Verification of Causal Relationships between *Listeria monocytogenes* Isolates Implicated in Food-Borne Outbreaks of Listeriosis by Randomly Amplified Polymorphic DNA Patterns

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Food and clinical isolates of *Listeria monocytogenes* recovered from four different outbreaks of listeriosis were analyzed by their PCR-based randomly amplified polymorphic DNA (RAPD) patterns to verify their causal relationships. The generation of DNA fingerprints by PCR-based RAPD analysis is a fast and sensitive method for the epidemiological tracking and identification of bacteria implicated in food poisoning outbreaks. The *L. monocytogenes* strains used in the study were obtained from the following four outbreaks: California, 1985, Mexican-style cheese; Canadian Maritime Provinces, 1981, coleslaw; Canada, 1989, brie cheese; and Canada, 1989, alfalfa tablets. RAPD profiles were generated by using random 10-mer primers for at least one food and one clinical isolate recovered from each outbreak. Identical profiles for 20 different primers were observed for each pair of food and clinical isolates from two of the four outbreaks. Isolates from the outbreak involving alfalfa tablets exhibited identical patterns for 19 primers; however, primer OPA-1 produced one additional 1.8-kb fragment, designated OPA-1-1.8, that was found in the food isolate but not in the corresponding clinical isolate. Hybridization analysis revealed that the absence of the OPA-1-1.8 polymorphic fragment in the clinical isolate was due to a deletion of at least 1.8 kb. Loss of the OPA-1-1.8 polymorphic fragment could not be induced by infective passage of the *L. monocytogenes* isolate from the alfalfa tablet through a mouse or by growth of this isolate under selective conditions. This suggests that the isolate recovered from the food was not identical to the isolate recovered from the patient. The ability to produce unique RAPD patterns allows for the discrimination between isolates even if they are of the same serotype and multilocus enzyme electrophoretic type.

*Listeria monocytogenes* is a food-borne pathogen that is ubiquitous in nature. As a result, *Listeria* contamination occurs quite often in a wide range of foods, such as raw and pasteurized milk, soft cheeses, ice cream, coleslaw, raw meat, fermented sausage, and raw fish (10, 13, 14, 32). Consumption of contaminated food can cause severe and often fatal infections in susceptible people. Several outbreaks of listeriosis in the past few years have increased the need for rapid, more sensitive methods for the detection and identification of *L. monocytogenes* strains associated with food-borne illness (13, 14, 17, 32, 33).

The length of time for the clinical symptoms of listeriosis to become apparent may take from a few days to several weeks after the ingestion of the contaminated food product. This factor, in combination with the lack of sensitive methods of identification, increases the difficulty in tracking and confirming the source of a food-borne infection. The typing methods used in the past have lacked the ability to consistently verify that two different bacterial isolates are actually the same strain. Newer techniques have a greater ability to differentiate two closely related isolates. Current methods include serotyping, phage typing, multilocus enzyme electrophoresis, randomly amplified polymorphic DNA (RAPD) analysis, and whole genome restriction digests (1, 3, 4, 7, 9, 10, 15, 18, 20, 21). RAPD analysis provides a fast and simple method for differentiating bacterial strains (6, 7, 12, 19, 20, 35).

RAPD polymorphisms can be used to discriminate between two otherwise indistinguishable isolates of the same serotype (7, 19). The establishment of identical RAPD patterns allows for the confirmation of a bacterium recovered from a food source that is suspected of being the causal agent in a food poisoning outbreak. Various genetic events give rise to polymorphisms, including primer-template mismatches, deletions or insertions within the primer-binding site, and deletions or insertions between two primer-binding sites, which can increase the fragment size or prevent amplification (if the distance between two primer-binding sites is greater than 4.0 kb).

One of the largest documented listeriosis outbreaks occurred in 1985 in the state of California. Jalesco cheese, a Mexican-style soft cheese, was implicated in the epidemic. *L. monocytogenes* was recovered from a sample of a patient’s refrigerated Mexican-style cheese as well as from unopened packages of cheese recovered from the factory. It was speculated that the source of *L. monocytogenes* was raw milk, which was mixed with pasteurized milk during the cheese-making process. The *L. monocytogenes* isolates recovered from the cheese and the patients were serotype 4b and electrophoretic type (ET) 1 (17). Another outbreak of listeriosis, which occurred in 1981 in Nova Scotia, Canada, implicated coleslaw as the vehicle of infection. *L. monocytogenes* was recovered from a sample of a patient’s refrigerated coleslaw and from unopened packages obtained from the factory. Further investigations revealed that the cabbage used for processing was contaminated with manure fertilizer obtained from a flock of sheep with listeriosis. The *L. monocytogenes* isolates recovered from the coleslaw and the patient were again serotype 4b and ET 1 (31). One isolated case of listeriosis that occurred in Canada involved the consumption of brie cheese (10). *L. monocytogenes* isolates, serotype 1/2b, ET 22 (28), were recovered from the patient and unopened packages of the brand of soft cheese that he had consumed (11). A second isolated case

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TABLE 1. *L. monocytogenes* strains used for RAPD analysis

<table>
<thead>
<tr>
<th>Year</th>
<th>Source</th>
<th>No.</th>
<th>Sero-type</th>
<th>ET</th>
<th>No.</th>
<th>Sero-type</th>
<th>ET</th>
</tr>
</thead>
<tbody>
<tr>
<td>1981</td>
<td>Coleslaw</td>
<td>PP-2</td>
<td>4b</td>
<td>1</td>
<td>PP-37</td>
<td>4b</td>
<td>1</td>
</tr>
<tr>
<td>1985</td>
<td>Mexican-style cheese</td>
<td>PP-22</td>
<td>4b</td>
<td>1</td>
<td>PP-23</td>
<td>4b</td>
<td>1</td>
</tr>
<tr>
<td>1989</td>
<td>Brie cheese</td>
<td>PP-395</td>
<td>1/2b</td>
<td>22</td>
<td>PP-447</td>
<td>1/2b</td>
<td>22</td>
</tr>
<tr>
<td>1989</td>
<td>Alfalfa tablets</td>
<td>PP-295</td>
<td>4b</td>
<td>1</td>
<td>PP-448</td>
<td>4b</td>
<td>1</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

**Bacterial strains and media.** The *L. monocytogenes* strains used in the study and their sources are listed in Table 1. These isolates were obtained from the Health Protection Branch of Health Canada and the Laboratory Center for Disease Control Canada. Multilocus enzyme electrophoresis typing of strains was conducted under the same conditions in the one laboratory (28). Strains were cultured on tryptic soy broth (TSB; Sigma, St. Louis, Mo.), *Listeria* enrichment broth (LEB; Difco, Detroit, Mich.), or brain heart infusion (BHI) broth (Difco), as noted below.

The bacteria to be used for mouse inoculations were cultured in BHI broth at 37°C for 20 h. Cells were washed with potassium phosphate-buffered saline (0.01 M; pH 7.4) and were resuspended in sterile water to approximate concentrations of 10^10 CFU/ml.

**Cell lysis.** The *Listeria* strains were grown at 37°C in TSB to an optical density at 600 nm of 1.5. A 250-µl aliquot of the culture was pelleted by centrifugation at 12,000 x g for 5 min, and the medium was discarded. The cell pellet was washed with saline (0.9% [wt/vol] NaCl) and was centrifuged. The bacterial pellet was then resuspended in 100 µl of 1 x PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl, 4 mM MgCl₂, 100 µM (each) deoxynucleotide triphosphate, 0.2 µM primer, 1 µl of cell lysate, and 1 U of *Tag* DNA polymerase (GIBCO BRL, Grand Island, N.Y.). RAPD amplification conditions were modified slightly from those of Williams et al. (35). RAPD amplifications were carried out by using a Hybaid TR1 thermal cycler. The cycling parameters were 45 s at 94°C, 1 min at 36°C, and 2 min at 72°C for a total of 45 cycles. RAPD products were analyzed by using a 1.0% agarose gel and TBE (89 mM Tris-borate, 2 mM EDTA [pH 8.2]) buffer at 100 V for 45 min.

**Dot blot and Southern hybridizations.** A 10-µl aliquot of unrestricted lysate was denatured by the addition of 10 µl of 0.8 M NaOH at 37°C for 10 min. A 90-µl aliquot of water was added to the denatured lysate mixture, which was then neutralized by the addition of 100 µl of 2 M ammonium acetate. The 200-µl solution was transferred to a nylon membrane (Micron Separations, Inc., Westborough, Mass.) with the Bio-Dot microfiltration unit (Bio-Rad, Rockville Centre, N.Y.). DNA was immobilized on the nylon membrane by baking the membrane at 125°C for 10 min and the DNA was used for the dot blot hybridization.

**Chromosomal DNA for Southern hybridization was prepared** as described by Pitcher et al. (30). Purified chromosomal DNA was then digested with *Apo*I (New England Biolabs, Beverly, Mass.), and the restricted fragments were separated in a 1.0% agarose gel and TBE buffer. The DNA was denatured by soaking the gel in 4 volumes of 1.0 M NaCl-0.5 M NaOH twice for 10 min. The gel was neutralized by soaking it in 4 volumes of 0.5 M Tris-HCl (pH 7.5)-1.5 M NaCl twice for 10 min. The DNA was transferred to a nylon membrane by capillary transfer blotting and was then immobilized on the membrane by baking at 125°C for 30 min.

**The RAPD product to be used as a probe was excised from** the agarose gel and was purified by using the USBioClean MP DNA purification kit (United States Biochemicals, Cleveland, Ohio). The purified DNA was used as a template for a second PCR, with the same primer used in the initial amplification, to incorporate digoxigenin-11-dUTP. Conditions were identical to those of the initial PCR, except that the concentration of dUTP was reduced to 80 µM and 20 µM digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, Ind.) was added. The digoxigenin-11-dUTP-labelled RAPD band was excised from a 1.0% low-melting-point agarose TBE gel (International Biotechnologies Inc., New Haven, Conn.), and a 100-µl aliquot of water was added to the excised DNA. The mixture was boiled for 10 min, and a 10-µl aliquot was used as a probe for dot blot and Southern hybridizations. The molecular weight markers (bacteriophage λ HindIII) were 3' end labelled by using the Klenow fragment of DNA polymerase I and dUTP-11-digoxigenin. The hybridization and nonradioactive detection were conducted according to the protocol provided with the alkaline phosphatase-based Genius 3 Nucleic Acid Detection Kit (Boehringer Mannheim).

**Mouse inoculations.** Five-week-old mice were injected intraperitoneally with a 250-µl aliquot of a 10-mg/ml solution of carragenan type II (Sigma). After 24 h, three mice were inoculated intraperitoneally with 10^5 CFU of *L. monocytogenes* and two mice were inoculated perorally with 10^5 CFU of *L. monocytogenes*, administered with a sterile 20-gauge 1.5-in. (3.8-cm) animal-feeding needle. The three mice that were inoculated intraperitoneally died within 48 h postinoculation.
The two mice that were inoculated perorally were killed by carbon dioxide asphyxiation after 72 h.

The brain, liver, and spleen from each mouse were aseptically removed by dissection, macerated, and individually cultured in 10 ml of TSB at 37°C for 12 h. The cultures were then streaked on *Listeria* agar (Oxford formulation; Unipath Ltd., Basingstoke, United Kingdom). *Listeria* colonies were identified by blackening the agar after 18 h of incubation at 37°C.

**RESULTS**

Food and clinical isolates from four outbreaks of listeriosis were analyzed in the study. At least one isolate was recovered from each of the suspected foods and was hypothesized to be the causal strain responsible for the corresponding outbreak. The isolates from the outbreak in 1981 involving coleslaw, the one in 1985 involving Mexican-style cheese, and the one in 1989 involving alfalfa tablets were serotype 4b, ET 1. There was one additional isolate, PP-861, recovered from the alfalfa tablets that was serotype 4b, ET 6 (28), which distinguished this isolate from the clinical isolate. The isolates from the outbreak in 1989 involving brie cheese were serotype 1/2b, ET 22 (28) (Table 1).

A total of 20 different 10-mer primers (kit A) were used for the RAPD analysis of the food and clinical *L. monocytogenes* isolates that were believed to be identical within each of the four outbreaks. Eighteen of the primers produced clear, reproducible patterns for each isolate. OPA-6 produced only one or two faint bands that were not consistently observed in repeated analysis. OPA-19 generated patterns that varied in the number of fragments produced for an individual lysate from one reaction to the next. The number of RAPD bands produced for a given primer ranged from 2 to 12, with molecular sizes ranging from 0.1 to 4.0 kb. The RAPD patterns generated with most primers were consistently produced for isolates of the same serotype. These results correspond to those of previous studies, which reported that a selected group of primers yields RAPD patterns that are the same for representatives within a given serotype (7), while other primers yield more discriminating patterns that differ between strains within a serotype (20). For example, primer OPA-1 differentiated the serotype 1/2b isolates from the serotype 4b isolates. The difference in the OPA-1 RAPD patterns for each serotype was exemplified by the presence of the 0.8- and 0.85-kb bands unique to the serotype 1/2b isolates (Fig. 1, lanes 2 to 3) and the 0.3-kb band unique to the serotype 4b isolates (Fig. 2, lanes 2 to 9). RAPD analysis of the isolates from the outbreak involving alfalfa tablets demonstrated the ability of RAPD analysis to discriminate between *L. monocytogenes* serovars of the same enzyme ET as well as between serovars of different ETs. For example, primer OPA-1 (Fig. 2) clearly differentiated the food isolates PP-295, PP-296, PP-297, PP-298, PP-299, and PP-397 from the clinical isolate PP-448, all of which were serotype 4b and ET 1. Primer OPA-1 also differentiated the alfalfa tablet isolate PP-861, also serotype 4b, but ET 6 (10), from the other alfalfa tablet isolates, all of which were serotype 4b, ET 1.

Figure 1 shows that identical RAPD patterns were produced for the food and clinical isolates involved in the outbreak involving brie cheese. Isolates recovered from that outbreak were serotype 1/2b and ET 22. Primer OPA-1 generated identical patterns for the food isolate PP-395 and the clinical isolate PP-447. A total of five bands ranging in size from 0.8 to 1.4 kb were generated for each isolate for OPA-1.

The RAPD patterns observed for the isolates from the outbreaks involving Mexican-style cheese and coleslaw were identical for the corresponding pairs of food and clinical isolates within each outbreak. For example, primer OPA-13 generated three distinct bands, approximately 0.9, 1.1, and 1.9 kb in size, that were identical for both the Mexican-style cheese isolate PP-22 and the clinical isolate PP-23 (Fig. 3). The RAPD patterns for the coleslaw isolate and the related clinical isolate were identical for all 20 primers. Primer OPA-20, for example, generated clear, identical patterns for the coleslaw isolate PP-2 and the clinical isolate PP-37 (Fig. 4). Bands of very low intensity were not consistently reproducible and were not taken into consideration when the RAPD patterns were compared. Primitives OPA-1 through OPA-20 generated identical patterns for the Mexican-style cheese and coleslaw isolates, and therefore could not further segregate the two isolates, further supporting their identities.

RAPD analysis of the food and clinical isolates from the outbreak involving alfalfa tablets revealed that at least two different strains of *L. monocytogenes* were present in the alfalfa...
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![RAPD Patterns of L. monocytogenes Alfalfa Food Isolates and Clinical Isolate](image)

**FIG. 2.** RAPD patterns for *L. monocytogenes* alfalfa food isolates PP-85, PP-295, PP-296, PP-297, PP-298, PP-299, and PP-397 and the corresponding clinical isolate PP-448. Lanes 1, 26, 27, and 52, bacteriophage λ DNA digested with *Eco*RI and *Hind*III (selected molecular size markers [in kilobases] are noted on the left); lanes 2 to 9, OPA-1; lanes 10 to 17, OPA-2; lanes 18 to 25, OPA-3; lanes 28 to 35, OPA-4; lanes 36 to 43, OPA-5; lanes 44 to 51, OPA-7; lanes 2, 10, 18, 28, 36, and 44, PP-448; lanes 3, 11, 19, 29, 37, and 45, PP-397; lanes 4, 12, 20, 30, 38, and 46, PP-861; lanes 5, 13, 21, 31, 39, and 47, PP-298; lanes 6, 14, 22, 32, 40, and 48, PP-296; lanes 7, 15, 23, 33, 41, and 49, PP-297; lanes 8, 16, 24, 34, 42, and 50, PP-298; lanes 9, 17, 25, 35, 43, and 51, PP-299.

Isolate PP-397 was serotype 4b and ET 1, which were the same as those for the clinical isolate PP-448, while isolate PP-861 was serotype 4b, ET 6. In addition to isolates PP-397 and PP-861, five additional isolates recovered from the same primary enrichment from the alfalfa tablets were analyzed (Table 1). The RAPD patterns for all five additional isolates, PP-295, PP-296, PP-297, PP-298, and PP-299, were identical to that for isolate PP-397. The patterns for isolate PP-861 were very similar to those for the other alfalfa tablet isolates, but they were not identical (Fig. 2). Isolate PP-861 generated the following polymorphisms in the RAPD patterns: OPA-1, 1.3 kb; OPA-2, 1.2 kb; OPA-7, 0.7 kb; OPA-9, 0.5 kb; OPA-16, 2.3 kb. Thus, the RAPD patterns discriminated isolate PP-861 from the other food isolates within the same serotype.

Identical RAPD patterns for food isolates PP-397, PP-295, PP-296, PP-297, PP-298, PP-299 and the clinical isolate PP-448 were observed for primers OPA-2 through OPA-5 and OPA-7 through OPA-20; OPA-6, as mentioned previously, did not generate bands for any of the isolates from the outbreak involving alfalfa tablets. The RAPD patterns produced by primer OPA-1 contained a single 1.8-kb polymorphism, designated OPA-1-1.8, that was present in all of the alfalfa tablet isolates except isolate PP-861 (Fig. 2).

Polymorphisms in RAPD patterns can arise from point mutations within the primer-binding site as well as insertions or deletions between primer-binding sites. In the outbreak involving alfalfa tablets, clinical isolate PP-448 differed from the implicated food isolate by the presence of the OPA-1-1.8.

![RAPD Patterns of Mexican-Style Cheese Isolate](image)

**FIG. 3.** RAPD patterns for the Mexican-style cheese isolate PP-22 and the corresponding clinical isolate PP-23. Lanes 1 and 22, bacteriophage λ DNA digested with *Eco*RI and *Hind*III (selected molecular size markers [in kilobases] are noted on left); even-numbered lanes represent the patterns produced by isolate PP-22; odd-numbered lanes represent the patterns produced by isolate PP-23. The primers OPA-11 through OPA-20 were used to generate these RAPD profiles for each pair of isolates and are shown in sequential order.
polymorphism. In order to determine the nature of the OPA-1-1.8 polymorphism, dot blots and Southern hybridizations were performed on cell lysates and genomic digests, respectively. Ten food and four clinical isolates from all four outbreaks were probed in the hybridizations with the L. monocytogenes food isolate PP-397 generated OPA-1-1.8. Digoxigenin-11-dUTP, incorporated into the fragment by PCR amplification, was used for colorimetric detection of the hybrids. Dot blot hybridizations revealed that the probe bound only to the food isolates PP-295, PP-296, PP-297, PP-298, PP-299, and PP-397, while the other eight isolates were negative for probe binding (data not shown). These were the only alfalfa tablet isolates that contained the OPA-1-1.8 RAPD fragment.

Genomic DNA digested with Apol was used for the Southern hybridization. OPA-1-1.8 hybridized only to a single fragment with a molecular size of approximately 1.8 kb; this fragment was found in the food isolate PP-397 but not in the clinical isolate PP-448 (Fig. 5). These results indicate that the additional band is not a result of a small genetic difference between the food and clinical isolates, such as a point mutation or a deletion within the primer-binding site, but rather is a deletion of at least 1.8 kb. It is only coincidental that the size of the Apol restriction fragment is identical to the size of the OPA-1-1.8 RAPD product.

The food isolate in the outbreak involving alfalfa tablets may have given rise to the clinical isolate by a spontaneous deletion, which may have included one or both of the OPA-1-binding sites, during its cycle of infection or subsequent isolation on microbiological medium. Experimental conditions were established in an attempt to simulate the selection conditions during isolation that may have induced this deletion. Food isolate PP-397 was cultured at 37 and 42°C in either TSB or LEB for 12 h and was subcultured for an additional 12 h. This subculturing step was repeated 10 times for a total of approximately 120 generations. RAPD analysis of 10 isolates recovered after 120 generations revealed no loss of the OPA-1-1.8 polymorphism. Mice were infected with strain PP-397 in order to simulate infective passage. Three mice were infected intraperitoneally, and two mice were infected perorally. L. monocytogenes was recovered from the livers, spleens, and brains of the mice infected intraperitoneally and perorally. Two isolates recovered from the livers, spleens, and brains of a mouse infected intraperitoneally and another mouse infected perorally were tested by RAPD analysis. Each of the isolates recovered carried the OPA-1-1.8 polymorphism (data not shown).

**DISCUSSION**

Food and clinical isolates of L. monocytogenes from four different outbreaks were tested by RAPD analysis to determine whether the original identification of the causative strain recovered from the food was correct. Reproducible, identical RAPD patterns were observed for each related food and clinical isolate in the outbreaks involving Mexican-style cheese, coleslaw, and brie cheese. Eighteen of 20 primers generated clear patterns; primers OPA-6 and OPA-19 gave inconclusive results. The deleted band was not detected among isolates from outbreaks involving alfalfa tablets and coleslaw.

**FIG. 4.** RAPD patterns for the coleslaw isolate PP-2 and the corresponding clinical isolate PP-37. Lanes 1 and 22, bacteriophage λ DNA digested with EcoRI and HindIII (selected molecular size markers [in kilobases] are noted on the left); even-numbered lanes represent the patterns produced by isolate PP-2; odd-numbered lanes represent the patterns produced by isolate PP-37. The primers OPA-11 through OPA-20 were used to generate the RAPD profiles for each pair of isolates and are shown in sequential order.

**FIG. 5.** Southern hybridization of Apol-digested genomic DNA with the digoxigenin-labelled 1.8-kb polymorphism produced by alfalfa tablet isolate PP-397 with OPA-1. Lane 1, bacteriophage λ DNA digested with HindIII; lane 2, strain PP-397; lane 3, clinical isolate PP-448.
results. Our data substantiate the results of restriction endonuclease analysis conducted by Wesley and Ashton (34) suggesting that the food isolates from the coleslaw and Mexican-style cheese were the causal sources for the 1981 Canadian and the 1985 Californian outbreaks, respectively. The RAPD analysis of the isolates implicated in the outbreak involving brie cheese also resulted in identical RAPD profiles for the food and clinical isolates, substantiating the previous confirmation of that causal source (31).

RAPD analysis did not differentiate the food isolates from the outbreaks involving coleslaw and Mexican-style cheese. These data are contradictory to the results obtained by Wesley and Ashton (34), suggesting that the two isolates were different strains. Differences in restriction patterns can result from similar genetic changes that produce RAPD polymorphisms, such as deletions or insertions between restriction sites and point mutations within the restriction site. One additional hypothesis for differences in the restriction patterns is the possibility of either the acquisition or the loss of a DNA methyltransferase. DNA methyltransferase genes are often incorporated into bacteriophages (16) and plasmids (23). The loss or gain of a lysogenic phage or plasmid containing a methyltransferase gene may occur under different environmental conditions. This type of change may result during the cycle of infection or isolation from the contaminated food. Two isolates of the same strain, growing under different conditions, may become divergent in their methylation patterns because of the different selective pressures. Changes in the methylation pattern from one isolate to the next may result in different restriction patterns. The sources of the differences in restriction patterns are unknown, and without further analysis, the genetic basis for these differences remains unclear. The number or extent of genetic differences which define two isolates of being two different strains is not easily resolved (8). However, the detection of relatively subtle differences will increase as the sensitivity of subtyping assays increases. It is also possible that the primers used for RAPD analysis did not bind to areas adjacent to or within the polymorphism, and no difference between isolates was detected.

RAPD analysis of the food and clinical isolates from the outbreak involving alfalfa tablets resulted in patterns that were identical for all primers tested except OPA-1. Polymorphism OPA-1-1.8 was observed only in the food isolates. Bands of very low intensity varied between reactions. The inability to consistently visualize these bands may have been due to the limited sensitivity of ethidium bromide staining. Another possible explanation for the lack of reproducibility of these very low intensity bands is that the primer may have preferentially bound to primary target sites (exact matches) during the initial amplification, thereby creating more of these target sites for later amplification cycles, resulting in bands of greater intensity. This essentially titers out primer that would have bound to the secondary target sites (one or more mismatches) generating the lower-intensity bands. In addition, the fidelity of primer binding is dependent on PCR conditions, such as the magnesium chloride concentration. An increase in the magnesium chloride concentration to 4 mM resulted in an increase in the number and intensity of RAPD bands (24). The increase in the number of RAPD bands is a result of the primer binding to targets with one or more mismatches. An increase in magnesium chloride of less than 4 mM resulted in the disappearance of the OPA-1-1.8 polymorphism produced by OPA-1 with the alfalfa tablet isolates, serotype 4b, ET 1 (data not shown).

The nonisotopically labelled primer OPA-1-1.8 bound to only the food isolates in both the dot blot and Southern hybridizations under stringent hybridization conditions, suggesting that the OPA-1-1.8 polymorphism comprises DNA that is completely absent from the clinical isolate. This observation negates the possibility that the polymorphism was the result of a small genetic change such as a point mutation within the primer-binding site. The possibility of a larger genetic alteration such as an insertion or deletion between primer-binding sites is unlikely. Theoretically, even under high-stringency conditions, a probe comprising a sequence of only 100 consecutive nucleotides identical to the target sequence should show a positive result for the clinical isolate (5).

These hybridization data suggest that the OPA-1-1.8 polymorphism is a segment of DNA that is unique to the food isolates PP-295, PP-296, PP-297, PP-298, PP-299, and PP-397. Experimental tests were conducted in an attempt to induce this deletion. L. monocytogenes often contains plasmids (27), which may be a possible source for RAPD bands. The loss of plasmids can occur quite readily, thereby explaining the loss of a RAPD fragment. Isolates recovered from the alfalfa tablets were repeatedly subcultured in TSB or LEB in an attempt to induce the deletion of the OPA-1-1.8 polymorphism. TSB was used as a growth medium to determine whether the cause of this deletion was a simple case of repeated culturing under nonselective conditions. LEB was used as a selective medium in an attempt to cure the strain of any plasmids resulting from the presence of acriflavine, a known plasmid-curing agent (11). Temperatures of 37 and 42°C were also tested as possible selective agents that might induce plasmid loss. RAPD analysis of the individual isolates after 120 generations resulted in patterns that were identical to the original patterns for isolates containing the OPA-1-1.8 polymorphism. Although this does not eliminate plasmid curing as the source of the polymorphism, it suggests that plasmid loss may not be as frequent or as easily induced by most isolation protocols for L. monocytogenes as has been suspected.

A second hypothesis suggests that the deletion of this sequence may have been selected for during the infective process. The transformation of specific types of Pneumococcus species during infection demonstrates the induction of inheritable and specific alterations in cell structure and function. The attenuated, nonencapsulated, avirulent variant was transformed in vitro to a fully encapsulated, virulent strain, which was indicated by the resulting infectivity (2). Another possibility is the transposition of an insertion element. Insertion elements have been directly linked to the reversible expression of a virulence antigen in Citrobacter freundii, which occurs at a high frequency (26). A high rate of antigenic variation has been observed in Borrelia hermsii, resulting in frequent changes of serotype within one strain. Antigenic variation in B. hermsii is a direct result of DNA sequence rearrangement (22). This type of frequent genetic recombination is a possible source of DNA loss. Examples of virulence and antigen switching such as these support the possibility of an infection-induced change within the alfalfa tablet isolate containing the OPA-1-1.8 fragment, giving rise to the clinical isolate.

In an attempt to simulate infection conditions, mice were immunocompromised and inoculated intraperitoneally and perorally. Mice inoculated intraperitoneally developed symptoms of listeriosis and died within 48 h, confirming the virulence of the isolates. The brains, livers, and spleens were removed from the mice, and L. monocytogenes was successfully isolated from all three organs from mice inoculated intraperitoneally and perorally. However, all isolates carried the OPA-1-1.8 polymorphism. This suggests that the cause of the deletion may not be due to the selective agents present during
infection. Although the food and clinical strains are closely related, they appeared to be distinct and stable strains.

Previous RAPD typing studies have involved the use of only one primer to type *Listeria* strains (19, 20), and the discriminating ability of primer HLWL74 is limited. Many of the RAPD profiles were similar, differing only in one or two bands of a total of seven or eight bands. The primer discriminates among most isolates of different serotypes; however, two groups of isolates, each group having four different serotypes, had identical RAPD patterns. This indicates the need for multiple primers when typing *L. monocytogenes* isolates. We have previously demonstrated that the use of as few as two primers clearly discriminates between isolates of different serotypes (7). The use of multiple primers generates a greater number of discriminatory bands, increasing confidence in the accuracy of the results.

Preliminary tests in our laboratory of various lysis methods have revealed that the boiling of cells may not provide sufficient lysis. The use of boiled lysates generated variable results from one amplification to the next (data not shown). We have also seen variable results in RAPD patterns when different numbers of cells were used for the lysis. Variability in the efficiency of cell lysis leads to variability in the amount of template available for primer binding. Changes in template concentration result in the appearance and disappearance of RAPD bands (24, 25). The method for RAPD amplifications of Mazurier et al. (19) did not include cell lysis prior to PCR amplification. Cells were added directly to the reaction tube and were heated at 94°C for 4 min (19). Differences in the amount of cells lysed from one reaction to the next could be another possible source of the absence of one or two RAPD bands.

RAPD analysis provides a method for the classification of *L. monocytogenes* strains into groups more specific than serotypes and possibly even more specific than ET. RAPD analysis performed with selected primers can identify and differentiate closely related strains of *L. monocytogenes*. Previous studies have demonstrated that clinical isolates of *L. monocytogenes* serotypes 1/2a, 1/2b, 1/2c, 4a, and 4b can be further subdivided into 45 ETs (29). RAPD profiles clearly differentiated the two isolates PP-397 and PP-861, which were of the same serotype but of a different ET. Five of a total of 20 primers generated different RAPD patterns for each isolate. Twenty-five percent of all primers used were able to discriminate between two strains of different ETs. Furthermore, 1 of 20 primers discriminated between two isolates that were of the same ET.

If the possible number of RAPD patterns were estimated on the basis of an average of six bands per primer and differences in RAPD patterns were scored on the basis of only a single band change, there would be a possible total of 720 different patterns for one primer. If changes in more than one band or band sizes were scored, the total number of possible bands would increase significantly. The large number of potential polymorphic bands suggests that RAPD fingerprinting is a very sensitive method of isolate discrimination.

The isolate recovered from the alfalfa tablets was previously assumed to be the causative agent. However, results of RAPD analysis conflicted with this initial indictment. Although the food isolate was clearly virulent, the actual food source that gave rise to this case of listeriosis remains to be identified. It is also possible that the causative strain was present in, but not recovered from, the alfalfa tablets. RAPD analysis of the food and clinical isolates from the outbreaks involving Mexican-style cheese, coleslaw, and brie cheese substantiated the original confirmation of the causal agent implicated in each outbreak. The RAPD technique provides a fast and simple method for the epidemiological confirmation of food-borne bacterial outbreaks.

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