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Molecular Epidemiology of Two International Sprout-Borne *Salmonella* Outbreaks

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Sprout-borne *Salmonella* outbreaks in Finland have increased during the last 10 years. The latest two were caused by *Salmonella enterica* serovar Bovismorbificans (antigenic structure 6,8:r:1,5) in 1994 and *S. enterica* serovar Stanley (4,5, 12:d:1,2) in 1995. In this study, the restriction fragment length polymorphism of genomic DNA after pulsed-field gel electrophoresis (PFGE) and antimicrobial resistance profiles of the outbreak and nonoutbreak strains were compared. In each separate outbreak, the PFGE patterns of the outbreak strains (40 strains of *S. enterica* serovar Bovismorbificans and 28 strains of *S. enterica* serovar Stanley) after digestion of genomic DNA with restriction enzyme *Xba*I were indistinguishable from each other but differed clearly from those of the nonoutbreak strains (26 strains of *S. enterica* serovar Bovismorbificans and 40 strains of *S. enterica* serovar Stanley). The restriction enzyme *Xho*I did not differentiate the outbreak and nonoutbreak strains. The *S. enterica* serovar Stanley strains associated with the outbreak also had a unique antimicrobial resistance pattern, whereas all *S. enterica* serovar Bovismorbificans strains, both outbreak and nonoutbreak strains, were sensitive to all antimicrobial agents tested. Thus, the molecular typing confirmed that the *S. enterica* serovar Bovismorbificans outbreak isolates from humans and sprout salad were identical and strongly supported the epidemiological finding that *S. enterica* serovar Stanley outbreak isolates also originated from contaminated alfalfa seeds. It also confirmed that the sources of similar outbreaks in Sweden in 1994 caused by *S. enterica* serovar Bovismorbificans and in the United States in 1995 caused by *S. enterica* serovar Stanley and the source of the Finnish outbreaks were common.

Sprouts have become an important cause of outbreaks of food-borne salmonellosis (4–6, 8, 9). The most recent sprout-borne outbreak caused by alfalfa seeds contaminated with *Salmonella enterica* subsp. *enterica* serovar Newport (abbreviated here as *S. enterica* serovar Newport occurred in the winter of 1995 and 1996 in Oregon and British Columbia (2).

In Finland, eight sprout-borne *Salmonella* outbreaks have occurred since 1980 (the statistics of the Laboratory of Enteric Pathogens [LEP], National Public Health Institute, Helsinki, Finland). In the 1990s, both the outbreak frequency and the number of people infected have been increasing. Thus, in four sprout-borne outbreaks during the past 5 years 100 to 300 cases of salmonellosis have been identified in each outbreak. In contrast, during the whole previous decade there were only four similar outbreaks in Finland, with 30 to 50 cases identified in each outbreak.

The last two sprout-borne *Salmonella* outbreaks took place in 1994 and 1995 in Finland. *S. enterica* serovar Bovismorbificans was identified in 210 subjects (6, 9) and *S. enterica* serovar Stanley was identified in 114 subjects (4, 5). These serovars are normally very rarely detected in Finland: less than 10 cases a year, mainly in subjects who have recently returned from abroad. Furthermore, there are no known reservoirs for these serovars in Finnish production animals. The finding of these serotypes has commonly been associated with travel abroad, mostly to Mediterranean countries (*S. enterica* serovar Bovismorbificans) and to Thailand (*S. enterica* serovar Stanley).

It is often difficult to evaluate only by serotyping which strain is actually associated or which strain is not associated with the outbreak. This is especially the case if an isolate is from a subject with a recent history of travel. Simultaneous outbreaks may also occur in other countries. Along with these two outbreaks in Finland there was an outbreak caused by *S. enterica* serovar Bovismorbificans in Sweden with 282 infected subjects (6, 9) and an outbreak caused by *S. enterica* serovar Stanley in Arizona and Michigan in the United States with 128 infected subjects (5).

In sprout-borne *Salmonella* outbreaks the suspected vehicle of the bacteria has mostly been traced by epidemiological means only; the culturing of salmonella from sprouts or seeds under suspicion has commonly failed (4–6, 8, 9). In the eight sprout-borne outbreaks that occurred in Finland after the 1980s, culturing of the organism from samples of the implicated seeds and/or sprouts has succeeded only twice (statistics of LEP). This was also the case in the latest two outbreaks described here. The culturing of *S. enterica* serovar Bovismorbificans from the implicated alfalfa sprouts succeeded (9), but that of *S. enterica* serovar Stanley failed and the culturing of the organism from seeds failed in both cases (1, 4). In the outbreak caused by *S. enterica* serovar Stanley the fact that the outbreak ended only after the industrial sprouting of alfalfa seeds was prohibited gave evidence that the seeds were the source.

We examined the restriction fragment length polymorphism of DNA after pulsed-field gel electrophoresis (PFGE) of outbreak and nonoutbreak *S. enterica* serovar Bovismorbificans and *S. enterica* serovar Stanley strains in order to evaluate the molecular epidemiology and potential genetic variety of these *Salmonella* strains. The antimicrobial susceptibilities of the strains were also determined. The results obtained from studies with the Finnish outbreak strains were compared with those obtained with Swedish and U.S. outbreak isolates.

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TABLE 1. *S. enterica* serovar Bovismorbificans and Stanley strains examined by PFGE and antimicrobial susceptibility testing

Salmonella serovar and period	Country of origin	Yr of isolation	No. of strains examined
S. enterica serovar Bovismorbificans (n = 66)			
Outbreak (n = 40)	Finland	1994	39[a]
	Sweden	1994	1
Nonoutbreak (n = 26)	Finland	1994	1
	Finland	1993	3
S. enterica serovar Stanley (n = 68)			
Outbreak (n = 28)	Finland	1995	24
	United States	1995	4[d]
Nonoutbreak (n = 40)	Finland	1995	3
	Finland	1994	2
	Finland	1993	2
	Finland	1992	1
	Thailand	1995	5
	Thailand	1994	3
	Thailand	1993	5
	Thailand	1992	2
	Other countries	1995	13[e]
	Indonesia	1994	1
	Other countries	1993	2[f]
	Indonesia	1992	1

[a] One isolate from alfalfa sprouts and one from sprout salad containing alfalfa sprouts.
[b] Strains were from Greece (three strains); Portugal, Spain, and the United States (Two strains from each country); and Bahrain, Belgium, Czech Republic, England, Germany, Malcedonia, Malaysia, Poland, Thailand, and Tunisia (one strain from each country).
[c] Strains were from Greece, Hungary, and India.
[d] Data are from reference 5.
[e] Strains were from Greece, Pakistan, Russia, and Spain (two strains from each country) and from the Dominican Republic, Israel, Japan, the United Arab Emirates, and the United States (one strain from each country).
[f] One strain each from India and Bangladesh.

MATERIALS AND METHODS

Definitions. A *Salmonella* outbreak strain was defined as a strain belonging to a certain serotype of *Salmonella* which was isolated from a subject with no history of travel at the time of the outbreak. A nonoutbreak strain was defined as a strain belonging to the same serotype as the outbreak strain but which was isolated from a subject outside the outbreak period or from subjects who had recently returned from a trip abroad.

***Salmonella* strains.** The Finnish clinical isolates (Table 1; mainly from stool samples) were isolated in microbiological laboratories in various parts of Finland and were submitted to LEP, National Public Health Institute, for serotyping, which was subsequently carried out by standard techniques (3, 7, 10). The strains were stored at −70°C in sterile skim milk until they were further analyzed by PFGE and by antimicrobial susceptibility testing.

***S. enterica* serovar Bovismorbificans.** Sixty-six isolates were examined in all. Of these, 37 (32 representing the peak period of the outbreak from March to June 1994 and 5 representing the period of the outbreak of August and September 1994) of the total 210 isolates associated with the Finnish outbreak (9) and 26 isolates representing a nonoutbreak period were analyzed by PFGE after the digestion of chromosomal DNA with the *Xba*I restriction enzyme. Thirty of these strains, including outbreak and nonoutbreak strains, were also analyzed after digestion with the *Xho*I restriction enzyme. In addition, one *S. enterica* serovar Bovismorbificans isolate from onion-alfalfa salad from the Swedish outbreak was obtained (kindly sent by Gunilla Lindberg and Yvonne Andersson from the Swedish Institute for Infectious Disease Control, Stockholm, Sweden), and two isolates were obtained from the Finnish National Veterinary and Food Research Institute, Helsinki, Finland (kindly sent by Raili Schildt); one of these latter two isolates was from salad containing alfalfa sprouts (isolated by Maija Hatakka of the Food and Environment Laboratory of the City of Vantaa, Vantaa, Finland, from a sample taken on 28 March 1994), and the other was from alfalfa sprouts (isolated by Anna Pitkälä, from the Environment Laboratory of the City of Helsinki, Helsinki, Finland, from a sample taken on 7 April 1994).

***S. enterica* serovar Stanley.** Sixty-eight isolates were examined in all. Of these, 24 (18 representing the peak period of the outbreak from March to June 1994 and 6 representing the period of the outbreak from July to October 1994) of the total 114 isolates associated with the Finnish outbreak (5) and 40 isolates representing a nonoutbreak period were analyzed by PFGE after the digestion of chromosomal DNA with the *Xba*I restriction enzyme (Table 1). Five of these strains, including outbreak and nonoutbreak strains, were also analyzed after digestion with the *Xho*I restriction enzyme. In addition, four *S. enterica* serovar Stanley isolates were from the U.S. outbreak (kindly sent by Joy G. Wells from National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Ga.).

PFGE. Preparation of cells and fragmentation of their genomic DNA by digestion with *Xho*I (Boehringer Mannheim, Mannheim, Germany) or *Xba*I (Boehringer Mannheim) were performed as described by Goering and Duensing (1a), with the following modifications. Cells from 10 ml of a logarithmic-phase culture and RNase (0.5 mg/ml) were mixed with low-melting-temperature agarose (SeA KEM ME; FMC BioProducts, Rockland, Maine), and cells were made into protoplasts with lysozyme. The agarose plugs were incubated in the presence of proteinase K (Boehringer Mannheim) at 60°C for 24 h and washed with TE buffer (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA) for 5 to 6 h, with the buffer exchanged every 30 min. The DNA fragments obtained by digestion were electrophoresed through 1% agarose in a pulsed field by using a Gene Navigator apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden) at 200 V for 20 h with a ramped pulse time of 20 to 80 s. A bacteriophage lambda ladder PFGE marker (New England BioLabs, Beverly, Mass.) was used as a standard for molecular size determinations. Strains with PFGE banding patterns differing from each other by no more than one band were considered to belong to the outbreak strains if the time of isolation also matched the time of the outbreak.

Antimicrobial susceptibility. The antimicrobial susceptibilities of all 134 strains were studied by the disk diffusion technique on a semisynthetic Iso-Sensitest medium by using the zone size criteria standardized for members of the family *Enterobacteriaceae* recommended by the disk manufacturer and based on breakpoints established by the Swedish Reference Group for Antibiotics (11). A panel of the following antimicrobial agents (AS Rosco, Taastrup, Denmark) was used: ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, imipenem, mecillinam, nalidixic acid, neomycin, sulfonamide, tetracycline, and trimethoprim.

RESULTS

***S. enterica* serovar Bovismorbificans.** In the preliminary study, the genomic DNAs of the 30 *S. enterica* serovar Bovismorbificans strains from different sources were tested with the *Xho*I and *Xba*I restriction enzymes. Since *Xho*I gave very little or no variation in the PFGE banding patterns of the strains (data not shown) in contrast to the PFGE patterns obtained with *Xba*I, *Xba*I was chosen for the further assays. The PFGE banding patterns of all 37 outbreak isolates from Finns were identical, with eight distinct fragments in the separation area (the uppermost bands and bands smaller than 49 kb are not included) (Fig. 1, lanes 1 to 4). The *S. enterica* serovar Bovismorbificans strain cultured from salad containing alfalfa

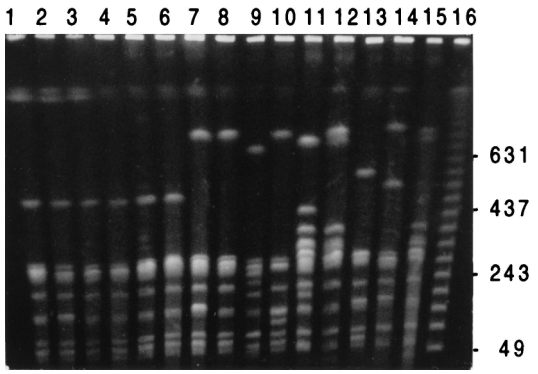

FIG. 1. PFGE banding patterns of genomic DNA of *S. enterica* serovar Bovismorbificans strains after digestion with the *Xba*I restriction enzyme. Lanes 1 to 7, outbreak strains from Finland (lanes 1 to 4), Sweden (lane 5), sprout salad (lane 6), and sprouts (lane 7); lanes 8 to 15, nonoutbreak strains, from Greece (1993), Finland (1994), Bahrain (1994), Thailand (1994), Sweden (1993), Finland (1993), India (1993), and Sweden (1993), respectively; lane 16, molecular size markers (in kilobases).

TABLE 2. Antimicrobial susceptibilities of *S. enterica* serovar Bovismorbificans and Stanley strains

Salmonella serovar	Yr of isolation	No. of strains	Susceptibility[a]										
			Amp	Cef	ChI	Cip	Imi	Mec	Nal	Neo	Sul	Tet	Tri
S. enterica serovar Bovismorbificans	1994	60[b]	S	S	S	S	S	S	S	S	S	S	S
	1993	6[c]	S	S	S	S	S	S	S	S	S	S	S
S. enterica serovar Stanley	1995	40[d]	S	S	S	S	S	S	S	R	R	R	R
	1985	6[e]	S	S	S	S	S	S	S	S	S	S	S
	1995	3[f]	S	S	S	S	S	S	S	S	R	R	S
	1994	4[g]	S	S	S	S	S	S	S	S	S	S	S
	1994	1[h]	S	S	S	S	S	S	S	S	R	R	S
	1993	9[j]	S	S	S	S	S	S	S	S	S	S	S
	1992	1[k]	R	S	R	S	S	S	S	R	R	R	R
	1992	3[l]	S	S	S	S	S	S	S	S	S	S	S

[a] S, sensitive; R, resistant. The following antibiotics were used in the susceptibility test: ampicillin (Amp), ceftriaxone (Cef), chloramphenicol (Chl), ciprofloxacin (Cip), imipenem (Imi), mecillinam (Mec), nalidixic acid (Nal), neomycin (Neo), sulfonamide (Sul), tetracycline (Tet), and trimethoprim (Tri).
[b] Forty outbreak strains (including a Swedish isolate and 2 isolates from sprouts and sprout salad) and 20 nonoutbreak strains from 16 countries (see Table 1).
[c] Three Finnish and three foreign (Greece, Hungary, and India) nonoutbreak strains.
[d] Twenty-four Finnish and 4 U.S. outbreak strains and 12 strains from eight countries: Finland, Greece, Russia, and Spain (2 strains from each country) and the Dominican Republic, Israel, Pakistan, and the United Arab Emirates (one strain from each country).
[e] Four Thai, one U.S., and one Finnish nonoutbreak strains.
[f] One Japanese, one Pakistani, and one Thai nonoutbreak strains.
[g] Two Finnish and two Thai nonoutbreak strains.
[h] One Thai nonoutbreak strain.
[i] One Indonesian nonoutbreak strain.
[j] Five Thai, two Finnish, and two Indian nonoutbreak strains.
[k] One Finnish nonoutbreak strain.
[l] Two Tahi and one Indonesian nonoutbreak strains.

sprouts had a PFGE pattern which was indistinguishable from the patterns of the outbreak strains (Fig. 1; compare lane 6 to lanes 1 to 4). The pattern for the *S. enterica* serovar Bovismorbificans strain isolated directly from the alfalfa sprouts differed, however, from the patterns of the outbreak strains. Instead of a band situated at approximately 437 kb, a band at approximately 631 kb was seen (Fig. 1; compare lane 7 to lanes 1 to 4). The strain obtained from the Swedish outbreak (isolated from onion-alfalfa salad) also had a PFGE pattern identical to those of the Finnish outbreak strains (Fig. 1; compare lane 5 to lanes 1 to 4). One isolate from Greece (isolated in 1993) had a banding pattern identical to that of the strain isolated from alfalfa sprouts from the outbreak period (Fig. 1; compare lane 8 to lane 7), and another nonoutbreak stain isolated in Finland in 1993 differed by only one band from the patterns of the outbreak strains (Fig. 1; compare lane 13 to lanes 1 to 4). In addition, seven strains which by definition were nonoutbreak strains, since although they were isolated during the outbreak, the subjects had recently returned from a trip abroad, also had *Xba*I banding patterns indistinguishable from those of the outbreak strains (data not shown). In contrast, the other 17 nonoutbreak strains had variable PFGE patterns, with 9 to 10 fragments that were clearly distinguishable from the fragments in the patterns of the outbreak strains (Fig. 1, lanes 9 to 12 and lanes 14 and 15, respectively). The banding patterns of these 17 nonoutbreak strains differed from those of the outbreak strains by more than two bands.

All 66 strains were sensitive to all antimicrobial agents tested (Table 2).

***S. enterica* serovar Stanley.** *Xho*I restriction enzyme, which was used in the preliminary experiments, gave identical banding patterns for all five *S. enterica* serovar Stanley strains tested, outbreak and nonoutbreak strains alike (data not shown). On the contrary, *Xba*I digestion led to clearly different kinds of banding patterns for the isolates from different sources (Fig. 2). The PFGE banding patterns of all 28 outbreak strains (24 from Finns and 4 from Americans) were identical, with 12 distinct fragments (Fig. 2, lanes 6 to 10). Ten of the nonoutbreak strains from subjects who had recently returned from a trip broad had *Xba*I banding patterns indistinguishable from those of the outbreak strains (data not shown). Thirty of the nonoutbreak strains showed banding patterns clearly different from those of the outbreak strains (Fig. 2; compare lanes 1 to 5 and 11 to 14 to lanes 6 to 10). The number of fragments in the nonoutbreak strains varied from 11 to 14, and the banding patterns differed from the patterns of the outbreak strains and from each other usually by more than three bands.

Thirty-eight strains (24 Finnish and 4 American outbreak strains) and the 10 nonoutbreak strains mentioned above) which had identical banding patterns by PFGE were also indistinguishable by their antibiogram patterns (Table 2). They

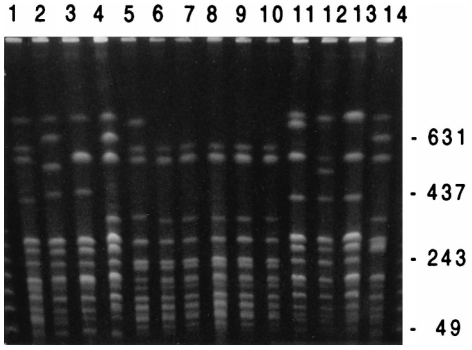

FIG. 2. PFGE banding patterns of genomic DNA of *S. enterica* serovar Stanley strains after digestion with the *Xba*I restriction enzyme. Lanes 6 to 10, outbreak strains from the beginning (lanes 6 and 7), middle (lanes 8 and 9), and end (lane 10) of the outbreak; lanes 1 to 5, Finnish nonoutbreak strains (1992 [lane 1], 1993 [lane 2], 1994 [lane 3], and 1995 [lanes 4 and 5]); lanes 11 to 14, nonoutbreak strains from Thailand (lanes 11 [1995], 12 [1994], and 14 [1992] and Bangladesh (lane 13 [1993]). Numbers on the right side are molecular size markers (in kilobases).

were all resistant to neomycin, sulfonamide, tetracyclines, and trimethoprim but were sensitive to ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, imipenem, mecillinam, and nalidixic acid. Two Finnish strains which were isolated in September and October 1995 had identical antibiograms, but their PFGE patterns different from those of the outbreak strains. Among the remaining 26 nonoutbreak isolates, four different antibiogram patterns were seen, and these also differed from the antibiogram patterns of the outbreak strains.

DISCUSSION

The PFGE experiments confirmed the epidemiological finding that the outbreak strains of *S. enterica* serovar Bovismorbificans obtained in 1994 originated from a common source, as did the outbreak strains of *S. enterica* serovar Stanley obtained in 1995. Both the *S. enterica* serovar Bovismorbificans and *S. enterica* serovar Stanley strains that were associated with the outbreak had indistinguishable PFGE patterns after *Xba*I digestion of their genomic DNAs. In addition, the *S. enterica* serovar Stanley outbreak strains with identical PFGE patterns also had identical antimicrobial resistance patterns. The sensitivity test did not discriminate among the *S. enterica* serovar Bovismorbificans strains at all: All strains, whether they were outbreak or nonoutbreak strains, were sensitive to the antimicrobial agents tested. The sprouting of contaminated seeds was prohibited in April 1994 and May 1995, and at the same time the sprouting plants were disinfected. However, some isolates with the outbreak PFGE and antibiogram patterns emerged as late as September in the case of *S. enterica* serovar Bovismorbificans and October in the case of *S. enterica* serovar Stanley.

The banding patterns of the nonoutbreak strains, whether they were known for certainty to be of foreign origin or not, usually differed by more than three bands from the banding patterns of the outbreak strains. In total, 22 different patterns could be detected among 30 *S. enterica* serovar Stanley nonoutbreak strains, whereas 11 different patterns could be detected among 18 *S. enterica* serovar Bovismorbificans nonoutbreak strains. This suggests that the *S. enterica* serovar Stanley strains are genetically more variable than the *S. enterica* serovar Bovismorbificans strains when the *Xba*I restriction enzyme is used for the digestion of genomic DNA.

The *S. enterica* serovar Bovismorbificans strain isolated from the salad containing alfalfa sprouts had a PFGE banding pattern indistinguishable from that of the outbreak strain. Interestingly, the PFGE banding pattern of the *S. enterica* serovar Bovismorbificans strain isolated directly from alfalfa sprouts about a week later differed by one band from the PFGE banding patterns of the outbreak strains isolated from patients and from salad. However, it is most obvious that the strain belonged to the outbreak strains, although no strains with identical PFGE banding patterns were found among the infected subjects. This finding is in agreement with the guidelines suggested by Tenover et al. (12), stating that differences of several bands in PFGE banding patterns are needed to consider an isolate unrelated to the outbreak strains. Thus, we believe that our results confirm the previous epidemiological finding that alfalfa sprouts were the source of the outbreak.

In the outbreak caused by *S. enterica* serovar Bovismorbificans, the alfalfa seeds were imported from Australia via Sweden to Finland (6, 9). Previously, however, no *S. enterica* serovar Bovismorbificans isolates from subjects after a trip to Australia were detected in Finland. In the outbreak caused by *S. enterica* serovar Stanley, the implicated alfalfa seeds were imported via a Dutch shipper who was reported to have mixed the seeds from lots imported from Italy, Hungary, and/or Pakistan (5). Two *S. enterica* serovar Stanley strains were isolated from two Finnish travelers after their trip to Pakistan at the same time when the outbreak occurred in 1995. One of the strains had an antibiogram identical to those of the outbreak strains, but the antibiogram of the other strain was different, since it was resistant only to sulfonamide and tetracyclines. These two strains were, however, not genotypically identical to the outbreak strains. In contrast, the Finnish *S. enterica* serovar Bovismorbificans and *S. enterica* serovar Stanley isolates from the outbreaks were genotypically identical to corresponding Swedish and U.S. isolates. Also, the antibiograms of these isolates were indistinguishable. This confirms the finding also shown by epidemiological means that the same contaminated lot of seeds was indeed the source of these infections in these countries.

An interesting finding in this study was that the PFGE and antimicrobial resistance patterns of the 7 *S. enterica* serovar Bovismorbificans and 10 *S. enterica* serovar Stanley strains which were isolated from patients who became ill after a recent trip abroad (to Belgium, Greece, Portugal, Spain, and the United Kingdom in the *S. enterica* serovar Bovismorbificans outbreak and to the Dominican Republic, Greece, Israel, Pakistan, Russia, Spain, and the United Arab Emirates in the *S. enterica* serovar Stanley outbreak) were indistinguishable from the outbreak strains. This finding suggests that these patients got their infection from contaminated sprouts in Finland instead of from some other source in a foreign country. Indeed, when asked, most of the patients remembered having eaten sprouts in Finland after their trips.

The findings of this study indicate that PFGE after *Xba*I digestion of genomic DNA is a powerful tool for tracing the origin of *Salmonella* contamination and revealing which *Salmonella* infections are caused by outbreak strains and which ones are not. Nowadays, people are increasingly taking holidays in foreign countries and various food items are crossing national borders, and *Salmonella* outbreaks are also very likely to do likewise. These two international outbreaks clearly point out the need for an applicable molecular typing method such as PFGE, which was used successfully in this study. Furthermore, conventional antimicrobial susceptibility testing may also give valuable additional information about the source of *Salmonella* infections, as it did in this study, especially in the outbreak caused by *S. enterica* serovar Stanley.

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