%) of 35 samples of in-field melons and from 7 (20.6%) of 34 samples of packed cantaloupes (Fig. 1); these results were considered indicative of a higher frequency of fecal contamination. The frequency of Salmonella detected seems consistent with the U.S. Food and Drug Administration findings in their surveys of imported and domestic produce (18) and with those of Castillo et al. (3), who analyzed cantaloupe samples from Mexico and United States. However, Castillo et al. used a sponge method for collecting samples from the surface of the melons, whereas in the present study, samples were collected from skin and pulp. Whether these sampling methods differ in effectiveness for recovering Salmonella from the rough cantaloupe surface is unclear and needs further research.
Characterization of oriC gene amplification. One of each type of sample that was positive for the amplified oriC fragment was sent to be sequenced and identified. All the sequences obtained were identical to the sequence of the oriC gene of Salmonella Typhimurium in GenBank (accession no. J01808), positions 167 to 250 without the oligonucleotide sequences.

The use of contaminated irrigation water, poor hygienic practices of workers, neglect of good manufacturing practices at the packing plant could favor the presence of Salmonella on the final product. The most likely sources of Salmonella contamination on production and postharvest cantaloupes are the irrigation water and workers’ hands. Identification of potential sources of product contamination could permit a science-based design of safety controls to be used for growing and packing melons.

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REFERENCES


