Isolation of *Salmonella* Enteritidis Phage Type 30 from a Single Almond Orchard over a 5-Year Period

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ABSTRACT

In 2001, *Salmonella* Enteritidis phage type (PT) 30 was isolated from drag swabs of 17 61-ha almond orchards on three farms linked to an outbreak of salmonellosis associated with consumption of raw almonds. The objective of this study was to evaluate the long-term persistence of *Salmonella* Enteritidis PT 30 in one of the almond orchards associated with the outbreak. Swabs (gauze saturated with full-strength sterile evaporated skim milk and attached to string) were pulled along the orchard floor in a standardized manner for 55 m. At each sample time, two pooled samples (four swabs each) were collected from each orchard quadrant. Swabs were enriched for *Salmonella* using a delayed secondary enrichment procedure developed for isolation of *Salmonella* from poultry houses. Suspect *Salmonella* isolates were selected, confirmed, serotyped, and phage typed, and pulsed-field gel electrophoresis (PFGE) patterns were determined after cleavage with *Xba*I and *Bln*I. *Salmonella* was recovered infrequently from pooled samples collected from January through July (3 of 56 samples, 5.3%). In general, *Salmonella* isolation frequency per sample time increased during and immediately after the harvest, when large amounts of dust were generated in or near the orchard: August, 4 (20%) of 20 samples; September, 13 (20%) of 64 samples; October, 27 (42%) of 64 samples; November, 4 (25%) of 16 samples; and December, 2 (25%) of 8 samples. All 53 *Salmonella* isolates during the 5 years were identified as *Salmonella* Enteritidis PT 30, and two PFGE patterns that differed by the presence of an approximately 40-kb fragment were identified. These data demonstrate the potential for long-term environmental persistence of *Salmonella* in almond orchards.

Salmonella is common in the environment, but the primary habitat of this pathogen is the intestinal tract of birds, reptiles, animals, humans, and occasionally insects *(11).* The potential for populations of *Salmonella* to multiply in the environment in the absence of an animal host has been discussed *(8, 27),* but environmental persistence of *Salmonella* is more commonly attributed to its broad host range and continual reintroduction of the organism *(27).* The ability of *Salmonella* to survive for long periods in dried foods and feed *(2, 25),* animal production facilities *(6),* and water, soil, and manure *(14)* has been well documented.

An outbreak of *Salmonella* Enteritidis phage type (PT) 30 infection in 2000 and 2001 was linked to consumption of raw almonds that were harvested in the fall of 2000 *(10).* *Salmonella* Enteritidis PT 30 was isolated from open and sealed bags of bulk almonds, and the implicated lot was traced to one California almond processor. In traceback investigations 7 to 8 months after the 2000 harvest, *Salmonella* Enteritidis PT 30 was isolated from drag swabs of almond orchard floors on three geographically linked farms

in a 25-km^2 area. Samples of farm irrigation water, airplane spray equipment, migratory waterfowl, and rodents all were negative for *Salmonella.* None of the farms had used manure or biosolids in the previous 5 years, and no livestock or poultry farms were adjacent to or near the almond farms *(3).* A source for the contamination was not identified during the outbreak investigation, but the isolation of *Salmonella* from such a large geographic area suggested a widely distributed contamination source *(3).*

These naturally contaminated almond orchards provided a unique opportunity to study the environmental persistence of *Salmonella* in this area. *Salmonella* Enteritidis PT 30 was isolated over a period of 5 years from a single almond orchard.

MATERIALS AND METHODS

A modified drag swab and a delayed secondary enrichment procedure developed for isolation of *Salmonella* Enteritidis from poultry houses *(18, 21, 22)* were used successfully to isolate *Salmonella* Enteritidis from almond orchards during the traceback investigation of the salmonellosis outbreak that occurred in 2000 and 2001 *(10).* This same procedure was used to isolate *Salmonella* from the almond orchard examined in the present study. Unless otherwise noted, all media were obtained from Difco (Becton Dickinson, Sparks, Md.).

Swab preparation. Drag swabs were prepared by folding two pieces of gauze (10 by 10 cm; Nu Gauze general-use sponges, Johnson & Johnson, New Brunswick, N.J.) in half and tying one end of a 1-m piece of cotton string around the center. Swabs were

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TABLE 1. *Weekly isolation of* Salmonella *Enteritidis PT 30 from one almond orchard during August, September, and October, 2002 through 2006*

| | No. of samples positive for *Salmonella*[a] | | | | | | | | | | |
| | August | | | | September | | | | October | | |
Year	Week 1	Week 2	Week 3	Week 4	Week 1	Week 2	Week 3	Week 4	Week 1	Week 2	Week 3
2002	0/4	1/4		2/4[b,c]			3/8[b]	1/8	4/8[c,d]		
2003					0/8		0/8		6/8[b,c]	0/8	
2004			1/8[b]		1/8[b]	2/8	3/8	3/8	1/8	4/8[c,e]	7/8[c,f]
2005											2/8
2006											3/8

[a] Data are the number of samples positive for *Salmonella* Enteritidis PT 30/total number of samples obtained.
[b] All *Salmonella*-positive pooled swab samples were detected by delayed secondary enrichment.
[c] Sampling week in which at least 50% of the samples were positive.
[d] Three of four *Salmonella*-positive pooled swab samples were detected by delayed secondary enrichment.
[e] One of four *Salmonella*-positive pooled swab samples were detected by delayed secondary enrichment.
[f] Two of seven *Salmonella*-positive pooled swab samples were detected by delayed secondary enrichment.

autoclaved and transferred aseptically to individual sterile plastic 532-ml Whirl-Pak bags (Nasco, Modesto, Calif.). A 12-ml volume of full-strength sterile evaporated skim milk (Nestle, Solon, Ohio) was added aseptically to each bag, and swabs were frozen and stored at −20°C. Swabs were thawed before use.

Environmental drag swabbing. Before swabbing the orchard, individuals collecting the samples covered their street shoes with disposable 6-mil plastic tie boot covers (Nasco). Boot covers were changed between collection of samples in each orchard quadrant. Latex gloves were worn and were sprayed with 70% ethanol and allowed to air dry. Gauze swabs were spread open with gloved hands and dragged by the end of the string diagonally along the orchard floor. Swabs were dragged beside (not behind) the individual collecting the sample.

Swabbing began 15 trees into the north and south end of the orchard, starting from the center row. Each drag swab was pulled diagonally across three rows of trees, behind one tree, and diagonally back across three rows of trees, for a total distance of approximately 55 m. A second swab was dropped to the ground a further five trees into the orchard and pulled in the same pattern. The third and fourth swabs were obtained in the same manner 13 rows to the east or west of the starting row, and these four swabs were pooled to form one sample. The swabbing pattern was repeated at 13-tree intervals further to the east or west to obtain a second pooled sample. Two pooled samples were collected from each quadrant (northeast, northwest, southeast, and southwest) of the orchard. Drag swabs were collected from August 2002 through October 2006.

Samples were placed on ice packs and delivered to the California Animal Health and Food Safety Laboratory (CAHFS) at the University of California (Davis, Calif.) for analysis.

***Salmonella* delayed secondary enrichment procedure.** Pooled swabs were enriched and *Salmonella* isolates were serotyped by CAHFS (Davis, Calif.). Tetrathionate broth (480 ml) was added to the pooled sample (four swabs per sample), mixed gently, and incubated aerobically at 42°C for 18 to 24 h. A sterile cotton swab was used to streak brilliant green agar (with 20 μg/ml novobiocin) and xylose lysine tergitol 4 agar plates, which were incubated aerobically for 24 or 48 h at 37°C and examined for the presence of suspect *Salmonella* colonies. At least three colonies per plate were confirmed using triple sugar iron or API 20E strips. Samples positive for *Salmonella* were confirmed with polyvalent sera and then sent for serotyping. The original enrichment broth was held for a further 5 to 7 days at room temperature (23 to 25°C). If plates from the initial enrichment were negative for *Salmonella,* the original broth was mixed, and after 5 to 7 days 1 ml was transferred to 10 ml of fresh tetrathionate broth and incubated for 18 to 24 h at 37°C. After incubation, the broth was streaked as described above, and suspect *Salmonella* colonies were treated as described above.

Phage typing. Phage typing was conducted at the U.S. Department of Agriculture Animal and Plant Health Inspection Service (National Veterinary Services Laboratory, Ames, Iowa).

Pulsed-field gel electrophoresis. The PulseNet pulsed-field gel electrophoresis (PFGE) protocol for *Salmonella* was used with the *Xba*I and *Bln*I restriction enzymes *(17)*. Gels were run on a CHEF Mapper II system (Bio-Rad, Hercules, Calif.).

Weather data documentation. Precipitation data and soil temperatures recorded at weather stations 124 Panoche and Panoche 2 W were accessed online from the California Irrigation Management Information System (CIMIS) and the National Climatic Data Center of the National Oceanic and Atmospheric Administration (NOAA) as previously described *(25)*.

RESULTS AND DISCUSSION

The orchard selected for sampling in the present study produced samples that were positive for *Salmonella* Enteritidis PT 30 in separate studies conducted by both the California Department of Health Services (CDHS) and the University of California at Davis in July and August 2001, respectively *(10)*. In 2002, this orchard (12 pooled swab samples) and three surrounding orchards (8 pooled swab samples per orchard) were swabbed in the first, second, and fourth weeks of August 2002 (Table 1). The selected orchard was the only one for which a positive sample was identified (weeks 2 and 4). From September 2002 through October 2006 this single 61-ha orchard was swabbed multiple times during the harvest months (August, September, and October) and occasionally during the rest of the year.

The selected orchard had alternating rows of Carmel and Monterey variety almond trees that were 12 years old

TABLE 2. *Monthly isolation of* Salmonella *Enteritidis PT 30 from a naturally contaminated almond orchard, 2002 through 2006*

	No. of samples positive for *Salmonella*[a]												
Year	Jan	Feb	Mar	Apr[b]	May	June	July	Aug	Sep	Oct	Nov	Dec	Total
2002								3/12	4/16	4/8[c]	2/8		13/44
2003	0/8		0/8		0/8				0/16	6/16	2/8	2/8	10/72
2004		0/8			2/8	1/8	0/8	1/8	9/32	12/24[c]			25/96
2005										2/8			2/8
2006										3/8			3/8
Total	0/8	0/8	0/8		2/16	1/8	0/8	4/20	13/64	27/64	4/16	2/8	53/228

[a] Data are the number of samples positive for *Salmonella* Enteritidis PT 30/total number of samples obtained.
[b] No orchard swabbing was conducted in April.
[c] Sampling month in which at least 50% of the samples were positive.

in 2001. The Carmel almonds were routinely harvested in September, and the Monterey almonds were harvested in October. Almonds were harvested from surrounding orchards as early as August, and all harvest activity on the farm was completed by the end of October.

The normal average rainfall recorded by NOAA in this region during August, September, and October is 1.2, 9.91, and 12.70 mm, respectively. Because almonds are harvested from the ground, dry conditions are favorable. As harvest time approaches, irrigation of the almond trees is reduced or stopped, which contributes to dry conditions on the soil surface, especially in the drive rows between the trees. The Cerini clay loam soil in the selected orchard made it impossible to harvest product if rainfall was sufficient to thoroughly wet the ground.

Typical almond tree density in the San Joaquin Valley is 225 trees per hectare but can be as few as 135 trees per hectare in fertile loam soils *(9)*. Surface (flood or furrow), sprinkler, and microdrip or microsprinkler irrigation methods may be used *(16)*. For all but two of the outbreak-associated orchards, the tree density was 375 trees per hectare (109 tree rows east to west and 208 tree rows north to south in the 61-ha orchard), and microsprinkling was used for irrigation. These management techniques resulted in a well-developed canopy that effectively shaded the orchard in the summer and early fall. Consistently wet soil covered with moss was observed at the tree line, and very dry soil and no vegetation were observed in the rows between the trees.

A total of 23% of all pooled swab samples (53 of 228 samples) collected from 2002 through 2006 were positive for *Salmonella* (Table 2). The technique used to enrich cultures for *Salmonella* included a delayed secondary enrichment that was used when the initial enrichment culture was negative. Of the 53 samples positive for *Salmonella,* 20 (38%) were identified only after the secondary enrichment. For several sample dates, *Salmonella* was detected only after the secondary enrichment (Table 1). Isolation after the secondary enrichment procedure suggests that extra time was needed to adequately resuscitate the organism.

The frequency of *Salmonella* isolation from the orchard at any given sampling date differed (Table 2). No *Salmonella* was recovered on single sample dates in January, February, or March. During the months of May, June,

and July, *Salmonella* was occasionally isolated (3 of 32 pooled samples). Isolation of *Salmonella* was more frequent in August (20% of samples positive for *Salmonella*), September (20% positive), October (42% positive), November (25% positive), and December (25% positive).

Isolation of *Salmonella* from pooled swabs during the harvest months of August, September, and October are shown by week in Table 1. Although overall isolation rates were higher during this time, the number of positive samples on any test date varied from week to week and year to year. The frequency of isolation was particularly low in 2003 (only one of four test dates yielded a positive sample) when compared with 2002 and 2004. Negative samples may reflect the absence of viable cells of the target organism but also may be due to the inadequacy of the isolation procedure. Development and/or improvement of techniques used to isolate human pathogens from the environment are very challenging because reliably contaminated samples are difficult to find and laboratory-grown cultures are unlikely to mimic the state of environmental isolates. Isolation methods for *Salmonella* in environmental samples were not compared in this study. Therefore, some samples containing viable *Salmonella* may have been scored as negative because our enrichment method was unable to recover these cells.

In 2003, an additional experiment was conducted that involved all of the orchards of interest in the original 2001 outbreak investigation. At the end of June and in early July 2001, the CDHS used the drag swab method to identify orchards that harbored *Salmonella (3)*. A total of 17 61-hectare almond orchards were swabbed by dragging two swabs from opposite corners of each orchard for a distance of approximately 15 m diagonally into the field from the corner. Drag swabs (four) from each orchard were combined into a single pooled sample and submitted for enrichment, and five of these pooled samples were positive for *Salmonella* Enteritidis PT 30, including the orchard of focus in the current study. The 17 orchards initially swabbed in 2001 were retested at the end of June and in early July 2003 using the protocol employed by the CDHS. None of the orchards were positive for *Salmonella* at that time. It is not known whether *Salmonella* would have been isolated using these methods at this time of year in other years of the study.
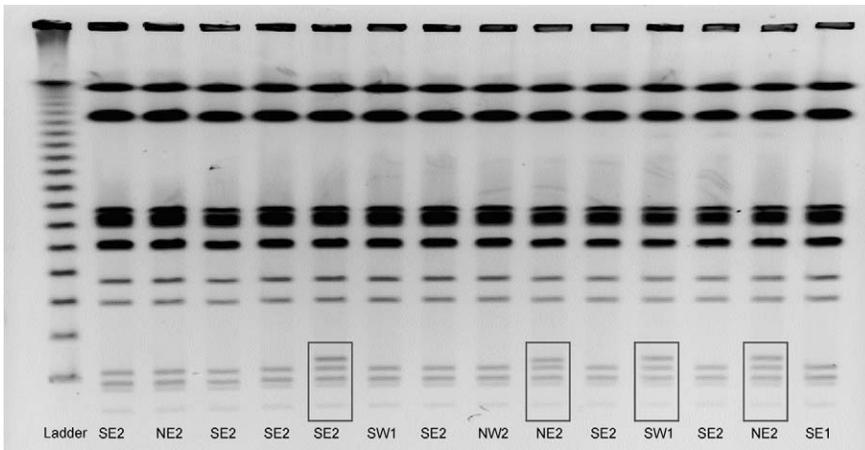
FIGURE 1. *Comparison of* Salmonella *Enteritidis PT 30* Xba*I-digested PFGE patterns from isolates obtained from various almond orchard quadrants.*

The increase in the number of *Salmonella*-positive almond orchard drag swabs during the harvest months coincided with an increase in harvesting equipment activity within the orchard. The shaking of trees, formation of windrows, and sweeping of almonds for transport out of the orchard involve considerable equipment activity, which disturbs and redistributes the top layer of soil from the drive row between the trees. This soil redistribution likely facilitated more uniform sampling. A greater adherence of soil to the swabs was observed during sampling in these months. The increase in *Salmonella* recovery from these swabs, therefore, may have been due simply to increased amounts of soil trapped by the swabs when dragged along dry versus wet ground.

However, the highest percentage of positive swabs (seven of eight) coincided with a rain event on 19 October 2004 while Monterey almonds were in windrows on the orchard floor. At this time, swabs picked up little visible soil or dust as they were dragged along the orchard floor and were similar in appearance to swabs collected at other times of year when the orchard was wet. A total of 20.8 mm of rain was recorded by the CIMIS for that date. Swabs were collected in the middle of the storm after water had pooled on the orchard surface, and areas sampled included those surrounding the windrowed almonds. High and average air temperatures were 15 and 13°C, respectively, on

that date, as recorded by the CIMIS at station 124 Panoche. Growth of *Salmonella* in the presence of wet almond hulls and shells has been documented at temperatures of 15°C (lower temperatures were not evaluated) *(25)*. The higher number of *Salmonella*-positive samples obtained on this October date may have been a consequence of *Salmonella* growth in the piles of wet almonds.

No attempt was made to correlate the isolation of *Salmonella* from the almond orchard to contamination of almond kernels. However, because soil is mixed with the almonds as they are harvested, some degree of contamination probably occurs. Almonds harvested from this area were all processed using methods that have been validated to reduce *Salmonella.* When Kokal and Thorpe *(12)* examined the incidence of *Escherichia coli* on soft-shelled almonds at various stages of production and harvest, they found an increase in incidence of this indicator organism as the almonds were harvested. Only 1% of the almonds on the tree were positive for *E. coli.* The proportion of almonds positive for *E. coli* gradually increased with each successive harvest step (shaking trees to detach nuts, sweeping, picking up, storage, weighing, and precleaning), up to 40% before hulling *(12)*.

All 53 *Salmonella* isolates collected from the selected orchard (2002 through 2006) were typed as *Salmonella* Enteritidis PT 30. No other *Salmonella* serovar was isolated. All isolates were subjected to PFGE with the *Xba*I restriction enzyme (Fig. 1). A subtle variation existed in the low-molecular-weight region (40 to 50 kb) of the *Xba*I-digested DNA separated by PFGE. An extra band at about 40 kb was observed in approximately 40% of the isolates and was found equally among isolates from each orchard quadrant. One *Salmonella* Enteritidis PT 30 isolate had a band at approximately 330 kb (not shown), indicating the possible presence of a plasmid. All orchard isolates were identical by their *Bln*I PFGE profiles (data not shown).

Isolates of *Salmonella* Enteritidis PT 30 were also compared by PFGE to human isolates of *Salmonella* Enteritidis PT 30 from the 2000 and 2001 outbreak and to strains of *Salmonella* Enteritidis PTs 4, 6a, 8, and 9c (Fig. 2; data not shown for PT 4 and PT 8). *Salmonella* Enteritidis PT 9c was associated with an outbreak linked to consumption of raw almonds that was reported in 2004 *(4)*.
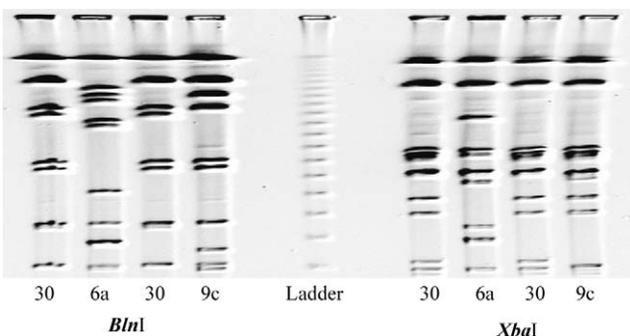

FIGURE 2. *Comparison of* Salmonella *Enteritidis strain PFGE patterns obtained with* Bln*I and* Xba*I: PT 30 (human isolate, almond-associated outbreak, 2001), PTs 6a and 30 (almond orchard, 2001), and PT 9c (human isolate, almond-associated outbreak, 2004).*

Only 2 (10%) of 20 human isolates of *Salmonella* Enteritidis PT 30 had the 40-kb fragment noted in >30% of the field isolates. PFGE patterns from *Salmonella* Enteritidis PT 30 and *Salmonella* Enteritidis PT 9c look similar following digestion with *Xba*I; however, upon digestion with *Bln*I, the PFGE profiles from almond and human isolates of *Salmonella* Enteritidis PT 30 were different from those of PTs 4, 6a, 8, and 9c. Studies are ongoing to determine the molecular basis for observed minor differences in PFGE patterns between the PT 30 and PT 9c strains.

In most studies where *Salmonella* has been isolated from the environment *(7, 20, 26)*, multiple serovars have been recovered, and these serovars are different at different times. Thirty-five different serovars were isolated from raw almonds during a 5-year survey of *Salmonella (5)*. These isolates were presumably acquired from the almond production environment.

In several studies where single *Salmonella* serovars have persisted in animal production environments for extended periods of time, e.g., pig farms *(1)*, pig production areas *(19)*, chicken farms *(15)*, calf units *(13)*, and calf-rearing areas *(24)*, the same serovars isolated from environmental samples were isolated from clinically diseased and asymptomatic farm animals. Thompson et al. *(23)* found *Salmonella* Bareilly isolates with a consistent phage pattern in soil samples taken from the same environment 4 years apart. These authors attributed this persistence to an infected rabbit colony, because the same strain was identified in on-site rabbit feces. *Salmonella* Enteritidis PT 4 persisted in a chicken farm environment for at least 26 months after depopulation and was isolated from a variety of wild animals, insects, litter, dried feces, and feed *(6)*. In the current study, no attempt was made to determine whether an animal source contributed to the persistence of *Salmonella* Enteritidis PT 30 in the orchard. During the original traceback investigation, however, fecal samples taken from numerous birds and rodents were negative for *Salmonella (3)*.

Salmonella Enteritidis PT 30 was isolated in this study from a single almond orchard during a 5-year period. If the outbreak almonds are included as evidence of contamination in 2000 (the harvest year) and isolation of *Salmonella* from this orchard in 2001 by our laboratory and the CDHS is counted *(10)*, then persistence could be considered to be at least 7 years. Environmental survival of *Salmonella* has been established *(14)*. Environmental survival may contribute to transmission and infection of a wide range of animal hosts and, thus, cyclic contamination of the environment *(27)*. The ability of stable dividing populations of *Salmonella* to exist in nonhost environments has been discussed for many years *(8, 14, 27)*. Therefore, the ability of *Salmonella* to multiply in wet almond hulls *(25)* should not be overlooked as a possible additional route of environmental persistence and contamination of almonds. In addition to rainfall, hulls on the ground can become wet from irrigation water when almonds fall prematurely or are missed during harvest.

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