Growth of *Salmonella* during Sprouting of Alfalfa Seeds Associated with Salmonellosis Outbreaks

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ABSTRACT

Growth of *Salmonella* was assessed during sprouting of naturally contaminated alfalfa seeds associated with two outbreaks of salmonellosis. *Salmonella* was determined daily in sprouts and sprout rinse water samples by a three-tube most probable number (MPN) procedure and a commercial enzyme immunoassay (EIA). Growth of *Salmonella* in the sprouts was reflected in the rinse water, and the MPNs of the two samples were generally in agreement within approximately 1 log. The results from EIA testing of sprouts and water samples were also in agreement. The pathogen was present in the seed at less than 1 MPN/g, and it increased in number to maximum population levels of 10^2 to 10^3 MPN/g in one seed lot and 10^2 to 10^3 MPN/g in the other seed lot. Maximum populations of the pathogen were apparent by day 2 of sprouting. These results show the ability of the pathogen to grow to detectable levels during the sprouting process, and they provide support for the recommendation to test the sprout water for the presence of pathogens 48 h after starting seed sprouting. The effectiveness of a 10-min, 20,000-µg/ml (ppm) calcium hypochlorite treatment of the outbreak-associated seeds was studied. For both seed lots, the hypochlorite treatment caused a reduction, but not elimination, of *Salmonella* contamination in the finished sprouts. These results confirm the need to test each production batch for the presence of pathogens, even after 20,000 µg/ml (ppm) hypochlorite treatment of seeds, so that contaminated product is not distributed.

Between 1988 and 1998, at least 10 salmonellosis outbreaks linked to the consumption of raw sprouts were documented internationally, 7 of them in the United States. Most involved alfalfa sprouts, but cress, mung bean, and clover sprouts were also implicated. Thirteen *Salmonella* serotypes were isolated from the clinical cases. For a review of these outbreaks, see Taormina et al. (1). *Salmonella* was determined daily in sprouts and sprout rinse water samples by a three-tube most probable number (MPN) procedure and a commercial enzyme immunoassay (EIA). Growth of *Salmonella* in the sprouts was reflected in the rinse water, and the MPNs of the two samples were generally in agreement within approximately 1 log. The results from EIA testing of sprouts and water samples were also in agreement. The pathogen was present in the seed at less than 1 MPN/g, and it increased in number to maximum population levels of 10^2 to 10^3 MPN/g in one seed lot and 10^2 to 10^3 MPN/g in the other seed lot. Maximum populations of the pathogen were apparent by day 2 of sprouting. These results show the ability of the pathogen to grow to detectable levels during the sprouting process, and they provide support for the recommendation to test the sprout water for the presence of pathogens 48 h after starting seed sprouting. The effectiveness of a 10-min, 20,000-µg/ml (ppm) calcium hypochlorite treatment of the outbreak-associated seeds was studied. For both seed lots, the hypochlorite treatment caused a reduction, but not elimination, of *Salmonella* contamination in the finished sprouts. These results confirm the need to test each production batch for the presence of pathogens, even after 20,000 µg/ml (ppm) hypochlorite treatment of seeds, so that contaminated product is not distributed.
alfalfa seeds showed increases of 2 to 4 logs in the sprouts (16, 18).

There is little information available in the published literature concerning the growth of pathogens during sprouting of naturally contaminated seeds or of the effectiveness of disinfection on such seeds. In this study we assessed the growth of Salmonella during sprouting of naturally contaminated alfalfa seeds associated with two outbreaks of salmonellosis and also tested the effect of 20,000 μg/ml (ppm) calcium hypochlorite treatment of the seeds on pathogen growth.

MATERIALS AND METHODS

Sprouting of alfalfa seeds. Alfalfa seeds associated with two different outbreaks of salmonellosis (seed lots A and B) were obtained from outbreak investigators. Seeds from each lot were sprouted in duplicate in glass jars as follows. The seeds (200 g per jar) were soaked in 1,000 ml sterile tap water for 3 h at ambient temperature and drained; then the jars were loosely capped, placed in a covered container, and incubated at 25°C for 3 days. The sprouts were rinsed once each day with 1,000 ml sterile tap water. Both the sprouts and the sprout rinse water from the duplicate jars were sampled each day for microbiological analysis. (On day 0, the soaked seeds and soak water were used for analysis.)

Microbiological analysis. Microbiological culture media were obtained from Difco (Becton Dickinson Microbiology Systems, Sparks, Md.). For aerobic mesophiles counts (aerobic plate counts), 10 g sprouts or 10 ml sprout rinse water was added to 90 ml Butterfield's phosphate buffer, pH 7.2. Sprouts were homogenized in a stomacher model 400 blender for 2 min at medium speed; sprout rinse water was swirled to mix. Tenfold serial dilutions of the mixtures were made in Butterfield's phosphate buffer. Dilutions were plated onto plate count agar in duplicate by the spread plate method, and the plates were incubated for 24 to 48 h at 30°C.

Salmonella was enumerated in the samples by a three-tube most probable number (MPN) method (5), incorporating standard enrichment and selection procedures for the pathogen (1), as follows. Ten grams of sprouts or 10 ml sprout rinse water was distributed into 90 ml lactose broth and incubated at 35°C. After 24 h, 0.1 ml from each pre-enrichment was transferred to 10 ml Rappaport-Vassiliadis broth and another 1 ml was transferred to 10 ml tetraphionate broth for incubation in a 42°C water bath (selective enrichment). After 24 h, the selective enrichment tubes were streaked onto xylose lysine deoxycholate, hektoen enteric, and bismuth sulfite plates, and randomly selected typical colonies were confirmed in triple sugar iron and lysine iron agar tubes.

Calcium hypochlorite treatment of seeds. Ten replicate jars of alfalfa seeds from each outbreak-associated lot were set up for sprouting. The seeds (200 g per jar) were rinsed with 500 ml sterile tap water and drained. To each of five of the replicates was added 500 ml of a freshly made solution of calcium hypochlorite (3% wt/vol, prepared from 65% available chlorine granules in sterile tap water [Aldrich Chemical Co., Inc., Milwaukee, Wis.]; equivalent to 20,000 μg/ml [ppm]), as recommended (10) (treated); 500 ml sterile tap water was added to each of the other five replicates (untreated controls). The pH of the sterile tap water was 8.6, and the pH of the calcium hypochlorite solution was 11.9. The seeds were swirled briefly to ensure wetting, allowed to stand for 10 min at ambient temperature, then drained and rinsed two times with 500 ml sterile tap water. The seeds were then soaked in 1,000 ml sterile tap water for 3 h at ambient temperature, drained, and incubated for 3 days of sprouting as described above. The Assurance Gold Salmonella EIA and confirmation were performed as described above after postenrichment of samples taken daily from each jar.

RESULTS AND DISCUSSION

Salmonella populations measured during sprouting of outbreak-associated seed lots A and B are illustrated in Figures 1 and 2, respectively. Levels of the pathogen (MPNs) in the sprouts and rinse water samples are indicated. For seed lot A, both of the duplicate jars of sprouts showed the presence of the pathogen during sprouting, and the data are shown in Figure 1 as replicate 1 and replicate 2. For lot B, only one of the duplicate jars showed the presence of the
pathogen during sprouting; therefore, the growth experiment was run a second time, again with duplicate jars. The results of the second run were the same as the first; i.e., only one of the duplicate jars was positive. These two experiments are shown in Figure 2 as trial 1 and trial 2.

Overall, the data obtained in this study support the recommendations made to the sprouting industry for testing of spent irrigation water from commercial sprout production batches for pathogen monitoring (21). Growth of *Salmonella* in the sprouts was reflected in the rinse water, and the MPNs of the two samples on any day were generally similar, i.e., within approximately 1 log. (An exception was the data from lot A, replicate 1 on day 1, in which the MPN determination of the water sample appeared to be anomalous; see Fig. 1.) The aerobic plate count data (Table 1) provide additional support for the recommendation: the average difference in APC (CFU/g) between sprouts and rinse water (both seed lots, all days of sprouting) was 0.61 ± 0.46. There was also agreement between the results of the sprouts and water sample testing for *Salmonella* detection by EIA (Table 2). These trends support the premise that the testing of the sprout rinse water can indicate the contamination status of the sprouts (4, 16, 21).

The EIA results showed the presence of the pathogen by day 1 of sprouting (Table 2), even in batches that were negative on day 0 (seed lot B, trials 1 and 2). There was one EIA-negative sample on day 0 that was positive by MPN; however, the rinse water sample from seed lot B, trial 1, was negative in the EIA (Table 2) but was positive by MPN, showing a log_{10} MPN of approximately −1 on day 0 (Fig. 2, triangles). Previous work has indicated the difficulty with using unsprouted seeds for pathogen detection (7), and these results support those observations. The increase in the pathogen population that occurs during sprouting improves detection.

Maximum population levels reached by *Salmonella* in both seed lots were apparent by day 2 (Figs. 1 and 2), which supports the recommendation that the testing of commercial sprout batches be conducted 48 h after starting seed sprouting (21). There were differences between the two lots of seeds in the *Salmonella* population levels attained, however. The pathogen was present at less than 1 MPN/g in seed lot A, and it increased in number to 10^2 to 10^3 MPN/g of sprouts. In seed lot B, a maximum population of approximately 10^4 MPN/g was reached in trial 1, but in trial 2 the population of *Salmonella* rose only to approximately 10^2 MPN/g. Additional studies using these and other naturally contaminated seed lots would be useful for assessing the range of variability in pathogen growth during sprouting. In none of the trials were the levels reached by the natural *Salmonella* populations as high as those obtained in previously reported pathogen growth studies using inoculated seeds; i.e., *Salmonella* Stanley at 10^7 CFU/g (8), or *E. coli* O157:H7 at 10^5 to 10^6 CFU/g (16, 18). In those previous studies, the pathogens were inoculated onto the seeds at levels three to four orders of magnitude greater than their presence in the naturally contaminated seeds used in this study, and it is possible that the higher pathogen loads on the seeds may account for their higher populations in the finished sprouts.

The effectiveness of a 10-min, 20,000-µg/ml (ppm)
TABLE 3. **Detection of Salmonella during sprouting of control and 20,000 µg/ml (ppm) hypochlorite-treated alfalfa seeds associated with salmonellosis outbreaks**

<table>
<thead>
<tr>
<th>Seed lot</th>
<th>Seed treatment</th>
<th>Sample</th>
<th>Detection on day of sprouting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 1</td>
</tr>
<tr>
<td>A</td>
<td>None</td>
<td>Sprouts</td>
<td>2/5</td>
</tr>
<tr>
<td></td>
<td>Hypochlorite</td>
<td>Sprouts</td>
<td>1/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Totals</td>
<td>1/10</td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>Sprouts</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Hypochlorite</td>
<td>Sprouts</td>
<td>1/5</td>
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<tr>
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<td>Water</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Totals</td>
<td>1/10</td>
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</table>

Notes:

- Colonies isolated from sample and confirmed as *Salmonella*.
- Number of samples positive/number of samples tested.

Calcium hypochlorite treatment of the outbreak-associated seeds was studied. In previous studies, this treatment produced a 4-log reduction of *E. coli O157:H7* inoculated onto alfalfa seeds (10). However, *E. coli O157:H7* was not completely eliminated; i.e., it could still be detected after enrichment of the treated alfalfa seeds (9, 10, 17) and even grew to levels as high as 10^7 CFU/g in the finished sprouts (9). Nevertheless, its use as a seed treatment by the sprout industry is recommended (10, 21), because more effective treatments have not been identified. No published studies have shown the effect of this treatment on *Salmonella*, on naturally contaminated seeds, or on pathogen growth through the sprouting process.

Both sprouts and rinse water samples from control and hypochlorite-treated seed lots A and B were tested daily during sprouting by EIA and confirmed for the presence of *Salmonella*. The EIA results were not always consistent with the *Salmonella* confirmation results for seed lot A, although agreement between the two methods was better for seed lot B. For seed lot A (80 samples, including sprouts and water, treated and untreated), the EIA was positive for 63.75% of the samples tested, but in only 48.75% of the samples was *Salmonella* confirmed. For seed lot B (80 samples), the EIA produced 32.5% positives, and *Salmonella* was confirmed in 31.0%. For all samples from both seed lots, the EIA showed a false-positive rate of 11.25% and a false-negative rate of 3.1%. There were no EIA false negatives, however, from day 2 of sprouting and beyond, the recommended sampling time for pathogen monitoring (21). Nevertheless, because of the discrepancies between the two detection methods, only the confirmatory results are shown in Table 3.

Results from the water samples were in agreement with those of the corresponding sprout samples, with a few exceptions (Table 3). In three cases, sprout samples were positive for *Salmonella*, but the corresponding water samples were negative (seed lot A, day 0, no treatment; seed lot A, day 0, hypochlorite treatment; seed lot B, day 0, hypochlorite treatment). In one case, a water sample was positive, but the corresponding sprout sample was negative (seed lot A, day 1, hypochlorite treatment). Nevertheless, by day 2 of sprouting, the recommended sampling time for pathogen detection (21), there was 100% agreement between the two samples. For both outbreak-associated seed lots, the hypochlorite treatment resulted in a lower frequency, but not elimination, of *Salmonella* contamination in the finished sprouts. Hypochlorite treatment of seeds produced a 50 and 66% reduction in the number of positive 3-day sprout samples from seed lots A and B, respectively.

The hypochlorite-treated samples were generally revealed as positive by day 2 of sprouting, and the frequency of positives did not change beyond day 2. For seed lot A, the treated samples showed a delay in the appearance of positive reactions, compared to the controls. Whereas there was no further increase in positives by day 1 for the controls, this was not the case until day 2 for the treated samples. These results provide additional support for the recommended sampling time (day 2) for monitoring alfalfa sprout production for pathogen detection (21).

This study confirms the need for microbiological testing of each production batch by commercial sprouters, even after 20,000 µg/ml (ppm) hypochlorite treatment of seeds, so that contaminated product is not distributed. Despite the use of a calcium hypochlorite treatment prior to sprouting, seed lot A was the source of an outbreak of salmonellosis in 1999 (13). Had the sprout batches been tested for the presence of pathogens, the illnesses may have been prevented. The need for additional research to find more effective seed decontamination treatments and pathogen control procedures during sprouting is also demonstrated by this study.

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**REFERENCES**


