Research Note

Two Processing Methods for the Isolation of Salmonella from Naturally Contaminated Alfalfa Seeds

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ABSTRACT

Two processing methods were examined for the recovery of Salmonella from naturally contaminated alfalfa seed. Seed samples, from each of three investigations, were processed by sprouting and shredding before preenrichment and culture. In lot A, Salmonella serotype Newport was isolated from 3 of 30 sample units with the sprouting method and 2 of 30 with the shredding method. In lot B, three serotypes in various combinations were isolated from 10 of 30 sample units with the sprouting method and 9 of 30 with the shredding method. In lot C, Salmonella group C 1 was isolated from 27 of 30 sample units with the sprouting method and 24 of 30 with the shredding method. Additionally, serotype Newport was found in one lot C sample unit. Using shredded seed data, a most probable number (MPN) for Salmonella contamination per lot was calculated. Serotype Newport was estimated at 0.07 MPN/100 g in lot A; the concentration for three serotypes was estimated to be 0.36 MPN/100 g in lot B; Salmonella group C 1 was estimated at 1.8 MPN/100 g in lot C. Our success in isolating Salmonella from alfalfa seeds was likely attributed to the volume of material tested and the quick acquisition of the seeds after the outbreak was identified. Shredding the seeds was easier and yielded definitive results more quickly than sprouting.

Outbreaks of Salmonella related to the consumption of sprouts have been reported both in the United States and internationally (6, 8, 10, 11, 14). California has experienced several outbreaks connected with the consumption of alfalfa/clover sprouts. In some of the outbreaks, Salmonella was isolated from seed and/or sprouts. In other cases the outbreak was confirmed with epidemiological data (9). Understanding lot number assignments at the distribution point is crucial to the trace-back for a regulatory agency. It is common practice for seed from multiple growers to be mixed and then assigned a lot number prior to distribution (3). This practice results in dilution of a potential pathogen in the lot.

Isolation of a microbial pathogen from seed is laborious, but it is a necessary function for laboratories supporting epidemiological investigations or a regulatory agency. In the course of our routine duties evaluating the microbiological content of outbreak-linked seed product, we initially sprouted seed to mimic the natural process of growth (4). However, we also had generated preliminary data on other methods (4).

In this report, we provide new data on the isolation of Salmonella following the sprouting or shredding of alfalfa seed. After data collection, we estimated the concentration of Salmonella per lot by calculating a most probable number (MPN) per 100 g of seed. This report may aid other agencies faced with the testing of seed product.

MATERIALS AND METHODS

History of seeds. Naturally contaminated alfalfa seeds were acquired from three sprout surveillance or outbreak investigations. Information was not available on storage conditions for seeds within the distribution centers. Lot A came from the 1996 outbreak of Salmonella serotype Newport in Oregon and British Columbia (14). Lot B was a surveillance sample obtained from a California sprout grower. Lot C was linked to outbreaks in Oregon and Washington (6). All samples were collected and submitted to the Microbial Diseases Laboratory for Salmonella testing by the California Department of Health Services Food and Drug Branch. Seeds delivered to the laboratory were stored at room temperature in a tamper-proof cabinet.

Sample, sample unit, and total number surveyed. A sample was composed of one bag of alfalfa seed weighing approximately 500 g; a sample unit consisted of 100 g from each sample bag. Thirty sample units per lot were tested for Salmonella after shredding the seeds. An additional 30 sample units of seed per lot were sprouted, and 100 g of sprouted product from each sample unit were tested for Salmonella. Either 100 g of sprouts or shredded seeds were diluted to a 1:9 ratio of weight to liquid for the pre-enrichment phase. In total, 3,000 g of seed per method by three lots by two methods yielded an evaluation of 18,000 g of seed. We processed 30 samples per lot from which two sample units were taken. This converts to 60 sample units per lot × three lots, to equal an analysis of 180 sample units split between the two methods.

Macroscopic examination. Two hundred alfalfa seeds from each lot were randomly taken from one sample bag and examined under a dissecting scope at 7× under bright light. Each seed was

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turned using a platinum needle. Whole seeds were scored as uninjured. Injured seeds were determined by the presence of cracks or breaks in the seed coat (15). The number injured out of 200 seeds evaluated per lot was converted to a percentage injured per lot.

Sprouting method. The basic procedure for sprouting seed was previously described (4). Minor modifications included (i) spreading the water-soaked seeds with a sterile spoon up the sides of the gauze layered beaker to maximize air exposure, (ii) incubating the beaker of wet seeds for 3 days instead of 4, and (iii) shaking the 1:9 dilution (900 ml) of sprouts and lactose broth by hand 25 times in 7 s in a 1-ft arc. The 1:9 dilution was incubated at room temperature for 1 h before adjusting the pH with 5 N NaOH (Spectrum, New Brunswick, N.J.) to \(6.8 \pm 0.2\). Unless otherwise noted, all microbiological media were obtained from Difco (Detroit, Mich.).

Shredding method. The shredding method (4) was previously described. The only modification included shaking the 1:9 dilution (900 ml) of shredded seeds and lactose broth by hand 25 times in 7 s in a 1-ft arc. The 1:9 dilution was then incubated at room temperature for 1 h before adjusting the pH to \(6.8 \pm 0.2\).

Enrichment, isolation, and identification. Preenrichment, enrichment, and plating for Salmonella followed the U.S. Food and Drug Administration’s *Bacteriological Analytical Manual* (1). Typical *Salmonella*-like colony types from each plating medium were picked in duplicate to triple sugar iron agar, lysine iron agar, and motility indole ornithine medium. Each colony pick was serogrouped in a slide agglutination test using triple sugar iron growth and grouping antisera (Microbial Diseases Laboratory, Berkeley, Calif.). Colony picks that agglutinated with antisera were further confirmed to the genus level with biochemical tests. Representative isolates were sero typed by the Enteric Bacteriology unit of the Microbial Diseases Laboratory.

MPN and confidence limits. The shredded seed data were used to estimate the concentration of *Salmonella* in each lot. Each 100-g aliquot was viewed as the equivalent of a single test tube in a 30-tube, single dilution MPN procedure for *Salmonella*. The Thomas formula (13) was used to calculate the MPN/100 g. The formula is \(MPN/g = P(NT)^b\) where \(P\) = no. of positive results, \(N\) = quantity of samples (in grams) in all negative tubes, and \(T\) = total quantity of sample (in grams) in all tubes. Confidence limits were calculated following Cornish and Fisher (2) using the U.S. Environmental Protection Agency’s MPN calculator version 4.04 (7).

RESULTS

Alfalfa seeds from each lot were kidney-shaped and varied in color (yellow, tan, green, dark brown, black). Seeds in lot A were more shriveled than seeds in B or C. Injury was estimated to be 5, 9.5, and 7.5% for lots A, B, and C, respectively.

The number of positive sample units by method and the data for the 180 sample units (90 samples) tallied into paired outcomes per lot number are presented in Table 1. The paired data were variable in all three lots for the isolation of *Salmonella* from samples processed by seed sprouting or shredding. For example, from lot A, 0 of 30 samples gave a positive positive by both methods; in other words, no two 100-g sample units taken from the same sample bag yielded a positive *Salmonella* isolation. For lot B, four sam-

<table>
<thead>
<tr>
<th>Lot no.</th>
<th>Sprouting</th>
<th>Shredding</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>3/30</td>
<td>4/30</td>
</tr>
<tr>
<td>B</td>
<td>10/30</td>
<td>15/30</td>
</tr>
<tr>
<td>C</td>
<td>27/30</td>
<td>22/30</td>
</tr>
<tr>
<td>Total</td>
<td>40/90</td>
<td>35/90</td>
</tr>
</tbody>
</table>

In some cases, two serotypes of *Salmonella* were isolated per sample unit.
TABLE 2. Variable distribution of Salmonella serotypes within lot B

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Salmonella serotype(s) isolated&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>62, 82, 85</td>
<td>Tennessee and Cubana</td>
</tr>
<tr>
<td>63, 67, 90</td>
<td>Havana</td>
</tr>
<tr>
<td>65, 66</td>
<td>Tennessee</td>
</tr>
<tr>
<td>68, 84</td>
<td>Havana and Cubana</td>
</tr>
<tr>
<td>74, 80, 83</td>
<td>Cubana</td>
</tr>
<tr>
<td>76, 78</td>
<td>Tennessee and Havana</td>
</tr>
</tbody>
</table>

<sup>a</sup> Combined serotype data for both methods of seed processing.

ples were positive by the two methods. For lot C, 22 samples were positive for both methods.

Variable distribution of specific Salmonella serotypes within lot B was observed (Table 2). One or two Salmonella serotypes appeared randomly among the 15 positive samples. For example, serotypes Tennessee and Cubana were isolated in samples 62, 82, and 85, whereas serotype Havana was the only Salmonella isolated from samples 63, 67, and 90.

Using data from the shredded seeds, the relative concentration of Salmonella in each lot was calculated and expressed as an MPN (Table 3). There were two different serotypes isolated from some sample units in lot B and from one sample in lot C; therefore, individual serotype MPNs do not equal the Salmonella positive MPNs for each respective lot. The 95% confidence limits show a narrow spread due to the calculation being based on a 30-tube method.

DISCUSSION

Microscopic examination of 200 seeds per lot showed the majority of seeds were uninjured. The International Specialty Supply and the U.S. Department of Agriculture are seeking a method for removal of injured seed from each lot before it is sold. These agencies suggest that seeds with >5% injury level (cracked seed coat) should not be used for spraying product for human consumption because injured seeds may not be effectively sanitized (12). Using this criterion for future decision making means that two lots (B, C) would have been diverted to an agricultural use, but lot A might have been released for spraying for human consumption.

The two methods for processing naturally contaminated alfalfa seeds prior to preenrichment had merits and deficits. Sprouting is time consuming and labor intensive. However, in situations where only a small volume of seed is available for testing, the spraying process provides a natural means for amplifying the normal flora and pathogen (5). Shredding seeds to a coarse powder prior to preenrichment is time efficient and requires fewer laboratory supplies. Results prove that Salmonella can be isolated after seeds are shredded and enriched; the 1:9 dilution probably reduces or eliminates toxicity associated with the seed coat (5).

Either method for processing seeds prior to preenrichment for Salmonella proved satisfactory. Overall, we tested 18,000 g of seed during this evaluation. The authors recognize that the amount of dry weight for seeds shredded and tested was not equivalent to the wet weight for the germinated sprouts tested. The 100 g wet weight for germinated sprouts converts to approximately 33 g of dry seeds. In view of this information, it could be surmised that the spraying procedure is possibly better for detecting low contamination levels in a smaller volume of seed.

Data support the concept of variable concentrations and random distribution of Salmonella within small batches of seed; one can presume that a similar distribution of the pathogen existed in each lot. Rarely was Salmonella detected in two 100-g sample units by both methods, except in lot C where the concentration of Salmonella was higher. Surprisingly, more than one serotype was present in lots B and C. The presence of different serotypes, within a sample bag and between sample units, demonstrates the difficulty that public health officials may encounter when trying to link an outbreak to a specific seed lot number. This is particularly true if a laboratory only examines a small volume of product. The Salmonella serotype Newport MPN of 0.07 in lot A may explain the difficulty many laboratories had in 1996 in culturing this pathogen following the outbreak. This is our second report using seed from lot A; the data show that Salmonella can remain viable in seed that has been stored for 2 years at room temperature in the dark. This further highlights the fact that seed age does not diminish the hazard to the public. Further laboratory testing is needed to answer many of the remaining questions surrounding the relationship of Salmonella to the seed and the seed flora.
ACKNOWLEDGMENTS

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