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Investigations Related to the Epidemic Strain Involved in the French Listeriosis Outbreak in 1992

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Two hundred seventy-nine cases of human listeriosis (92 pregnancy-related cases and 187 non-pregnancy-related cases) caused by a serovar 4b and phagovar 2389:2425:3274:2671:47:108:340 strain were identified in France between March and December 1992. Epidemiological investigations included a case-control study (not described here) and microbiological analyses of foods. Results of the case-control study and characterization of food isolates identified pork tongue in jelly, a ready-to-eat meat product, as the major vehicle of this outbreak, and to a lesser extent, delicatessen products contaminated secondarily during handling in food stores. As far as serotyping, phage typing, DNA macrorestriction pattern analysis (obtained by pulsed-field gel electrophoresis [PFGE]), and ribotyping are concerned, this epidemic strain is phenotypically and genomically closely related to strains responsible for major outbreaks of listeriosis previously observed in Europe and North America. The epidemic strain sensu stricto as defined by PFGE (2/1/3) displayed the same serovar, phagovar, ribovar, and *Apa*I and *Not*I PFGE patterns as the epidemic strains from outbreaks in Switzerland, California, and Denmark, but it consistently showed differences in the *Sma*I PFGE profile. This information greatly contributed to the identification of the major food vehicle (pork tongue in jelly) and further allowed exclusion of other foods (cheese) as possible sources of this major listeriosis epidemic.

With demonstration of the food-borne transmission of listeriosis during the 1981 outbreak in Nova Scotia (40), which was subsequently documented in several additional outbreaks and sporadic cases (2, 15, 17, 22, 24, 26, 27, 40, 41), *Listeria monocytogenes* and listeriosis have become a major concern for food industries and public health authorities in Europe and North America. Consequently, surveillance systems, mainly laboratory based, were established in a number of industrialized countries (37) to monitor the occurrence of human listeriosis, to detect outbreaks, and to evaluate the impact of control strategies.

In France, the surveillance system used in 1992 was established in 1987 and comprised two National Reference Centers (NRC); *Listeria* strains were serotyped in the NRC located in Nantes (A. L. Courtieu) and then phage typed and characterized by molecular methods in the NRC of the Pasteur Institute. Regular exchanges of strains and information between these two laboratories allowed a timely follow-up of the evolution of the disease in humans for many years. Listeriosis was characterized in France during previous years by a background level of sporadic cases with small outbreaks superimposed (13, 14, 23, 38, 39). This pattern has been observed in other countries (37).

With this surveillance system, a major outbreak of 279 cases caused by a serovar 4b and phagovar 2389:2425:3274:2671:47:108:340 strain was observed in 1992. After an alert in May, a network encompassing the Direction Générale de la Santé (Ministry of Health), the Réseau National de Santé Publique, the Direction Générale de l'Alimentation (Ministry of Agriculture), and the Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes (Ministry of Economy) was constituted at national and departmental levels to investigate the outbreak (8). Results from the case-control study and typing of *L. monocytogenes* strains isolated from food sampled at various stages (process, transport, distribution, and patients' refrigerators) implicated contaminated pork tongue in jelly as the major vehicle of the outbreak (19).

This paper deals with laboratory investigations related to the detection and characterization of the epidemic strain. The description of the outbreak and the epidemiological investigations conducted in order to identify the food vehicle will be described in a separate publication.

MATERIALS AND METHODS

Origin of strains. In 1992, 758 strains of human origin were collected by public and private medical laboratories. Strains were isolated by the routine method used by each laboratory. During the period from July to December 1992, 14,479 strains of nonhuman origin were sent for typing by 81 departmental veterinary laboratories, 6 fraud repression laboratories, and 91 private food hygiene laboratories. One thousand two hundred twenty-five strains were recovered from a food environment, 11,343 strains were recovered from food (203 from milk, 2,544 from cheeses, 5,262 from delicatessen products, 2,191 from meat and meat

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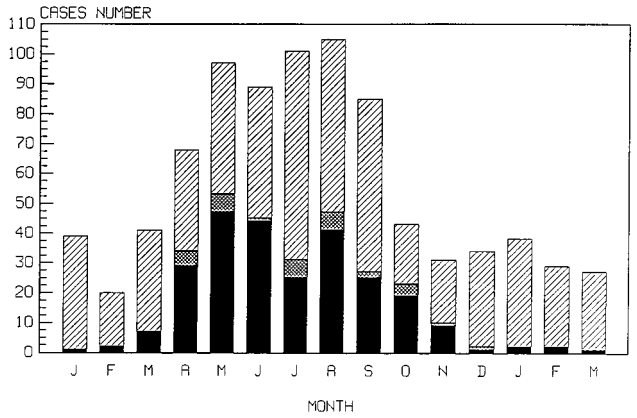

FIG. 1. Monthly distribution of listeriosis cases in France from January 1992 to March 1993. ■, epidemic cases sensu stricto (serovar 4b, phagovar 2389:2425:3274:2671:47:108:340, pulsovar 2/1/3); ▨, cases of serovar 4b, phagovar 2389:2425:3274:2671:47:108:340, and pulsovars different from 2/1/3; ▨, sporadic cases.

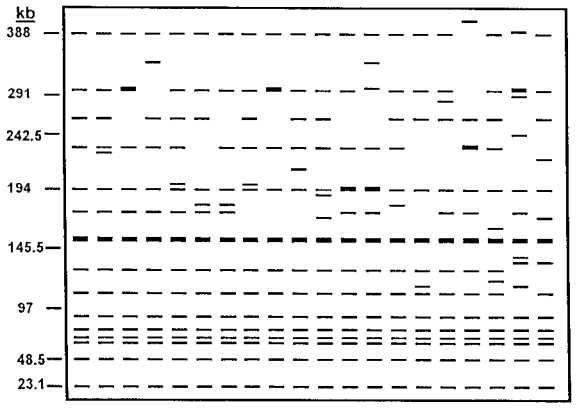

FIG. 2. DNA macrorestriction patterns of serovar 4b and phagovar 2389:2425:3274:2671:47:108:340 strains after cleavage with *Apa*I and separation by PFGE.

products, and 1,143 from various other food products), and 142 strains were recovered from animal specimens; 1,769 strains were received without information about the source. Because foods were frequently contaminated with different strains of *L. monocytogenes*, 1 to 12 colonies per contaminated food were sent, which explains the large number of isolates received.

Definition of the epidemic strain. The strain responsible for this unusual increase in the number of cases belonged to serovar 4b and phagovar 2389:2425:3274:2671:47:108:340. It is therefore designated the epidemic strain. Epidemic strains belonging to pulsovar 2/1/3 are called epidemic strain sensu stricto.

Detection of the epidemic strain. Strains were first screened by serogrouping with unabsorbed and diluted sera prepared according to the reference method (42), in order to identify serogroup 4 strains. These strains (and non- or autoagglutinable ones) were phage typed with the international set of *L. monocytogenes* bacteriophages (except phage 3551) (36).

Characterization of the epidemic strain. *L. monocytogenes* strains belonging to the phagovar 2389:2425:3274:2671:47:108:340 were characterized by DNA macrorestriction patterns obtained after digestion with *Apa*I, *Sma*I, or *Not*I and separation of the generated fragments by pulsed-field gel electrophoresis (PFGE) with a previously described protocol (4). A diagram of representative DNA macrorestriction patterns was created as previously described (6). Ninety-five strains belonging to different pulsovars were further analyzed by ribotyping with the pBA2 probe and restriction with *Eco*RI and *Hin*dIII (21).

RESULTS

Detection of the epidemic strain. (i) Human strains. (a) Early warning. In May 1992, the NRC of the Pasteur Institute noted an unusual increase in the number of clinical isolates of *L. monocytogenes* phagovar 2389:2425:3274:2671:47:108:340 and alerted the Direction Générale de la Santé and the Réseau National de Santé Publique. Thirty-two cases of listeriosis caused by this particular strain had been observed since January (January, 1 case; February, 2 cases; March, 6 cases; April, 23 cases) (Fig. 1). The annual number of cases caused by this strain in previous years ranged from 6 to 27 (unpublished data). Because of the involvement of a single strain, an emerging epidemic caused by a common source was strongly suspected.

(b) Outbreak. Two hundred seventy-nine human cases of listeriosis caused by the epidemic strain as defined by serovar and phagovar (37% of the total number of cases observed in 1992 and 40% of those observed for the May-December period) were recorded. This outbreak constituted a noticeable increase in the number of cases superimposed on the background level of sporadic cases. Thirty-three percent were pregnancy-related cases, and 67% were non-pregnancy-related cases. The epidemic curve (Fig. 1) was irregular, with peaks in May, June, and August. A wide geographical distribution was observed (79 departments), with higher incidences in central and eastern France (19).

(ii) Strains isolated from food and their relative environment. The epidemic strain was isolated from 220 food products as follows: delicatessen products, 135; cheeses, 40; meat and meat products, 31; milk, 1; other foods, 13; and environmental samples, 40. Fifty-eight of these 260 samples were contaminated with at least two different *Listeria* strains. In cases in which multiple isolates with the epidemic serovar and phagovar were received from one food sample, one strain was selected for further characterization by molecular typing methods.

(iii) Strains isolated from animal specimens. Five epidemic strains were isolated from 142 animal specimens.

Molecular characterization of epidemic strains. (i) Human strains of the outbreak. Twelve unique *Apa*I, 6 unique *Sma*I, and 3 unique *Not*I DNA macrorestriction profiles were observed among the 279 human strains. Twenty different combinations of profiles were detected. Examples of various *Apa*I profiles are diagrammed in Fig. 2. Two hundred forty-seven (89%) of the 279 human strains displayed exactly the same combination of *Apa*I, *Sma*I, and *Not*I profiles, named pulsovar 2/1/3 according to previous nomenclature (4, 7). The strains with these characteristics (2/1/3) will be referred to hereafter as epidemic strain sensu stricto (Fig. 3). DNA patterns differing by one band or more with a single restriction enzyme were considered different from the epidemic strain sensu stricto. With ribotyping, 41 strains (29 strains belonging to pulsovar 2/1/3 and 12 strains belonging to 11 other pulsovars) clustered in ribovar EM1 and HM1 after cleavage with *Eco*RI and *Hin*dIII, respectively (Fig. 4).

(ii) Strains isolated from food and their relative environment. Two hundred six epidemic strains isolated from foodstuffs and 33 strains isolated from a food environment were characterized by DNA macrorestriction patterns. Twelve, 10, and 8 different profiles were distinguished after cleavage with *Apa*I, *Sma*I, and *Not*I, respectively (Fig. 2 and 3), leading to the identification of 24 different pulsovars constituted by 1 to 171 strains. Ten of these pulsovars were common to human and food isolates, whereas 14 were detected only in food or a food environment. The epidemic strain sensu stricto as previously defined (DNA profile 2/1/3) was recovered from 154 foods of the following types and frequencies (in parentheses): delicatessen products, including pork tongue in jelly (112), other meat products (19), cheeses (12), and miscellaneous foods

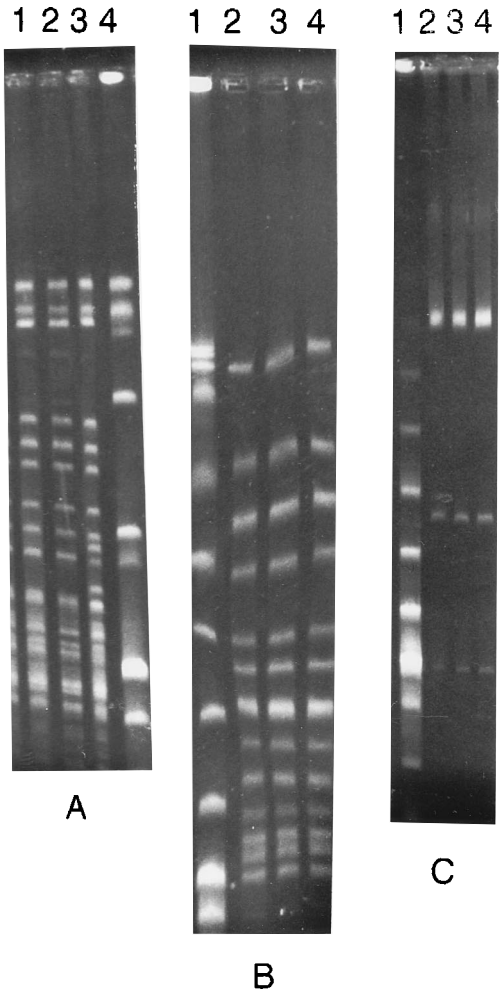

FIG. 3. DNA macrorestriction patterns after cleavage with *Apa*I, *Sma*I, and *Not*I of serovar 4b, phagovar 2389:2425:3274:2671:47:108:340, and pulsovar 2/1/3 and 2/26/3 strains. (A) *Sma*I patterns. Lanes: 1, CLIP 21640 (human; pulsovar 1); 2, CLIP 28476 (pork tongue in jelly; pulsovar 1); 3, CLIP 25703 (cheese; pulsovar 26); 4, lambda concatemers. (B) *Apa*I patterns. Lanes: 1, lambda concatemers; 2, CLIP 21640 (human; pulsovar 2); 3, CLIP 28476 (pork tongue in jelly; pulsovar 2); 4, CLIP 25703 (cheese; pulsovar 2). (C) *Not*I patterns. Lanes: 1, lambda concatemers; 2, CLIP 21640 (human; pulsovar 3); 3, CLIP 28476 (pork tongue in jelly; pulsovar 3); 4, CLIP 25703 (cheese; pulsovar 3).

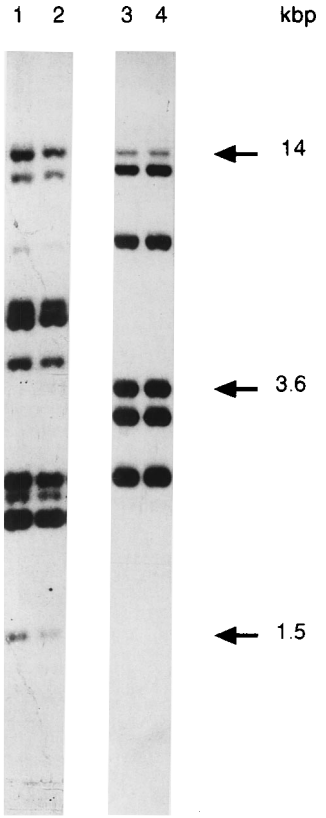

FIG. 4. Ribovars after cleavage with *Eco*RI (lanes 1 and 2) and *Hin*dIII (lanes 3 and 4) of serovar 4b and phagovar 2389:2425:3274:2671:47:108:340 strains. Lanes: 1 and 3, CLIP 22335 (human), ribovars EM1 and HM1; 2 and 4, CLIP 27993 (pork tongue in jelly), ribovars EM1 and HM1.

(11). Eighty-three percent of the epidemic strains isolated from the delicatessen belonged to the pulsovar 2/1/3. Foods were collected at various stages of the food-processing chain, including from slaughterhouses, processing plants, retailers (packaged foods and foods sliced at the counter), and patients' refrigerators. Among food samples from the 45 refrigerators belonging to patients involved in the epidemic, 8 were contaminated with this strain (hams and sausage, 4; cooked meat, 3; cheese, 1). The epidemic strain sensu stricto was isolated from 17 environmental samples.

Delicatessen items contaminated with the epidemic strain sensu stricto included products in jelly, including pork tongues, various hams (raw and cooked), various patés, "rillettes," and sausages. Most of these products were sampled in at least 30 food stores in retail stands. All epidemic strains isolated from pork tongue in jelly of brand A belonged to pulsovar 2/1/3 (seven samples from unopened packages, and six samples previously sliced at the counter). With regard to the food environment, the epidemic strain sensu stricto was recovered from seven samples from retail stands as well as from the plant which processed the incriminated pork tongue in jelly (four strains). Besides pork tongue in jelly and delicatessen sliced at the counter, 17 other processed pork products in their original packages were contaminated with the epidemic strain sensu stricto.

Besides the epidemic strain sensu stricto, the pulsovar 2/26/3 was the most frequent combination identified. This combination was recovered for 28 strains isolated only from cheeses and cheese-processing plants. Fifty-eight percent of the epidemic strains from these origins belonged to the pulsovar 2/26/3. Pulsovars 2/26/3 and 2/1/3 differed slightly (Dice index, 0.92 for *Sma*I profiles) (Fig. 1). Interestingly, no human case of listeriosis caused by this strain was detected during the outbreak. The 22 remaining pulsovars consisted of 1 to 10 strains.

Ribotype hybridization patterns of 54 strains with food and environmental origins (27 strains belonging to the epidemic pulsovar and 27 strains belonging to 20 minor pulsovars) displayed ribovars EM1 and HM1 with *Eco*RI and *Hin*dIII, respectively (Fig. 4).

(iii) Strains isolated from animal specimens. Among the five epidemic strains isolated from animals, four were epidemic strain sensu stricto (pulsovar 2/1/3).

DISCUSSION

Phenotypic typing methods (serotyping and phage typing) have been used for routine surveillance of human listeriosis in France for 7 years and have been proved to be appropriate to

detect outbreaks as soon as they emerge. These methods were essential for the screening of the epidemic strain: serogrouping allowed elimination of the high proportion of serovar 1/2 strains which usually contaminate foods, and phage typing proved to be a useful tool for identifying epidemic strains. Despite their limitations, these methods are timesaving and inexpensive and therefore are especially adapted to the analysis of a large number of strains in a short period (in this case, 15,000 strains in 7 months). In addition, results are rapidly obtained by these typing methods (a few minutes for serogrouping and 24 to 48 h for phage typing). These features are especially important for such investigations because of the need to identify epidemic cases of infection quickly, allowing subsequent interviews of patients and controls (who rely on memory regarding food habits) and allowing an immediate search for contaminated foods. Substantial progress in the field of typing has been made during the last few years with the introduction of molecular methods. Previous studies indicated that molecular typing is highly discriminative for *L. monocytogenes* serovar 4b (1, 4, 6, 7, 12, 20, 21, 25, 28–30, 32, 33, 44) and more precisely for strains of phagovar 2389:2425:3274:2671:47:108:340. This phagovar was divided into nine and five groups by DNA macro- and microrestriction patterns, respectively (4, 12). This is further documented in this investigation, in which a total of 34 pulsovars were identified within this phagovar. Eighty-nine percent of the human epidemic strains belonged to the pulsovar 2/1/3, thus demonstrating the emergence of a single clone responsible for the outbreak. Other DNA patterns observed within epidemic human strains could result from recent mutations during the outbreak, isolation procedures, or storage of cultures or, in contrast, could represent the usual sporadic cases superimposed on the epidemic. No correlation with clinical forms, geographic distribution, or date of isolation was demonstrated for these atypical strains. The exact number of cases involved in this outbreak is therefore uncertain (ranging from 247 according to a strict analysis of DNA macrorestriction patterns to 279 according to phagovar). Until now epidemic cases were defined by phagovars (2, 17, 40), but the recent introduction of molecular typing methods should lead to a better definition and identification of such cases. In this study, the use of PFGE analyses with three different restriction enzymes allowed, for example, the clear exclusion of strains specifically recovered from cheeses and their environment (which differed from the epidemic strain sensu stricto only in the profiles obtained after digestion with *Sma*I) as vehicles of transmission of outbreak-related infections.

As far as serotyping, phage typing, DNA macrorestriction pattern analysis (PFGE), ribotyping (unpublished data), and multilocus enzyme analysis (2a) are concerned, this epidemic strain is phenotypically and genomically closely related to the strains responsible for the outbreaks in California, Denmark, and Switzerland and, to a lesser extent, to the second strain involved in the Swiss outbreak and the strain from a previous French outbreak (7). This observation confirms that most major listeriosis outbreaks were caused by a group of closely related strains, as previously demonstrated with DNA pattern analyses and multilocus enzyme analysis (1, 7, 33, 44). Despite numerous studies related to the molecular mechanisms of *L. monocytogenes* virulence (35), strain-specific differences in terms of epidemiogenicity are not known.

No human case of infection was caused by pulsovar 2/26/3 strains, which were exclusively isolated from cheeses and their relative environment during the outbreak. This could be an additional observation of *L. monocytogenes* strains that are very rarely pathogenic (or nonpathogenic) for humans, as previously suggested by the results of serotyping (13, 14), multilocus enzyme analysis (3, 32, 43), and ribotyping (21). Because virulence studies in mice with strains of various types (5, 9, 11, 16, 34) failed to identify such a correlation between low-virulence strains and certain typing characteristics, further studies with new methodologies are required to identify markers specific for these strains.

The conclusions of the case-control study were clearly correlated with the results from microbiological analyses (8, 18, 19). The epidemic strain was precisely identified on the basis of PFGE analyses soon after the beginning of the outbreak, and this rapidly eliminated cheeses as a possible vehicle, because most of the cheese isolates had a different pulsovar (2/26/3). This finding further focused investigations on delicatessen items. These results corroborated those of the first step of the case-control study (8). Later, the statistical association (odds ratio, 9.2; 95% confidence interval, 3.8 to 22.4) found between patients and consumption of pork tongue in jelly of brand A was supported by the detection of the epidemic strain sensu stricto in samples of this food (19). Pork tongue in jelly was not found in any of the patients' refrigerators because it is rapidly consumed; therefore, it could not be sampled. In addition, because of the lag time between the consumption of the contaminated foods and the subsequent diagnosis of the infection, the foods have usually disappeared from the patients' refrigerators. The role of the various delicatessen items contaminated with this strain sampled at retailers was documented by the last step of the case-control study: among patients who did not eat this pork tongue in jelly, listeriosis was associated with the consumption of foods handled in stands where pork tongue brand A was sold (18). However, the possible role of some packaged delicatessen items or rarely other products contaminated by the epidemic strain sensu stricto in this outbreak was not elucidated. Perhaps they had the same significance as foods contaminated with this strain in the previous years, whereas no outbreak was detected at that time, or perhaps they represented an amplification of food contamination by healthy carriers for example.

A surveillance system specific for listeriosis at the national level based on laboratory analyses of human isolates was of major importance in detecting the outbreak, first because of the very low attack rate and the wide geographical distribution and later to identify precisely epidemic cases and to detect foods contaminated with the epidemic strain. Routine surveillance of human cases of infection and outbreak investigations require a combination of different typing methods appropriate to each step of the analysis. According to our experience, phage typing as a phenotypic method and DNA macrorestriction patterns analysis (PFGE) as a genomic method appeared to be the most suitable and the most discriminative methods during this outbreak. Since 1989, various molecular typing methods have been applied to *L. monocytogenes*, including multilocus enzyme analysis (1, 31–33, 43), ribotyping (20, 21, 29), DNA micro- and macrorestriction pattern analysis (4, 12, 30, 44), and, more recently, random amplification of polymorphic DNA (RAPD) (10, 25), and these methods have proved to be useful during epidemiological investigations. For coordination of the use of these new methods and better handling of the large body of results accumulated during the past 10 years, a multicenter study on *L. monocytogenes* typing was initiated by the World Health Organization (Food Safety Unit, Geneva, Switzerland) in order to select and standardize the most appropriate methods and to define a common nomenclature for varieties. The results of this study, which involves 27 laboratories, will greatly help further epidemiological investigations toward a better understanding of food-borne transmission of listeriosis.

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