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Annaëlle Kérouanton, Sylvie M. Roche, Muriel Marault, Philippe Velge, Anne-Marie Pourcher, et al.. Characterization of isolates of Listeria monocytogenes from sludge using pulsed-field gel electrophoresis and virulence assays. Journal of Applied Microbiology, 2010, 108 (4), pp.1380-1388. 10.1111/j.1365-2672.2009.04531.x . hal-02659493

**HAL Id: hal-02659493**

**https://hal.inrae.fr/hal-02659493v1**

Submitted on 30 May 2020

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Journal of
Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

# Characterization of isolates of *Listeria monocytogenes* from sludge using pulsed-field gel electrophoresis and virulence assays

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**Keywords**
*Listeria monocytogenes*, PFGE subtyping, sludge, virulence assay.

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2008/0468: received 18 March 9 2009, revised 30 June 2009 and accepted 18 August 2009

doi:10.1111/j.1365-2672.2009.04531.x

## Abstract

**Aims:** To study the diversity and virulence of *Listeria monocytogenes* isolated from sludge.
**Methods and Results:** A total of 60 isolates of *L. monocytogenes* from sludge were characterized by serotyping, PFGE typing and using *in vitro* and *in vivo* virulence assays. The PFGE patterns were compared with those of food and human isolates to determine whether specific group clones are associated with environmental samples. The 60 isolates gave 44 different combined *Apa*I/*Asc*I PFGE patterns. The PFGE patterns of most isolates were similar or very similar to those of epidemic isolates. The majority (93%) of isolates were found to be virulent by plaque-forming assay and by mouse virulence assay.
**Conclusions:** Our findings suggest that *L. monocytogenes* strains found in non-sanitized sludge are virulent and represent a potential health hazard. Although no case of listeriosis related to sludge spread onto agricultural land has been reported, particular attention to this pathogen is needed.
**Significance and Impact of the study:** This is the first study dealing with the characterization of *L. monocytogenes* isolates from non-sanitized sludge samples by molecular typing methods and *in vitro* and *in vivo* virulence assays. Our findings provide relevant information for evaluating the health risks associated with spreading sludge onto agricultural land.

## Introduction

In France, around 900 000 ts dry matter of sludge are applied to agricultural land each year. Various treatment methods, including sedimentation, anaerobic digestion, liming and composting, are used to stabilize or treat sludge. However about 15% of sludge spread onto agricultural land is spread without any sanitation procedure except storage for 3–6 months.

Sludge can be regarded as a reservoir of pathogens including many zoonotic bacteria (De Luca *et al.* 1998; Sahlstrom *et al.* 2004). As a consequence, spreading sludge on to arable land entails a risk of crop contamination and the transmission of disease to people and animals.

One of these pathogens is *Listeria monocytogenes*. This Gram-positive, facultative, intracellular pathogen is capable of causing severe food-borne infections in animals and humans. In 2003, we showed that the density of *Listeria monocytogenes* in non-sanitized sewage sludge may be between 1 and 240 MPN $g^{-1}$ dry matter (Garrec *et al.* 2003).

A possible agricultural route for human exposure has been hypothesized through the ingestion of uncooked food crops grown in soil irrigated with contaminated water and/or fertilized with *Listeria*-contaminated manure or biosolids. Nevertheless, few studies have dealt with the

distribution and characteristics of environmental isolates of *Listeria monocytogenes* (Fugett *et al.* 2007; Lyautey *et al.* 2007a,b; Ho *et al.* 2007).

*Listeria monocytogenes* strains from sewage sludge have not been studied extensively, and typing of these environmental strains has often been limited to serotyping (De Luca *et al.* 1998; Lozniewski *et al.* 2001; Garrec *et al.* 2003). However, typing of *L. monocytogenes* from environmental samples is important for evaluating their diversity and virulence potential, for identifying sources of contamination and routes of spread and, finally, for determining any health risk.

Although the molecular typing of *L. monocytogenes* isolates from environmental samples has not been described, several phenotypic and genotypic typing methods have been extensively used to subtype *L. monocytogenes* from human clinical cases or food and food-processing environment samples. The methods used include phage typing, electrophoretic typing of esterases, multilocus enzyme electrophoresis (zymotyping), ribotyping, random amplification of polymorphic DNA and pulsed-field gel electrophoresis (PFGE) (Kerouanton *et al.* 1998). PFGE is recognized as being more discriminatory and reproducible than the other techniques.

The virulence of *L. monocytogenes* isolates is diverse (Farber and Peterkin 1991), and it would be useful to be able to determine the virulence of strains in the environment. To measure the virulence of *L. monocytogenes* strains, several methods have been described, including both *in vivo* and *in vitro* tests. Roche *et al.* (2001) developed a method – based on combination of a plaque-forming assay (PFA) on human colon adenocarcinoma cell line HT-29 and subcutaneous inoculation of mice – for quantification of the virulence of *L. monocytogenes* strains. The *in vitro* test allows the analysis of a large number of strains with a good reproducibility in a short time (24 h). Then, only the low-virulence strains need be confirmed by the *in vivo* test in mice. We used this methodology to determine the virulence of the strains isolated from the sludge.

The aims of this work were: (i) to study the diversity of environmental isolates of *L. monocytogenes* using PFGE and virulence assays, (ii) to compare the PFGE patterns with those of food and epidemic isolates and thereby assess whether there are relationships between the isolates from the various sources and (iii) to determine whether particular genetic groups were dominant among sludge isolates.

## Materials and methods

### Bacterial strains

A total of 60 strains of *L. monocytogenes* were analysed. The strains were isolated between 1999 and 2002 from different types of sludge collected from different sewage treatment plants (STP) in Angers and its vicinity, as previously described by Garrec *et al.* (2003). Isolates were obtained by monthly analyses of sludges. 'Clones' were avoided by the selection of only one colony per sludge sample.

*Listeria monocytogenes* isolates were preserved frozen at −80°C in cryobeads (AES, Combourg, France). For each isolate, one bead was transferred to Oxford agar (Difco, Becton Dickinson, Le Pont de Claix, France) and incubated overnight at 37°C prior to DNA extraction.

For the virulence assays, *L. monocytogenes* strains were maintained in a storage medium (Bio-Rad, Marne la Coquette, France) at 4°C, and then cultured in brain-heart-infusion broth (Difco) at 37°C for 8 h, and BHI agar (Difco) slopes were seeded with bacterial suspensions and incubated overnight at 37°C.

### Serotyping

Strains were serotyped according to the method of Kerouanton *et al.* (1998), using the procedures described by Seeliger (1979). Some serotypes were confirmed by an improved PCR method based on that published by Doumith *et al.* (2004).

### Pulsed-field gel electrophoresis

Bacterial cultures for DNA isolation were grown on TSA-YE plates at 37°C for 24 h. Bacterial DNA was prepared by extracting chromosomal DNA according to the CDC PulseNet standardized procedure for typing *L. monocytogenes* (Graves and Swaminathan 2001) and digested at 37°C for 4 h with two different macrorestriction enzymes, *Apa*I (Amersham Pharmacia Biotech, Orsay, France) or *Asc*I (New England Biolabs, Ozyme, Saint Quentin en Yvelines, France). Restriction fragments were separated in a 1% SeaKem Gold agarose (Cambrex Bio Science, Verviers, Belgium) gel, using the CHEF method in a CHEF-DRIII (Bio-Rad SA) apparatus. The following electrophoresis conditions were used: voltage, 6 V $cm^{-1}$; initial switch time, 4 s; final switch time, 40 s; run time, 21 h. *Asc*I-digested DNA from *L. monocytogenes* H2446 was included, as a reference, in all PFGE gels.

Banding patterns were visualized under UV light. The patterns were categorized and analysed by dendrogram analysis using the Dice coefficient and data clustering by the unweighted pair-group arithmetic averaging method with Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium). A tolerance of 1% in the band position was applied.

### Calculation of discrimination indices

For each serotype, the ability of the method to discriminate between subtypes was assessed by calculating Simpson's index of diversity (*D* value), as previously described (Hunter and Gaston, 1988).

### Quality assurance

Serotyping and PFGE typing analyses were performed in accordance with the standard NF EN ISO 17025 'General requirements for the competence of testing and calibration laboratories'.

### Virulence assay

*Plaque-forming assays*

The *in vitro* test of virulence based on PFA was conducted as previously described by Roche *et al.* (2001). Briefly, the human adenocarcinoma cell line HT 29 [European Collection of Animal Cell Cultures (ECACC) no. 85061109, Salisbury, UK] was used between passages 24 and 42. Cells were grown in 75 $cm^2$ plastic tissue culture flasks (Nunc, Life Technologies, Cergy Pontoise, France) in culture medium (Dulbecco's Modified Eagle medium 4·5 g $l^{-1}$ glucose, Life Technologies) supplemented with 10% (v/v) foetal calf serum (Life Technologies) and 2 mmol $l^{-1}$ L-glutamine (Life Technologies). Antibiotics (100 IU $ml^{-1}$ penicillin, 100 $\mu g$ $ml^{-1}$ streptomycin; Sigma) were routinely added to the culture medium except for the virulence assays. Cells were maintained in a humidified incubator at >90% relative humidity (Heraeus, les Ulis, France) at 37°C under 5% (v/v) $CO_2$. Trypsinized HT-29 cells ($3 \times 10^4$) were deposited in 96-well tissue culture plates (Falcon, AES, Combourg, France) and cultured in a culture medium with antibiotics to obtain a constant proliferation rate. The cells were incubated for 4 days to obtain almost confluent monolayers and then in the medium without antibiotics for 24 h. The *L. monocytogenes* strains were grown overnight on BHIA slopes, and bacterial concentrations were standardized turbimetrically and diluted appropriately in DMEM. Cells were infected with 2- to 7-log *L. monocytogenes* strains per well and incubated for 2 h at 37°C in a humidified incubator, then for 1 h 30 min in the presence of 100 $\mu g$ $ml^{-1}$ gentamicin (Sigma) in culture medium. Each well was then overlaid with an agarose gel containing 0·48% indubiose (Serva, BioWhittaker, Fontenay Sous Bois, France) in a culture medium supplemented with 10 $\mu g$ $ml^{-1}$ gentamicin. The same medium was then added to prevent cell starvation.

The numbers of plaques were counted with an inverted microscope after an overnight incubation. In each experiment, the *L. monocytogenes* EGDe was used as positive control and the *L. innocua* BUG 499 was used as negative control. Experiments were carried out in duplicate and repeated twice for each strain. Culturable strains deposited on 96-well tissue culture plates were counted on TSA plates. The results are expressed as the number of plaques obtained for 7-log *L. monocytogenes* cells deposited per well.

*Mouse virulence assays*

A mouse virulence assay was used to confirm the results obtained by PFA with nonpathogenic or weakly pathogenic strains of *L. monocytogenes*. This assay was conducted as previously described by Roche *et al.* (2001). Animals were handled humanely according to established protocols (SR-2005-03) under the responsibility of an authorized person (S. Roche, ''Certificat d'autorisation d'expérimenter sur des animaux vivants'', Agreement no. 37-017). Briefly, 7-week-old conventional Swiss female mice were used (Iffa-Credo, Saint-Germain-sur l'Arbresle, France). They were maintained on sterilized wood shavings with free access to water and sterilized food. Briefly, 4-log CFU was injected s.c. into the left hind footpad of five mice for each strain. The mice were maintained in a controlled atmosphere during the experiments and killed by cervical dislocation 3 days after s.c. inoculation. Spleens were removed and rapidly homogenized. Homogenized spleens were serially diluted, and viable bacteria were counted on TSA plates. The average numbers of bacteria per spleen were calculated.

## Results

### Serotyping

The 60 isolates were grouped into six serotypes. The most prevalent serotypes were 4b (31 strains), 1/2a (13 strains) and 1/2b (12 strains). The other four strains were serotype 3b or 3c (Table 1).

### PFGE

All strains were typeable by PFGE and we observed a great genetic diversity among the 60 environmental isolates. DNA digested with *Apa*I gave 12–18 fragments from 30 to 540 kb and DNA digested with *Asc*I gave 8–13 fragments, from 28 to 700 kb. A total of 39 different PFGE patterns (*D* = 0·97) were generated by *Apa*I; 16 of these patterns have not previously been described in PFGE database built in the AFSSA laboratory since 1998. *Asc*I gave 28 different PFGE patterns (*D* = 0·92), ten of which were novel in the AFSSA PFGE database. Using results with the two enzymes, we describe 43 different


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**Table 1** Results of typing of 60 *L. monocytogenes* isolates from sewage sludge

| Listeria strains | STP | Type of sample | Serotype | *Apa*I pattern | *Asc*I pattern | Virulence pattern: Plaque-forming assay (PFA)* | MVA Spleen† | Mice‡ | Status |
|---|---|---|---|---|---|---|---|---|---|
| *L. innocua* | | | | | | 0 | 0 | 0/5 | |
| EGDe | – | Animal | 1/2a | 5 | 128 | 5·51 ± 0·45 | 5·23 ± 0·64 | 5/5 | V |
| LSEA 00-25 | D | Stored sludge | 4b | 103b | 3a | 6·39 ± 0·03 | | | |
| LSEA 00-14 | C | Dewatered sludge | 4b | 106 | 3a | 4·94 ± 0·36§ | 0 | 0/5 | LV |
| LSEA 02-09 | C | Sludge | 4b | 109 | 3 | 4·15 ± 0·35 | 3·5 ± 0·51 | 5/5 | V |
| LSEA 00-26 | D | Stored sludge | 4b | 11 | 7 | 6·31 ± 0·09 | | | |
| LSEA 02-22 | B | Stored sludge | 4b | 37 | 38b | 6·28 ± 0·17 | | | |
| LSEA 01-153 | A | Compost | 4b | 37f | 38 | 6·15 ± 0·24 | | | |
| LSEA 01-184 | C | Dewatered sludge | 4b | 4a | 3 | 4·73 ± 0·05 | | | |
| LSEA 02-01 | C | Activated sludge | 4b | 4d | 3? | 5·37 ± 0·22 | | | |
| LSEA 02-10 | C | Dewatered sludge | 4b | 4d | 3 | 6·15 ± 0·29 | | | |
| LSEA 01-160 | B | Stored sludge | 4b | 4e | 3a | 6·33 ± 0·22 | | | |
| LSEA 02-12 | A | Activated sludge | 4b | 61 | 3 | 4·22 ± 0·20 | 4·2 ± 0·24 | 4/5 | V |
| LSEA 99-15 | C | Activated sludge | 4b | 61 | 3 | 4·81 ± 0·17 | | | |
| LSEA 99-38 | C | Sludge | 4b | 61 | 3? | 6·06 ± 0·16 | | | |
| LSEA 00-2 | C | Dewatered sludge | 4b | 61 | 3 | 2·90 ± 0·36 | 4·29 ± 0·34 | 3/5 | LV |
| LSEA 00-7 | C | Dewatered sludge | 4b | 61 | 3 | 2·70 ± 0·06 | 5·28 ± 0·28 | 5/5 | V |
| LSEA 00-11 | C | Dewatered sludge | 4b | 61 | 3 | 4·22 ± 0·12 | 4·77 ± 0·07 | 5/5 | V |
| LSEA 00-8 | C | Dewatered sludge | 4b | 61? | 3 | 4·40 ± 0·24 | 4·89 ± 0·09 | 5/5 | V |
| LSEA 02-04 | A | Activated sludge | 4b | 61a | 3 | 5·53 ± 0·29 | | | |
| LSEA 02-05 | A | Stored sludge | 4b | 61a | 3 | 5·31 ± 0·34 | | | |
| LSEA 02-11 | A | Stored sludge | 4b | 61a | 3 | 5·32 ± 0·07 | | | |
| LSEA 99-19 | C | Activated sludge | 4b | 61a | 3 | 6·14 ± 0·13 | | | |
| LSEA 99-37 | C | Dewatered sludge | 4b | 61a | 3 | 5·25 ± 0·25 | | | |
| LSEA 02-03 | C | Dewatered sludge | 4b | 61a | 3 | 5·25 ± 0·05 | | | |
| LSEA 01-03 | C | Dewatered sludge | 4b | 61c | 3 ou 3b | 6·42 ± 0·08 | | | |
| LSEA 00-16 | A | Stored sludge | 4b | 61e | 3a | 6·00 ± 0·07 | | | |
| LSEA 01-08 | C | Activated sludge | 4b | 61f | 3b | 5·66 ± 0·33 | | | |
| LSEA 01-183 | C | Sludge | 4b | 61g | 3 | 5·97 ± 0·16 | | | |
| LSEA 02-19 | C | Dewatered sludge | 4b | 67 | 36 | 5·98 ± 0·15 | | | |
| LSEA 99-17 | C | Activated sludge | 4b | 9a | 11 | 4·90 ± 0·20 | | | |
| LSEA 00-5 | C | Dewatered sludge | 4b | 9a | 4b | 4·24 ± 0·22 | 4·70 ± 0·43 | 5/5 | V |
| LSEA 01-05 | C | Activated sludge | 4b | 9a | 11 | 6·32 ± 0·06 | | | |
| LSEA 99-8 | C | Sludge | 3c | 24c | 20 | 5·04 ± 0·17 | 2·99 ± 0·93 | 5/5 | V |
| LSEA 99-23 | C | Activated sludge | 3c | 24c | 20 | 3·78 ± 0·12 | 4·49 ± 0·89 | 3/5 | LV |
| LSEA 99-7 | C | Sludge | 3b | 58b | 44b | 5·38 ± 0·22 | | | |
| LSEA 99-9 | C | Sludge | 3b | 58b | 102 | 5·00 ± 0·24 | | | |
| LSEA 02-07 | B | Stored sludge | 1/2b | 10b | 92a | 4·81 ± 0·14 | | | |
| LSEA 02-14 | B | Activated sludge | 1/2b | 10b | 92 | 5·58 ± 0·18 | | | |
| LSEA 02-23 | B | Activated sludge | 1/2b | 110 | 92 | 6·11 ± 0·25 | | | |
| LSEA 99-2 | C | Sludge | 1/2b | 34b | 55 | 5·60 ± 0·16 | | | |
| LSEA 99-43 | C | Activated sludge | 1/2b | 34b | 44c | 5·46 ± 0·17 | | | |
| LSEA 00-28 | C | Dewatered sludge | 1/2b | 34b | 35d | 6·09 ± 0·21 | | | |
| LSEA 02-21 | A | Activated sludge | 1/2b | 34e | 59d | 6·11 ± 0·19 | | | |
| LSEA 02-08 | C | Activated sludge | 1/2b | 34f | 44c | 5·08 ± 0·31 | | | |
| LSEA 01-182 | C | Activated sludge | 1/2b | 58a | 81 | 5·62 ± 0·37 | | | |
| LSEA 02-18 | C | Stored sludge | 1/2b | 58a | 81 | 5·96 ± 0·22 | | | |
| LSEA 01-06 | C | Dewatered sludge | 1/2b | 58f | 59b | 6·20 ± 0·20 | | | |
| LSEA 99-24 | C | Activated sludge | 1/2b | 88 | 25b | 6·30 ± 0·13 | | | |
| LSEA 00-24 | D | Stored sludge | 1/2a | 107 | 9a | 5·69 ± 0·16 | | | |
| LSEA 01-173 | B | Stored sludge | 1/2a | 108 | 20 | 4·63 ± 0·34 | | | |
| LSEA 01-159 | B | Activated sludge | 1/2a | 14 | 27 | 5·10 ± 0·47 | | | |
| LSEA 00-22 | B | Stored sludge | 1/2a | 14a | 27 | 5·53 ± 0·20 | | | |
| LSEA 01-185 | B | Stored sludge | 1/2a | 22a | 30a | 4·04 ± 0·55 | 5·08 ± 0·06 | 5/5 | V |

**Table 1** (Continued)

| Listeria strains | STP | Type of sample | Serotype | *Apa*I pattern | *Asc*I pattern | Virulence pattern: Plaque-forming assay (PFA)* | MVA Spleen† | Mice‡ | Status |
|---|---|---|---|---|---|---|---|---|---|
| LSEA 02-06 | B | Activated sludge | 1/2a | 22a | 30a | 3·55 ± 0·26 | 4·77 ± 0·47 | 5/5 | V |
| LSEA 02-17 | B | Activated sludge | 1/2a | 22a | 30a | 4·93 ± 0·15 | | | |
| LSEA 01-10 | A | Stored sludge | 1/2a | 36 | 37a | 5·51 ± 0·17 | | | |
| LSEA 99-4 | C | Sludge | 1/2a | 48 | 101 | 4·38 ± 0·16 | 3·67 ± 0·81 | 3/5 | LV |
| LSEA 01-186 | B | Activated sludge | 1/2a | 53 | 51b | 5·28 ± 0·11 | | | |
| LSEA 01-180 | A | Compost | 1/2a | 65c | 28 | 3·74 ± 0·11 | 4·79 ± 0·26 | 5/5 | V |
| LSEA 99-1 | C | Sludge | 1/2a | 90a | 68 | 4·83 ± 0·17 | | | |
| LSEA 99-16 | C | Activated sludge | 1/2a | 91a | 73a | 4·98 ± 0·20 | | | |

STP, sewage treatment plant; V, virulence; LV, low virulence; MVA, mouse virulence assay.

*Log numbers of plaques per $10^7$ CFU deposited. Values are means from three independent experiments performed in duplicate.

†Log numbers of *L. monocytogenes* recovered from spleens 3 days after s.c. injection of $10^4$ CFU in 50 $\mu$l into the left hind footpad of immunocompetent Swiss mice; values are for contaminated mice.

‡Ratio of infected mice to inoculated mice in the s.c. test.

§Plaques were only detectable after 48 h.

PFGE patterns, and 23 of which were novel in the AFSSA PFGE database.

Strains of serotype 4b were classified into 19 combined *Apa*I/*Asc*I PFGE patterns (Table 1). Two combined *Apa*I/*Asc*I PFGE patterns (61/3 and 61a/3) predominated, with six strains each. These two patterns are closely related (96% similarity). The next most prevalent combined *Apa*I/*Asc*I PFGE pattern was 9a/11, found for two strains. Strains of serotype 1/2a, gave 11 combined *Apa*I/*Asc*I PFGE patterns, with pattern 22a/30a being the most common (three strains). Serotype 1/2b strains gave ten PFGE patterns with only one *Apa*I/*Asc*I PFGE pattern (58a/81) being found for two strains. Both serotype 3c strains gave the same combined *Apa*I/*Asc*I PFGE pattern (24c/20). The other combined *Apa*I/*Asc*I PFGE patterns were each represented by only a single isolate.

The PFGE patterns obtained in this study were compared with those in the AFSSA LERQAP PFGE database. This database is essentially constituted by patterns obtained from food strains, food production environment and animal or human cases. The PFGE method is very discriminative for the study of *L. monocytogenes* strains, in particular for 1/2a strains, and more than 300 different patterns are recorded in this database for each enzyme tested, *Apa*I and *Asc*I. Some patterns obtained in this study appeared to be specific to sludge samples with 23 new combined PFGE patterns. Lyautey *et al.* (2007b) also found more than 50% of new combined PFGE patterns (13/21), when they analysed the PFGE patterns from *L. monocytogenes* isolates from surface water. In this study, there were however, eleven of the 43 patterns for *L. monocytogenes* isolates from sludge, which were the same as those for strains previously isolated from milk products, fish products, meat products, prepared food, the environment, or animal or human cases (Fig. 1). In particular, 64·5% (20/31) of the serotype 4b strains had combined *Apa*I/*Asc*I PFGE patterns similar or closely related to those for sporadic or epidemic human strains.

Different *L. monocytogenes* isolates with identical PFGE patterns were generally recovered on several occasions from the same STP. The isolates LSEA 99-8 and LSEA 99-23 were isolated from the sludge of the STP C at the 15-day interval, isolates LSEA 99-17 and LSEA 01-05 were isolated from the sludge of the STP C after an interval of 40 days, isolates LSEA 01-182 and LSEA 02-18 were isolated from the sludge of the STP C after an interval of 15 months, and isolates LSEA 01-185, LSEA 02-06 and LSEA 02-17 were isolated from the sludge of the STP B over a period of 75 days. The isolates with combined *Apa*I/*Asc*I PFGE patterns 61a/3 and 61/3 were isolated from the STP A and C over a period of 14 months.

*Virulence assay*

A virulence test combining a PF assay and SC inoculation of mice allowed us to estimate the level of virulence of the *L. monocytogenes* strains (Roche *et al.* 2001). For the screening assay, a cut-off at 3·3-log plaques was determined by CHAID (Kass 1980) and CART (Breiman *et al.* 1984) analyses to determine which strains ought to be considered as having potentially low-virulence. To avoid the elimination of low-virulence strains, we usually extended this value to 4·4 log in the screening assay. *In vivo* assays were performed only when results obtained from *in vitro* assays were inferior or equal to 4·4 log. *L. monocytogenes* strains were considered as low-virulence strains, when fewer than four mice were contaminated with a mean number of bacteria in the spleen inferior to 3·45 ± 0·77 log (Roche *et al.* 2001).


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| *Apa*I PFGE patterns | *Asc*I PFGE patterns | Human | Milk products | Fish products | Delicatessen /meat products | Prepared food | Environ-ment | Breeding |
|---|---|---|---|---|---|---|---|---|
| 34b | 55 | | | X | | | | |
| 37 | 38b | X | X | | X | X | X | |
| 61a | 3 | | X | X | X | X | | |
| 4 | 3 | X | X | X | X | | | |
| 9a | 11 | | X | | | X | | |
| 11 | 7 | X | X | | X | | X | X |
| 58b | 44b | | X | X | X | | | |
| 58a | 81 | | | | X | X | X | |
| 65c | 28 | | | X | X | X | | |
| 53 | 51b | X | X | X | X | X | X | X |
| 24c | 20 | | X | X | X | X | | X |

**Figure 1** Comparison of *Apa*I/*Asc*I PFGE patterns for *L. monocytogenes* strains isolated from sludge with those of the AFSSA LERQAP PFGE database.

Forty-five of the 60 strains tested (75%) gave more than log 4·40 plaques (log 4·40–log 6·39; see Table 1) and were therefore scored as virulent. The strains belonged to different serotypes, and there was no specificity: serotype 4b (24 isolates), 1/2a (eight isolates) 1/2b (11 isolates), 3b (two isolates) and 3c (one isolates).

Fifteen strains have to be considered as potentially of low-virulence. Among these, the strain LSEA 00-14 formed small plaques observed only after 48 h of incubation. A mouse virulence assay was used to confirm the virulence. Four strains were identified as low-virulence (LSEA 00-14, LSEA 00-2, LSEA 99-23 and LSEA 99-4, of serotypes 4b, 3c and 1/2a respectively). These four strains have very different PFGE patterns, two of which (LSEA99-4 pattern 48/101 and LSA 00-14 pattern 106/3a) were not found among virulent strains (more than log 4·40 plaques) or in the AFSSA LERQAP PFGE database.

## Discussion

*Listeria monocytogenes* is a Gram-positive rod that causes listeriosis, an invasive disease with the highest case-fatality rate of any food-borne illnesses (20–30%) (Farber and Peterkin 1991). This bacterium is widely distributed in the environment and may be found in decaying vegetation, soil and the agricultural ecosystem (Welshimer 1960, 1968; Welshimer and Donker-Voet 1971; Welshimer 1975; Seeliger 1979; Van Renterghem *et al.* 1991), in waste water (Watkins and Sleath 1981; Geuenich *et al.* 1985; Al-Ghazali and Al-Azawi 1986) and treated water (Al-Ghazali and Al-Azawi 1986; Paillard *et al.* 2005) and in sludge spread onto agricultural land (Watkins and Sleath 1981; Al-Ghazali and Al-Azawi 1986; Schwartzbrod *et al.* 1989; Lozniewski *et al.* 2001; Paillard *et al.* 2005).

Between 1999 and 2002, we isolated *L. monocytogenes* with a frequency of 88% in non-sanitized sludge intended for direct spreading. Although concentrations observed were relatively low—from 1 to 240 NPP $g^{-1}$ dry matter (Garrec *et al.* 2003)—the contamination of crops and possible spread of *L. monocytogenes* into the human food chain is a risk that should be considered.

Sixty *L. monocytogenes* isolates collected during this sampling programme were examined. They belonged to serotype 4b (52%), 1/2a (22 %), 1/2b (20%), 3b (3%) and 3c (3%). This agrees with the findings of De Luca *et al.* (1998), Lozniewski *et al.* (2001) and Paillard *et al.* (2005) who reported that serotypes 4b, 1/2a and 1/2b are common in sludge obtained from the STPs of Bologna (Italy), Nancy and the Bordeaux area (France), respectively.

The strain populations we analysed were genetically heterogeneous. The 60 isolates included 43 subtypes identified on the basis of combined PFGE patterns obtained with *Apa*I and *Asc*I restriction enzymes; these enzymes have been described as the most useful for generating a convenient number of readily discernible macrorestriction fragments with *Listeria* sp. (Howard *et al.* 1992). Such heterogeneity of environmental isolates was previously described by Lozniewski *et al.* (2001) and Fugett *et al.* (2007). Lozniewski *et al.* (2001) have first reported the characterization of 32 strains of *L. monocytogenes* from sludge, using serotyping, phage typing and PFGE typing, and have identified 22 different PFGE patterns. Fugett *et al.* (2007) reported on the characterization of several isolates of *L. monocytogenes* from human clinical cases, foods, farms, urban and natural environments and

showed that urban and natural environment isolates grouped into the three linages, I (41·25 %), II (53·75%) and III (2·5%) usually used to subtype this species and generally overrepresented among human isolates, food isolates and animal listeriosis cases, respectively.

Comparisons of PFGE patterns of *L. monocytogenes* from sludge with those of food and clinical isolates in the AFSSA LERQAP PFGE database indicated that of the 31 serotype 4b strains, 64·5% (20/31) gave a similar or closely related combined *Apa*I/*Asc*I PFGE pattern to those for human sporadic or epidemic human strains without any geographical or temporally link. This observation agrees with the results obtained by Fugett *et al.* (2007) who showed that some *L. monocytogenes* PFGE types are widely distributed and appear to be stable.

We investigated the virulence of the 60 *L. monocytogenes* isolates using both *in vitro* and *in vivo* assays. Fifty-six strains were considered as virulent (93%), and four were scored as low virulence (7%). The proportion of the strains exhibiting a high virulence was greater than that reported in other studies dealing with the characterization of environmental isolates of *L. monocytogenes* (Lyautey *et al.* 2007a,b), who investigated the prevalence and characteristics of *L. monocytogenes* environmental isolates from livestock, wildlife human faecal sources and surface waters, and showed that only nearly half of their strains exhibited a potential virulence linked to the production of functional internalin A. *L. monocytogenes* isolates excreting a truncated internalin A and were found to be less virulent than the others according to CaCo-2 cells. To confirm that pathogenic potential is directly conditioned by the efficiency of the cell wall-associated internalin A (Olier *et al.* 2005), it would be interesting to study the structure of the inlA gene of the strains described on this article to verify the hypothesis that low virulence strains have a truncated inlA gene.

Several authors (Farber and Peterkin 1991; Hof and Rocourt 1992) have reported that *L. monocytogenes* strains isolated from food industries exhibit heterogeneous levels of virulence and about 10–20% are weakly or nonvirulent as assessed by the experimental infection of immunocompromised or immunocompetent mice (Tabouret *et al.* 1991; Roche *et al.* 2001). The apparent rarity of low-virulence strains in environmental samples compared to the rate described for food isolates may be a consequence of the protocol used. Gracieux *et al.* (2003) suggested that *L. monocytogenes* strains exhibit different growth patterns on selective media according to their level of virulence, and low-virulence strains may be poorly detected by the two media used in this study (PALCAM and Rapid'L mono media).

In our study we recovered *L. monocytogenes* isolates with identical PFGE patterns on several sampling occasions from the same STP suggesting that: (i) these strains were widely excreted by population, or (ii) that these strains were able to proliferate or survive during the storage in a tank.

The survival of *L. monocytogenes* in such ecosystems has never been investigated; only in-vessel experimentation has been described (Garrec *et al.* 2005). Assuming that *L. innocua* and *L. monocytogenes* have the same behaviour, Girardin *et al.* (Girardin *et al.* 2005) introduced *L. innocua* into soil through organic fertilizers and assessed its persistence and transfer to plants (parsley) during cultivation over three successive years. They reported that *L. innocua* was not able to persist in the soil for more than 90 days and was not detected on parsley leaves beyond 30 days. They suggest that the rate of loss is mostly dependent on some intrinsic factor of the soil (type of soil, soil microbiota, temperature and humidity); however, this poor survival of *L. innocua* in soil was observed in a system in which there was no decaying vegetation or mud.

To our knowledge, this is the first report of the characterization of *L. monocytogenes* isolates from non-sanitized sludge samples by molecular typing methods and *in vivo* and *in vitro* virulence assays. Our findings provide relevant information for evaluating the health risks associated with spreading sludge on to agricultural land.

According to Girardin *et al.* (2005), the delay imposed by French regulations between the application of organic fertilizers and cultivation seems to be sufficient to prevent contamination of crops with *L. monocytogenes*. Nevertheless, because of the virulence status of strains isolated from sludge, sanitation is recommended before spreading on to agricultural land. This view is shared by Paillard *et al.* (2005), who recommended the hygienisation of sludge by composting under strictly supervised conditions or by liming. Currently, composting appears to be the most promising method for producing a hygienically safe product (Lemunier *et al.* 2005; Paillard *et al.* 2005) and may also be economically acceptable for small facilities (Pourcher *et al.* 2005).

## Conclusion

We report here that 64·5% of *L. monocytogenes* strains isolated from sludge have PFGE patterns similar or very similar to epidemic patterns and that 93·3% of the strains are virulent according to virulence testing combining *in vitro* and *in vivo* assays. This study suggests that the health risks associated with the presence of *L. monocytogenes* in sludge spread on to agricultural land should be assessed, especially in light of the continuing debate about the pertinence of regulations concerning the control of pathogens. This study provides relevant information for


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increasing the PFGE patterns database with environmental isolates to help better understand the diversity of this food-borne pathogen.

## Acknowledgement

This work was supported by the French Environment and Energy Agency (ADEME).

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