INTRODUCTION

Human listeriosis can occur either as a sporadic disease (probably most often of foodborne origin) or as an outbreak. Most cases of listeriosis are considered to be single cases. The incubation period and infective dose have not been firmly established, although they may be inversely related. Reported incubation times vary from a few days to 2–3 months. Outbreaks may therefore easily be missed. The annual rate varies from 2 to 15 cases per million population (Rocourt & Bille, 1997). However, the high-risk population is increasing (elderly, immunocompromised patients, transplant patients, HIV-positive patients), particularly in the Western hemisphere. Likewise, the potential sources of listeriosis from contaminated food have also increased because of major changes in food production, preservation and consumption. The identification and investigation of outbreaks of human listeriosis require knowledge of the microbiological characteristics of clinical and environmental isolates. Various methods have been developed for such investigations: serotyping (Seeliger & Hohne, 1979), bacteriophage typing (Loesser & Busse, 1990), rDNA fingerprinting (Graves et al., 1991), restriction fragment length polymorphism analysis (Ridley, 1995), multilocus enzyme electrophoresis (Selander et al., 1986), pulsed-field gel electrophoresis (PFGE) (Brosch et al., 1991) and randomly amplified polymorphic DNA patterns (Mazurier et al., 1992).

The Belgian Listeria Reference Centre receives between 30 and 50 human clinical strains of Listeria monocytogenes per year. The aim of this study was to investigate clonal relationships between clinical strains of L. monocytogenes isolated in Belgium in 2001. All strains were subtyped by PFGE. This technique was preferred because it has been established that PFGE is a highly discriminatory and reproducible method for subtyping L. monocytogenes (Brosch et al., 1996). In addition, this technique was chosen by the US PulseNet, a national network of public health laboratories that fingerprint foodborne bacteria, such as L. monocytogenes (Swaminathan et al., 2001).

METHODS

Forty-eight strains of L. monocytogenes isolated from human cases of listeriosis in Belgium in 2001 were available for investigation. The strains were serotyped according to the reference method (Seeliger & Hohne, 1979). Strains were further subtyped on the basis of arsenic and cadmium susceptibility according to McLauchlin et al. (1997) with minor modifications: the results were read after 48 h instead of overnight incubation because of delayed growth with some strains. The protocol of Graves & Swaminathan (2001) for subtyping L. monocytogenes by macrorestriction and PFGE was used. DNA restriction fragments in plugs were separated by electrophoresis on a Gene Navigator PFGE apparatus (Pharmacia-LKB). DNA restriction fragments were sized against lambda DNA ladder (Boehringer). L. monocytogenes strain ATCC 51777 was used as the standard/reference strain. Analysis of banding patterns was performed with ImageMaster video documentation system (Amersham Pharmacia Biotech) and ImageMaster 1D software, version 4.00 (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

Serotyping

Of the 48 strains of L. monocytogenes investigated, 26 belonged to serovar 4b, 18 to serovar 1/2a and four to serovar...
1/2b. This partition is not unusual because most (> 95 %) human infections are caused by strains belonging to serovars 4b, 1/2a and 1/2b (Graves et al., 1999). Therefore, serotyping alone is of limited value in epidemiological investigations.

**Arsenic and cadmium susceptibility**

Subtyping based on arsenic and cadmium susceptibility is easy to perform and can subdivide strains grouped within the same serovar. The results for 48 clinical strains of human origin are shown in Table 1. Strains belonging to serovar 4b could be subdivided into four biotypes: resistant to arsenic and cadmium (A^bC^b), resistant to arsenic and sensitive to cadmium (A^bC^s), sensitive to arsenic and cadmium (A^sC^b) and sensitive to arsenic and resistant to cadmium (A^sC^s). It is noteworthy that no arsenic-resistant strains were detected among members of serogroup 1/2. McLauchlin et al. (1997) also reported small numbers of serogroup 1/2 clinical isolates resistant to arsenic.

**Pulsotyping**

Strains of *L. monocytogenes* were grouped on the basis of serovar and arsenic-cadmium resistance for PFGE. All isolates displayed Ascl and Apal restriction endonuclease digestion profiles (Fig. 1). Ascl generated 6–12 major fragments ranging in size from approx. 25 to 675 kb, while Apal-digested DNA generated digestion profiles with 8–21 major fragments ranging in size from 20 to 860 kb.

PFGE data are presented in Table 1. For every serovar-metal resistance subtype, the numbers of different Ascl and Apal profiles are given and also the number of pulsotypes. Different profiles were defined by a change of at least one band (i.e. a missing band, an extra band or migration to a different position). Of the 48 clinical strains, 34 different Ascl profiles and 38 different Apal profiles were encountered, resulting in 39 different pulsovars (81 %). There were no common pulsosvars across the different serotype and metal-resistance groupings. The large number of different pulsovars indicated that a great variety of genetically distinct strains cause listeriosis in Belgium. It also points indirectly to a low degree of listeriosis outbreaks: only 14 strains of *L. monocytogenes* were involved in likely episodes of listeriosis (three episodes involved two cases, one episode comprised three and one episode comprised five clinical cases).

The high percentage of pulsovars in this study is in line with published data for clinical cases. Table 2 includes data from

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**Table 1. Differentiation of clinical strains of *L. monocytogenes* based on serovar, metal resistance and PFGE**

<table>
<thead>
<tr>
<th>Serovar</th>
<th>Metal resistance</th>
<th>Strains (n)</th>
<th>Different PFGE profiles (n)</th>
<th>Different pulsovars (n)</th>
<th>Ascl</th>
<th>Apal</th>
</tr>
</thead>
<tbody>
<tr>
<td>4b</td>
<td>A^bC^b</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>A^bC^s</td>
<td>11</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A^sC^b</td>
<td>9</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A^sC^s</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>1/2a</td>
<td>A^bC^b</td>
<td>7</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A^bC^s</td>
<td>11</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A^sC^b</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 1.** PFGE patterns for Ascl and Apal of representative *L. monocytogenes* isolates for each of the five identified listeria episodes. Lanes 1–5, isolates from episodes I–V; lane 6, reference strain ATCC 51777.
Three metal-resistance profiles were seen: ARCS (episode I), linked to strains of serovar 4b, and two to serovar 1/2a strains. The number of infected people was small. Three episodes were reported in Belgium. However, a general outbreak was not likely, as the biotype was isolated from more than one person in the community. Epidemiological evidence is generally flimsy at best. Based upon serotyping, metal-resistance profiles and pulsotyping, the Listeria Reference laboratory found clonal relatedness between strains, thereby suggesting the possibility of episodes of listeriosis. PFGE demonstrated the greatest discrimination of the three assays. Information on the patient (age, gender, one occasion, ice cream cake was identified as the food vehicle. Fig. 1 shows the representative profiles of each episode.

Epidemiological evidence for the episodes was not always apparent. The most prominent epidemiological evidence was obtained for two patients from episode I. Two strains (serovar 4b) were isolated from elderly patients at the same hospital on the same day. There was no obvious link with the third patient of episode I. She was younger (48 years old) and lived in another province. However, the same strain was isolated at the same time. The second episode is an example of an episode with no epidemiological evidence. The strains (serovar 4b) were isolated from a cancer patient (74 years old) and a neonate, 5 months, and 50 km apart. This incident underlines the persistence and/or spread of L. monocytogenes in the community. Episode III concerned two immunocompromised women aged 57 and 62. The same strain (serovar 1/2a) was isolated in different provinces, 6 weeks apart. No common source of infection was seen in episode IV. Five elderly men were infected by the same strain (serovar 1/2a). The strains were isolated over a period of 9 months, in four different provinces. One of the patients died of listeriosis complications. With episode V, the source of food contamination was traced. Two patients, one with meningitis and the other with gastroenteritis, attended a family party. Frozen ice cream cake was served, which subsequently was shown to contain L. monocytogenes (serovar 4b). The strains isolated from the food and from the two patients were identified as the same pulsovar.

It is clear from the description of these episodes that epidemiological evidence is generally flimsy at best. Based upon serotyping, metal-resistance profiles and pulsotyping, the Listeria Reference laboratory found clonal relatedness between strains, thereby suggesting the possibility of episodes of listeriosis. PFGE demonstrated the greatest discrimination of the three assays. Information on the patient (age, gender,

### Table 2. Differentiation of human clinical strains of L. monocytogenes using PFGE

Values in parentheses indicate percentages of isolates displaying a distinct pulsovar.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Strains (n)</th>
<th>Number of pulsovars (%)</th>
<th>Restriction enzymes employed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vela et al. (2001)</td>
<td>51</td>
<td>29 (57)</td>
<td>Apal, SmaI</td>
</tr>
<tr>
<td>Buchrieser et al. (1991)</td>
<td>14</td>
<td>13 (93)</td>
<td>Apal, SmaI, NolI</td>
</tr>
<tr>
<td>Brosch et al. (1991)</td>
<td>20</td>
<td>18 (90)</td>
<td>Apal, SmaI, NolI</td>
</tr>
<tr>
<td>Ojeniwi et al. (2000)</td>
<td>41</td>
<td>15 (37)</td>
<td>Apal</td>
</tr>
<tr>
<td>Gianfranceschi et al. (2002)</td>
<td>28</td>
<td>17 (61)</td>
<td>Apal, SmaI</td>
</tr>
<tr>
<td>Boerlin et al. (1997)</td>
<td>21</td>
<td>11 (52)</td>
<td>Apal, SmaI, XhoI</td>
</tr>
<tr>
<td>Louie et al. (1996)</td>
<td>51</td>
<td>22 (43)</td>
<td>Apal</td>
</tr>
<tr>
<td>Lozniewski et al. (2001)</td>
<td>11</td>
<td>10 (91)</td>
<td>Apal, SmaI</td>
</tr>
<tr>
<td>Nakama et al. (1998)</td>
<td>82</td>
<td>45 (55)</td>
<td>Apal, SmaI, AscI, Sse83871</td>
</tr>
<tr>
<td>This study</td>
<td>48</td>
<td>39 (81)</td>
<td>Apal, AscI</td>
</tr>
</tbody>
</table>

nine other studies. Great variation is seen in the number of pulsovars obtained in different studies. Several factors contribute to this phenomenon, (i) not all of the clinical strains used correspond to sporadic cases; (ii) the choice of macrorestriction endonucleases; and (iii) analysis protocol. The current work was the only study that used the PulseNet method described by Graves & Swaminathan (2001).

**The necessity of two restriction enzymes**

One hospital sent three strains of L. monocytogenes to the Listeria Reference Centre in a single month. The strains were isolated from elderly people, living in the same region. The three strains belonged to serovar 1/2b, an infrequent serovar among human clinical isolates. In addition, these strains presented the same metal-resistance profile (A<sup>B</sup>C<sup>R</sup>), suggesting an outbreak. PFGE, however, differentiated the three strains. Two of the three strains presented identical AscI profiles, but gave distinctive Apal profiles (four bands difference). On another occasion, two strains of L. monocytogenes serotype 4b (A<sup>A</sup>C<sup>R</sup>) isolated within the same period displayed identical Apal profiles but were differentiated by their AscI profiles, distinct by one band. Such a small difference indicates a single genetic event and thus suggests some relatedness. However, epidemiological data did not indicate an episode.

**Characteristics of listeria episodes**

PFGE identified five occasions when the same L. monocytogenes biotype was isolated from more than one person in Belgium. However, a general outbreak was not likely, as the number of infected people was small. Three episodes were linked to strains of serovar 4b, and two to serovar 1/2a strains. Three metal-resistance profiles were seen: A<sup>B</sup>C<sup>S</sup> (episode I), A<sup>A</sup>C<sup>R</sup> (episode IV) and A<sup>A</sup>C<sup>B</sup> (the remaining episodes). On nine other studies. Great variation is seen in the number of pulsovars obtained in different studies. Several factors contribute to this phenomenon, (i) not all of the clinical strains used correspond to sporadic cases; (ii) the choice of macrorestriction endonucleases; and (iii) analysis protocol. The current work was the only study that used the PulseNet method described by Graves & Swaminathan (2001).

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address, etc.) and on the isolation of the strain (time, hospital, biological fluid, etc.) alone is insufficient. More appropriate information must be gathered regarding the patients’ food habits by interviewing them. This information is not easy to obtain, especially in Belgium, where the Flemish- and French-speaking communities have their own health inspections. Furthermore, as identical strains are sometimes isolated after a relatively long interval of time, investigators may have difficulty identifying patient histories. On the other hand, the possibility of a coincidental episode must not be ignored. According to Autio et al. (2002), the recovery of indistinguishable pulsotypes may mislead the investigation towards establishing the vehicle of infection. Several food products may harbour identical strains without any form of cross-contamination or common infection source. Therefore, a suspected epidemic may actually consist of two or more independent sporadic cases. However, as long as there is a large variation of circulating pulsovars, the probability of dealing with a coincidental episode seems low.

Human listeriosis in Belgium involves a great variety of genetically different strains. However, on five occasions in 2001, indistinguishable strains were isolated from more than one patient, suggesting the existence of five different episodes of listeriosis. In four of the five episodes, the food vehicle was not determined, because of a lack of epidemiological evidence.

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REFERENCES


