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Microbiological Aspects of the Investigation That Traced the 1998 Outbreak of Listeriosis in the United States to Contaminated Hot Dogs and Establishment of Molecular Subtyping-Based Surveillance for *Listeria monocytogenes* in the PulseNet Network

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A multistate outbreak of listeriosis occurred in the United States in 1998 with illness onset dates between August and December. The outbreak caused illness in 108 persons residing in 24 states and caused 14 deaths and four miscarriages or stillbirths. This outbreak was detected by public health officials in Tennessee and New York who observed significant increases over expected listeriosis cases in their states. Subsequently, the Centers for Disease Control and Prevention (CDC) began laboratory characterization of clinical isolates of *Listeria monocytogenes* by serotyping and restriction fragment length polymorphism analysis using pulsed-field gel electrophoresis (PFGE). For the purpose of this investigation, outbreak-related isolates were defined as those that had a specific AscI-PFGE pattern and indistinguishable or highly similar (no more than 2 band difference in 26 bands) ApaI-PFGE patterns when their DNA was restricted by AscI and ApaI restriction enzymes. Timely availability of molecular subtyping results enabled epidemiologists to separate outbreak cases from temporally associated sporadic cases in the same geographic areas and facilitated the identification of contaminated hot dogs manufactured at a single commercial facility as the source of the outbreak. During the investigation of this outbreak, a standardized protocol for subtyping *L. monocytogenes* by PFGE was developed and disseminated to public health laboratories participating with CDC's PulseNet network; these laboratories were requested to begin routine PFGE subtyping of *L. monocytogenes*.

Listeria monocytogenes is a foodborne bacterial pathogen that has been estimated to cause severe disease in some 2,500 persons in the United States each year, with a 20% to 25% mortality rate (19). Most *L. monocytogenes* infections are thought to be sporadic (22, 28); however, more than 30 large foodborne outbreaks of listeriosis have occurred in North America and Europe since 1981 (32).

Of the six species in the genus *Listeria*, only *L. monocytogenes* is almost exclusively associated with human disease. *L. monocytogenes* is commonly found in soil, in water, and on plant material and is ubiquitously distributed in the environment (25). Its high salt tolerance, ability to grow at refrigeration temperatures, and propensity to be associated with biofilms (36) allow it to thrive in food-processing environments where such conditions exist.

Pregnant women, neonates, and elderly or immunocompromised adults are uniquely susceptible to listeriosis, which typically presents as septicemia, meningitis, or meningoencephalitis (12, 29, 31). In pregnant women, *L. monocytogenes* takes advantage of the natural localized immunosuppression at the maternal-fetal interface and causes abortions and stillbirths. A milder form of listeriosis that presents as febrile gastroenteritis was recognized in the 1990s (10, 26); this disease state is induced when otherwise healthy hosts consume large numbers of *L. monocytogenes* bacteria.

In November 1998, public health officials in four U.S. states (Tennessee, New York, Connecticut, and Ohio) alerted the Centers for Disease Control and Prevention (CDC) about significant increases in listeriosis cases reported in their states. Ribotyping of clinical isolates of *L. monocytogenes* from New York State that was performed at Cornell University indicated that a set of isolates had the same ribotype (35). CDC epidemiologists notified other public health departments about this situation and advised all state public health laboratories to forward all *L. monocytogenes* isolates to CDC for further characterization and subtyping (4). At that time, *L. monocytogenes* was not being routinely subtyped by public health laboratories participating in CDC's PulseNet, the national molecular subtyping network for foodborne disease surveillance (32). Between July 1998 and June 1999, CDC characterized and subtyped 447 clinical isolates of *L. monocytogenes*. In addition, as

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the epidemiologic investigation progressed and a specific food-processing facility was epidemiologically associated with the outbreak, food samples collected by the investigators were analyzed qualitatively and quantitatively for *L. monocytogenes*. The details of this laboratory investigation are described here; the epidemiologic and traceback aspects of the outbreak investigation were previously described (4).

MATERIALS AND METHODS

Maintenance and revival of *Listeria* isolates. Isolates of *L. monocytogenes* were stored frozen at −70°C in Trypticase soy broth and 20% glycerol for long-term storage. The isolates were revived from frozen stocks by plating onto 5% sheep blood agar plates (BBL, Cockeysville, MD) and incubating strains overnight at 35°C. For short-term storage, isolates were maintained in motility medium (Remel, Lenexa, KS).

Isolation of *L. monocytogenes* from clinical specimens. Specimens obtained from normally sterile sites (blood, cerebrospinal fluid, amniotic fluid, placenta, or fetal tissue) were subjected to blood culture or direct culture plating by standard microbiologic procedures (20).

Isolation of *L. monocytogenes* from foods. Food and environmental samples collected from a food-processing facility that was epidemiologically linked to the outbreak were examined for the presence of *L. monocytogenes* at the CDC laboratory. Food samples (25 g) were enriched in modified University of Vermont broth (BBL Cockeysville, MD) for 22 ± 2 h at 35°C and plated onto lithium chloride-phenylethanol-moxalactam (Difco, Detroit, MI) and modified Oxford (Oxoid, Hampshire, England) agars. The plates were incubated at 35°C and examined for the presence of *Listeria*-like colonies after 24 and 48 h. Also, 0.1 ml of the University of Vermont enrichment was added to 10 ml of Frasier broth, incubated at 35°C for 24 h, and plated onto lithium chloride-phenylethanol-moxalactam and modified Oxford agars. The agar plates were incubated at 35°C and examined after 24 and 48 h (9, 14, 15). Presumptive *Listeria* colonies on plates were further characterized and confirmed as described below.

Identification of *L. monocytogenes*. All *L. monocytogenes* isolates were biochemically characterized by recommended procedures (34). Acid production from the following substrates was evaluated: D-glucose, D-xylose, D-mannitol, lactose, sucrose, maltose, L-rhamnose, and α-methyl-D-mannoside. In addition, all isolates were confirmed as *L. monocytogenes* by the AccuProbe (GenProbe, San Diego, CA) test.

Quantification of *L. monocytogenes*. Food samples that yielded *L. monocytogenes* by the qualitative procedure described above were subjected to quantification tests by the three-tube most-probable-number (MPN) method and direct plating (21, 33).

Serotyping and restriction fragment length polymorphism analysis using PFGE subtyping. Serotyping was done by the method of Seeliger and Hohne (30). Pulsed-field gel electrophoresis (PFGE) subtyping was done using AscI and ApaI restriction endonucleases in accordance with the PulseNet standardized protocol (13).

Computer-assisted analysis of PFGE patterns and establishment of the PulseNet database of PFGE patterns. The PulseNet national pattern database was established using Molecular Analyst Fingerprinting Plus with Data Sharing Tools Version 1.6 (Bio-Rad Laboratories, Hercules, CA) as the software program for normalization, pattern analysis, and pattern matching. An AscI digest of a *L. monocytogenes* isolate assigned the number H2446 by the CDC National *Listeria* Reference Laboratory was used as the reference standard in establishing the database (13). A tagged image file format image of a gel run by the standardized protocol with this isolate run on both ends and in the middle was used to create the global reference standard for the database. All other tagged image file format images in the database are then normalized against this standard lane. The sizes of the bands used for normalization were determined in multiple electrophoresis runs alternating the H2446 standard with a lambda (λ) ladder (48.5 to 1,018.5 kb; New England Biolabs, Beverly, MA) and a high-molecular-weight standard (8.3 to 48.5 kb; Bio-Rad, Hercules, CA) in the lanes on the gels.

Pattern naming conversion in PulseNet. In the PulseNet standardized nomenclature, the first three characters identify the pathogen (GX6 = *L. monocytogenes*); the next three characters identify the restriction enzyme used to restrict the bacteria DNA (A16 = AscI; A12 = ApaI); and the last four numbers designate the sequentially assigned pattern number. For example, GX6A16.0001 would be equivalent to *L. monocytogenes*, restriction enzyme AscI, pattern number 0.0001. Two or more patterns combined are designated as a PFGE profile; e.g., GX6A16.0001-GX6A12.0001 is equivalent to *L. monocytogenes*, restriction enzymes AscI-ApaI, pattern numbers 0.0001-0.0001 (1).

TABLE 1. Clinical isolates of *L. monocytogenes* received from states during the period of the study and other characterizations

State	No. of isolates			
	Total	Serotype 4b	Outbreak PFGE[a] profiles	Other PFGE profiles
Alaska	1	1	1	
Alabama	2	1		2
Arizona	16	13	9	5
California	48	14	5	34
Colorado	8	7	4	2
Connecticut	43	20	2	33
Delaware	1	1		1
Florida	12	3	2	7
Georgia	36	19	4	29
Iowa	2	2	1	1
Illinois	16	10	3	11
Indiana	4	3	3	1
Kansas	1			1
Kentucky	1	1	1	
Massachusetts	16	12	5	9
Maryland	16	6	1	14
Maine	1			1
Michigan	15	11	9	6
Minnesota	19	8	2	16
Missouri	3	1		3
Nebraska	3	1		3
New York	39	26	12	22
Ohio	66	43	25	33
Oklahoma	12	8	1	9
Oregon	22	9	1	13
Pennsylvania	12	8	5	6
Rhode Island	5	2	1	3
South Carolina[b]	1	1	1	
Tennessee	16	7	6	7
Texas	6	5		5
Vermont	2	2	2	
West Virginia	2	2	2	
Total	447	247	108	277

[a] E_0, E_1, or E_3 profile.
[b] Patient was a resident of West Virginia.

RESULTS

Between 1 January 1998 and 31 July 1999, the CDC *Listeria* Laboratory received 447 clinical isolates of *L. monocytogenes* for confirmation and serotyping. After states reported significant increases in listeriosis cases to CDC in November 1998, CDC issued a request to all states to provide information about listeriosis cases in their states and send all isolates received after 1 July 1998 to the CDC Laboratory for serotyping and subtyping. Significantly more isolates were received in response to this request. The states from which the isolates were received during the period of this study, number of serotype 4b isolates, number of outbreak PFGE profiles, and number of other PFGE profiles are shown in Table 1.

Three highly related PFGE patterns associated with the 1998 outbreak were designated "the outbreak strain" (4). These three types were given the following PulseNet pattern designations: (i) GX6A16.0002-GX6A12.0002 (hereafter referred to as E_0), (ii) GX6A16.0002-GX6A12.0003 (hereafter referred to as E_1), and (iii) GX6A16.0002-GX6A12.0057

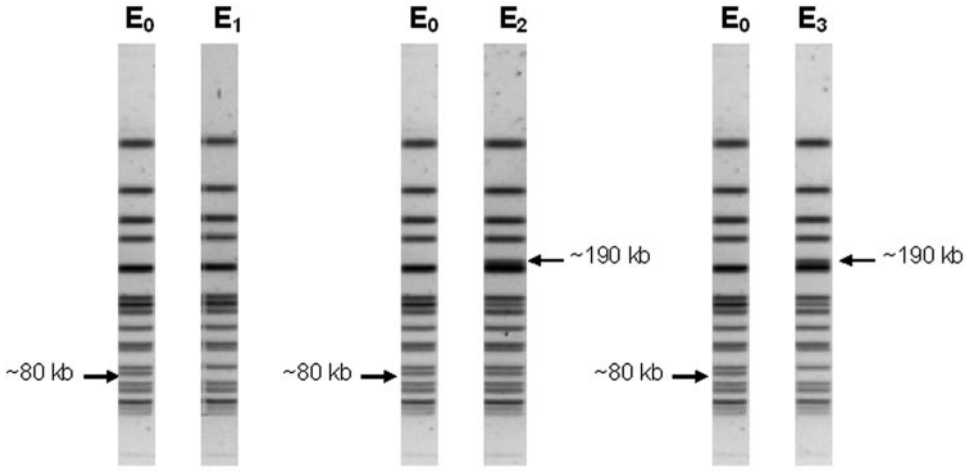

FIG. 1. Comparison of ApaI restriction digest of E_0 to E_1, E_2, and E_3 patterns.

(hereafter referred to as E_3) (Fig. 1). Compared with the E_0 pattern, E_1 was missing a DNA fragment of ~80 kb; E_3 was missing a DNA fragment of ~80 kb but had a fragment of ~190 kb that was missing from E_0. These differences were observed with ApaI restriction profiles.

A total of 108 isolates with E_0, E_1, or E_3 PFGE profiles were identified. All except one were serotype 4b; one isolate was untypeable. Four states (Arizona, Michigan, New York, and Ohio) accounted for more than 50% of the cases. Of the 108 isolates, 71% were PFGE type E_0, 27% were E_1, and 2% were E_3. Four states (Connecticut, Oklahoma, Oregon, and South Carolina) had cases only with the E_1 pattern, while nine states had cases only with the E_0 pattern. Both isolates with the E_3 pattern were isolates from patients in Tennessee. There were no temporal associations for E_0 and E_1 patterns.

The results of food testing are shown in Table 2. *L. monocytogenes* with PFGE profile E_0 was isolated from one open package of hot dogs obtained from a patient's refrigerator and one open package of deli meat from an institution. Deli meat from one patient's refrigerator yielded a nonserotypeable isolate with PFGE profile E_1. Additionally, a new profile, E_2, that had an additional fragment of ~190 kb (compared to profile E_0) was found in *L. monocytogenes* isolates from two open packages of hot dogs obtained from two different patients' refrigerators and from an unopened package of hot dogs obtained from the food-processing plant implicated as the source of the outbreak. Three intact packages of hot dogs obtained from this producer did not yield any *L. monocytogenes*. Of five deli meat samples obtained from the processing plant, three yielded *L. monocytogenes* serotype 1/2a with PFGE profile GX6A16.0014-GX6A12.0016.

When the numbers of *L. monocytogenes* bacteria in the positive food samples were quantified by the MPN and direct plating methods, we found that patterns associated with the 1998 outbreak strain were present at very low levels in the hot dogs (below the minimum quantifiable limit of the three-tube MPN method), while the serotype 1/2a strain was present in deli turkey meat samples at levels ranging from <0.3 to 2,200 CFU/g. Despite the high level of *L. monocytogenes* found in the deli turkey meat samples from the processing facility implicated as the source of the outbreak, we did not find any clinical isolates that matched the deli turkey isolates during the period of this investigation.

Of 447 isolates received, 339 were considered to be unrelated to the outbreak on the basis of PFGE subtyping; these isolates did not meet the definition of "the outbreak strain." Serotypes 1/2a (28%), 1/2b (26%), and 4b (41%) accounted for 95% of all isolates received at CDC; serotypes 1/2c, 3a, 3b, and 3c and a designation of nontypeable together accounted for only 5% of the isolates. There were a total of 144 AscI, 191 ApaI, and 230 AscI/ApaI patterns; therefore, the dual restriction enzyme system for *L. monocytogenes* provided greater discrimination between isolates than either enzyme used by itself. Because much of this work was done retrospectively and not in real time, small clusters of isolates that were identified were not followed up. For example, we found a cluster of 13 isolates that were serotype 4b and had a PFGE profile of GX6A16.0012-GX6A12.0007. These isolates were collected from seven states. The dates of collection ranged from 02/20/1998 and 07/19/1999; 10 of the isolates were collected between 10/05/1998 and 01/03/1999. Although this cluster was not investigated, it is reasonable to assume that a second smaller outbreak may have been simultaneously occurring.

DISCUSSION

This investigation underscores the importance of routine real-time subtyping of clinical isolates of *L. monocytogenes*. If PulseNet had implemented real-time PFGE subtyping of *L. monocytogenes* earlier, the outbreak might have been detected earlier and morbidity and mortality associated with this outbreak could have been substantially reduced. In 1996 and 1997, PulseNet was still in the process of training state public health laboratories to do routine subtyping of *Escherichia coli* O157 and submit the patterns to CDC for building a national database of PFGE patterns. Plans were under way to introduce standardized subtyping of *Salmonella* in PulseNet in 1998 and *L. monocytogenes* in 1999. However, large outbreaks of *Salmonella* sp. strain Agona in toasted oats cereal (5) and *Shigella sonnei* in imported parsley (7) and the listeriosis outbreak

TABLE 2. Plant Q products tested for *L. monocytogenes* from December 1998 to March 1999, organized by isolate serotype, product type, and sell-by date

Item	Sell-by date	Source	Package condition	Serotype	PFGE pattern		E pattern designation	Where tested	MPN (CFU/g)
					AscI	ApaI			
Franks	06/03/98	Patient's refrigerator[a]	Opened	4b	GX6A16.0002	GX6A12.0057	E_3	CDC	<0.3
Franks	11/25/98	Patient's refrigerator[a]	Opened	4b	GX6A16.0002	GX6A12.0004	E_2	CDC	<0.3
Franks	11/25/98	Patient's refrigerator[a]	Opened	4b	GX6A16.0002	GX6A12.0004	E_2	CDC	<0.3
Franks	12/16/98	Plant Q	Unopened	4b	GX6A16.0002	GX6A12.0004	E_2	CDC	<0.3
Franks	12/20/98	Patient's refrigerator[b]	Opened	4b	GX6A16.0002	GX6A12.0002	E_0	CDC	<0.3
Deli meat (turkey)	Unknown	Institution kitchen[b]	Opened	4b	GX6A16.0002	GX6A12.0002	E_0	USDA[d]	<0.3
Deli meat (chicken)	Unknown	Patient's refrigerator[c]	Opened	Nontypeable	GX6A16.0002	GX6A12.0003	E_1	CDC	<0.3
Deli meat (turkey)	12/23/98	Plant Q	Unopened	1/2a	GX6A16.0014	GX6A12.0016		CDC	2,200
Deli meat (turkey)	02/17/99	Plant Q	Unopened	1/2a	GX6A16.0014	GX6A12.0016		CDC	0.8
Deli meat (mixed)	02/17/99	Plant Q	Unopened	1/2a	GX6A16.0014	GX6A12.0016		CDC	<0.3
Franks	12/16/98	Plant Q	Unopened	No *L. monocytogenes* isolated				CDC	
Franks	12/23/98	Plant Q	Unopened	No *L. monocytogenes* isolated				CDC	
Franks	02/17/99	Plant Q	Unopened	No *L. monocytogenes* isolated				CDC	
Deli meat (turkey)	12/23/98	Plant Q	Unopened	No *L. monocytogenes* isolated				CDC	
Deli meat (turkey)	12/23/98	Plant Q	Unopened	No *L. monocytogenes* isolated				CDC	

[a] Patient symptomatic, no isolate recovered.
[b] Patient isolate and food isolate were indistinguishable by PFGE.
[c] No isolate; diagnoses confirmed by serology.
[d] USDA: U.S. Department of Agriculture.

recognized in 1998 forced PulseNet to accelerate the development, validation, and deployment of standardized protocols for the three additional bacterial pathogens in 1998. Fortunately, we had made significant progress in the development of a 1-day standardized PFGE protocol for *L. monocytogenes*. However, a standardized PFGE protocol had not been disseminated to the PulseNet participating laboratories. Only New York State and New York City contributed timely PFGE data during this outbreak investigation. Nearly all of the PFGE subtyping for this outbreak was done by the CDC laboratory. By the time the outbreak investigation was completed in March 1999, we had disseminated the standardized *Listeria* PFGE protocol, trained several participating laboratories in performing standardized PFGE subtyping of *L. monocytogenes*, and set up a national database of *Listeria* PFGE patterns.

During the 1-year period of this investigation, we identified 247 isolates of serotype 4b. Of those, 113 (46%) had the AscI pattern that was associated with the outbreak. Use of a second enzyme (ApaI) for restriction of the DNA allowed us to determine that 5 of the 113 isolates were unrelated to the outbreak patterns. This information helped the investigating epidemiologists in two ways: (i) it allowed them to separate outbreak-associated cases from geographically and temporally associated sporadic cases, and (ii) the epidemiologists were able to use the cases with PFGE profiles different from the outbreak patterns as controls for case-control studies conducted to identify specific foods associated with the outbreak.

As mentioned earlier, the E_0, E_1, and E_3 PFGE profiles have identical AscI patterns and exhibit minor differences (one or two fragments) in their ApaI patterns. PulseNet protocols require that even a single fragment difference between two PFGE patterns is significant and that the two patterns shall be assigned different pattern numbers. After PulseNet reported on the 77 E_0, 29 E_1, and 2 E_3 profiles, the epidemiologists concluded that all three PFGE patterns were from outbreak-related cases. One additional isolate had the E_1 PFGE profile and was epidemiologically linked to the outbreak, but its serotype could not be determined because it failed to react with any of the cellular (O) antisera. It is highly likely that this isolate was also serotype 4b. Thus, a total of 108 outbreak-associated cases were identified.

All three PFGE profiles from clinical isolates were observed in isolates from food. An additional fourth profile was found in food isolates. The E_0 profile was observed in an isolate from an open package of hot dogs and another from turkey deli meat; the E_1 profile was observed in an open package of deli chicken meat. The E_2 profile was observed in isolates from open packages of hot dogs found in a patient's refrigerator and in an unopened hot dog package obtained from the food-processing facility. The E_3 profile was found in an open package of hot dogs obtained from a patient's refrigerator, but this profile was not observed in any isolates from the food-processing facility. The close relatedness of *L. monocytogenes* isolates with E_0, E_1, E_2, and E_3 PFGE profiles and their isolation from either outbreak-associated cases or implicated food suggest that these strains may have shared a common ancestor that underwent

minor genetic modifications that resulted in the four PFGE profiles. If this hypothesis is valid, it would be reasonable to assume that the strain was resident in the implicated food-processing facility for a duration long enough to produce the genetic variants. *L. monocytogenes* has been known to be part of biofilms and to persist in food-processing environments for extended periods of time (36).

Evans et al. (11) recently reported on genetic markers unique to *L. monocytogenes* serotype 4b. They demonstrated that the strains involved in several foodborne listeriosis outbreaks in Europe and North America during the past 2 decades, including those in Nova Scotia (27), California (17), Switzerland (2), and France (pork tongue in 1992) (24), were very closely related, and they designated them epidemic clone I (ECI). However, in this 1998 to 1999 multistate outbreak traced to contaminated hot dogs, a different strain type of serotype 4b, with a genetic fingerprint rarely encountered before, was identified and designated ECII. As previously reported by Evans et al. (11), the ECII strains were markedly divergent in (or completely lacked) specific open reading frames that were probably involved in the expression of a cell surface component (16, 23).

It is tempting to make inferences about infectious doses from the finding of the outbreak strains in the implicated product at very low levels and the simultaneous finding of a different strain (different serotype) in the implicated food-processing facility's product at nearly a 1,000-fold higher level with no human illnesses attributable to it. However, such inferences must be made with caution. The samples were obtained from patients' refrigerators several weeks after their onset of illness. Because *L. monocytogenes* multiplies in hot dog packages at refrigeration temperatures and may have had enough time to reach stationary and death phases, the numbers of *L. monocytogenes* bacteria determined in the foods may not be indicative of the doses actually consumed by the patients.

PulseNet has proved extremely useful for detecting listeriosis clusters and making links between outbreak clusters and their food and environmental sources in epidemiologic investigations. PulseNet has been used for selecting out small and large clusters of listeriosis cases from background sporadic cases (3). Fewer than 2 years after the 1998 hot dog outbreak, PulseNet was used to link *L. monocytogenes* to raw milk from a manufacturing grade dairy during a small outbreak of listeriosis in Mexican immigrants caused by illicitly produced Mexican-style cheese. Isolates from 10 female patients, cheese samples from two stores, cheese retrieved from the home of a case patient, and raw milk from one local dairy had indistinguishable PFGE patterns by AscI and ApaI (6, 18). Because molecular subtyping was able to identify indistinguishable PFGE patterns, links among human disease, the cheese, and the source of the raw milk used to make the cheese were confirmed. PulseNet has been instrumental in every documented outbreak of listeriosis in the United States since the 1998 hot dog outbreak, including the large 2002 multistate outbreak associated with sliceable turkey deli meat (8).

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