Characterization of *Listeria monocytogenes* isolates from human listeriosis cases, Italy

**Running title:** Epidemiology of human listeriosis cases, Italy

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Abstract

The objective of this study was to characterize by serotyping, pulsed field gel electrophoresis and polymerase chain reaction (PCR) amplification of virulence genes and markers of epidemic clones (EC) I, II and III 54 human isolates from apparently sporadic cases of infection occurring in the Lombardy region and in the province of Florence, Tuscany, Italy, in the years 1996-2007. L. monocytogenes isolates were provided by the clinical microbiology laboratories of the Lombardy region and the “Careggi” Hospital of Florence, Tuscany, Italy. Serotyping, PFGE after digestion with enzymes AscI and ApaI, PCR amplification for inlA, inlC and inlJ genes and EC I, II and III markers were performed according to previously described procedures.

Twenty-five (46.3%) L. monocytogenes isolates were attributed to serotype 1/2a, 23 (42.6%) to serotype 4b and 6 (11.1%) to serotype 1/2b. Thirty-one AscI pulsotypes were recognized among the 54 human isolates. Eleven molecular subtype clusters, of which eight included indistinguishable pulsotypes and three closely related pulsotypes, were shared by two to seven isolates. Unique AscI pulsotypes were showed by 15 isolates.

Three groups of clustered isolates and two apparently sporadic isolates generated EC amplicons. All strains tested positive for the genes inlA, inlC and inlJ. Based upon the results of serotyping and molecular typing, 11 occasions were detected when strains of L. monocytogenes with the same subtype were isolated from more than one listeriosis case. A total of 39 out of 54 isolates (72.2%) were attributed to molecular subtype clusters. The results of the study suggest that routine subtyping of L. monocytogenes strains from human listeriosis cases could allow to more timely detect possible common source foodborne outbreaks and control ongoing food exposures, thus preventing more cases to occur.
Introduction

Listeria monocytogenes (L. monocytogenes) is the etiological agent of listeriosis, a foodborne disease occurring primarily in immunocompromised individuals causing septicemia and central nervous system infections and in pregnant women who may suffer of preterm delivery, miscarriage or stillbirth (8, 19). Healthy adults may suffer a febrile gastroenteritis after ingesting large numbers of L. monocytogenes cells (25).

Most industrialized countries, including those within the European Union (EU), have an annual incidence of listeriosis between 2-10 reported cases per million people per year (13). However, listeriosis has a high case fatality rate that can exceed 30% (8, 29). Recently, several European countries are experiencing an apparently increasing incidence of listeriosis, mainly among persons aged 65 years and older (6, 13).

In Italy, notification of listeriosis cases is mandatory since 1993, but transmission of data to the national level is done quarterly. A second syndrome-based surveillance system is in place, that covers infections of the central nervous system. A National Reference Laboratory, that receives strains and epidemiological and clinical information on a voluntary basis, is also present. The incidence of reported cases by the Ministry of Health is lower than most EU countries, being equal in recent years (2004-2006) to 0.8 annual cases per million inhabitants (12, 16). This low incidence must be considered with care because of poor sensitivity of the universal passive surveillance system and the difficulties posed by L. monocytogenes infection epidemiology and natural history.

Indeed, a recent report about a one-year (2002-2003) period of enhanced laboratory-based surveillance showed an incidence of 1.3 cases per 1,000,000 inhabitants (12).

Throughout Europe and the United States, the majority of cases of listeriosis reported to Public Health authorities are apparently not linked to a common source and are, therefore, defined as sporadic (26, 28). However, because of the unique epidemiological and clinical characteristics of human foodborne listeriosis, traditional epidemiologic surveillance systems alone are unable to detect most common source outbreaks, particularly when a limited number of cases is scattered over
a wide geographic area (26, 27, 30). Furthermore, when attempting to trace food exposures and
transmission routes through food-processing chains, a further challenge is posed by the wide
spectrum of *L. monocytogenes* strains of which many are virulent and associated with significant
morbidity and mortality, whilst others are avirulent and unable to establish an infection within
mammalian hosts (1, 20). Consequently, role of rapid and discriminatory subtyping methods, such
as different DNA-based methods, e.g. pulsed-field gel electrophoresis (PFGE) and detection by
polymerase chain reaction (PCR) and restriction-fragment length polymorphism (RFLP) analysis
or sequencing of some specific sequences, is essential for the epidemiological investigations on *L.
monocytogenes* and tracking of specific clones along the food processing chains (5, 27, 30).
Recently, a rapid PCR-based method for detection of epidemic clones (EC) I, II and III of *L.
monocytogenes* has been reported by Chen and Knabel (2). Moreover, Liu *et al.* (21) have described
a multiplex PCR assay where a combined application of *inlA, inlC* and *inlJ* gene primers makes it
possible to rapidly determinate potential virulence.

Our study is a retrospective subtyping analysis of 42 human isolates identified from apparently
sporadic cases of infection in the Lombardy region and in the province of Florence, Tuscany, Italy,
in the years 2006-2007. Twelve further isolates from human cases occurring in the same geographic
areas since 1996 were available and included in the study. The objectives were to assess molecular
clustering of *L. monocytogenes* isolates by using serotyping and PFGE and evaluate distribution of
EC markers and genetic determinants of virulence within the human isolates under investigation.

**Materials and Methods**

**Bacterial isolates**

Between January 2006 and October 2007, 34 human isolates from cases of listeriosis were collected
from the laboratories of diagnostic microbiology of the Lombardy region, Italy (Tables 1 and 2).
Eight further isolates were recovered in the same period from the “Careggi” Hospital of Florence,
Tuscany, Italy (Tables 1 and 2). Twelve sporadic human isolates of *L. Monocytogenes*, that had been identified by the same laboratories since 1996, were also included in the study (Tables 1 and 2). The two geographical areas were chosen based upon the willingness of the regional health authorities of Lombardy and the laboratory microbiologists of the “Careggi” Hospital of Florence to participate in a collaborative study on *L. monocytogenes* epidemiology. In Lombardy, a strengthening of the surveillance system was implemented in the period of the study.

Most strains had been isolated from blood or cerebrospinal fluid (CSF) of immunocompromised or elderly patients. Only three isolates were from maternal-neonatal infections. Each isolate in this study represents a single human listeriosis case. No case had been formally recognized as belonging to a foodborne outbreak or associated to a specific food vehicle during the conventional epidemiological investigations conducted by the Public Health Departments.

The International Life Sciences Institute North America *L. monocytogenes* outbreak and diversity strain sets were used as reference strains (11).

**Serotyping**

All strains tested were serotyped following the manufacturer’s instructions using commercial specific antisera (Denka Seiken, Tokyo, Japan).

**PFGE analysis**

PFGE was performed according to the PulseNet protocol with enzymes *Ase*I and *Apa*I (14).

Bacterial cultures were embedded in agarose, lysed, washed, digested with the restriction enzyme and electrophoresed on a Chef Mapper XA (BioRad Laboratories, Hercules, CA, US) at 6 V/cm for 22 h with switch times of 4 s to 40.01 s. *Xba*I digested DNA of *Salmonella enterica* serotype *Braenderup H9812* was used as the size reference standard. The profiles obtained were compared using the Applied Maths BioNumerics software package (version 5.1, Applied Maths, Saint-Martins-Latem, Belgium). Pattern clustering was performed by the unweighted pairs group algorithm and the Dice correlation coefficient with a tolerance of 1.5%. The results of the clustering
analysis were confirmed by visual comparison of the PFGE profiles. In a first round of typing, AscI was used. Two profiles obtained by AscI were classified as indistinguishable when the DNA fragment patterns matched each other completely, closely related if they differed by one to three bands, unrelated if they differed by more than three bands (26). Numbers were used to designate the AscI profiles. Closely related patterns were assigned an additional capital letter. Indistinguishable or closely related strains were subsequently cleaved with ApaI for clustering confirmation.

Multiplex PCR for detection of epidemic clones

The multiplex PCR protocol described by Chen and Knabel (2), with minor modifications, was used for identification of isolates belonging to *L. monocytogenes* epidemic clones I, II and III. Primer sequences were as described (2). In the PCR reaction mixture, 50 nM for the ECI marker, 90 nM for the ECII marker and 30 nM for the ECIII marker were added. PCR was performed in a volume of 25 µl containing 1.0 U Taq DNA polymerase (Promega), 1× PCR buffer, 200 µM dNTPs and 10 ng each *L. monocytogenes* strain DNA. The cycling program and electrophoresis conditions were as previously described (2). A 1 Kbp DNA ladder (Promega) was used as molecular size marker.

Multiplex PCR for detection of virulence-specific genes

A multiplex PCR including *inlA*, *inlC* and *inlJ* gene primers was performed as described by Liu *et al.* (21) with minor modifications. Briefly, genomic DNA from *L. monocytogenes* strains was obtained after treatment with lysozyme and proteinase K, resuspended in sterile distilled water and used immediately for PCR or stored at -20°C until use. Oligonucleotide primers for the *L. monocytogenes* internalin genes *inlA*, *inlC* and *inlJ* were used in a multiplex PCR format by using a GeneAmp PCR System 2400 (Perkin Elmer, Boston, MA, US) in a volume of 25 µl containing 1.0 U Taq DNA polymerase (Promega, Madison, WI, US), 1× PCR buffer, 200 µM dNTPs and 10 ng each *L. monocytogenes* strain DNA, together with 40 pmol each *inlA*, 30 pmol each *inlC* and 20 pmol each *inlJ* primers. The cycling program and electrophoresis conditions were as previously described (21). A 1 Kbp DNA ladder (Promega) was used as molecular size marker.
Results

A total of 25 clinical isolates (46.3%) were classified in the serotype 1/2a, 23 (42.6%) in the serotype 4b and 6 (11.1%) in the serotype 1/2b.

Thirty-one AscI pulsotypes were recognized among the 54 human isolates. Unique AscI pulsotypes were showed by 15 isolates, whereas the remaining ones were assigned to eleven clusters. Eight clusters – II, III, IV, V, VII, IX, X and XI – included isolates with indistinguishable pulsotypes and three clusters – I, VI and VIII - closely related pulsotypes (Table 1). Each cluster contained two to seven isolates (Table 1). ApaI profiles of the strains with indistinguishable AscI profiles showed no difference in number or position of bands in all clusters but I, where ApaI profiles of two isolates differed by two bands. Closely related isolates by AscI differed by one to three bands by ApaI.

A total of 15 L. monocytogenes isolates tested positive for the EC markers. Three groups of clustered isolates, including respectively, three isolates of serotype 1/2a, seven isolates of serotype 4b, and three isolates of serotype 4b, generated ECIII, ECI and ECII amplicons (Table 1). Furthermore, two apparently sporadic isolates showing serotype 4b yielded the ECII specific band (Table 2). Figure 1 shows the PCR products of some representative isolates of L. monocytogenes and the reference strains FSL-J1-110 (ECI), FSL N1-225 (ECII) and FSL R2-499 (ECIII). AscI PFGE patterns of the five strains positive for the ECII marker and the three strains positive for the ECIII marker are showed in Fig. 2. The two sporadic isolates belonging to the ECII were characterized by unique pulsotypes differing from each other and from the clustered isolates by more than three bands and, consequently, classified as unrelated according with the interpretative criteria adopted in this study.

PCR of virulence genes inlA, inlC and inlJ identified the expected bands of 800, 517 and 238 bp in all strains under study (Fig.3).

Clustering of L. monocytogenes isolates

Based upon the results of PFGE, 11 occasions were detected when strains of L. monocytogenes with the same subtype were isolated from more than one listeriosis case. A total of 39 out of 54 isolates
(72.2%) were attributed to molecular subtype clusters, with each cluster including from two to seven isolates. Eight clusters included isolates from the Lombardy region only, whereas three clusters contained isolates from the two different geographic areas under study, i.e. Lombardy and the province of Florence, Tuscany. With the exception of clusters IV and X, the strains of \textit{L. monocytogenes} within a single cluster had been isolated through an interval of time ranging from a minimum of 0 days (same day) to a maximum of two years. Cluster IV grouped together isolates identified in the years 1996 and 2005 and cluster X in the years 2000 and 2007. Epidemiological evidence was not apparent for any putative cluster. Tables 1 and 2 summarize the characteristics of \textit{L. monocytogenes} isolates associated or not to a cluster, respectively.

\textbf{Discussion}

Because of the complex epidemiology of \textit{L. monocytogenes}, a routine adoption of subtyping tools for identification and tracking of epidemic clones and outbreak strains is able to provide a great advantage to prevention and control activities. Indeed, a timely identification of foodborne outbreaks/epidemics and transmission routes will likely continue being a challenging task for Public Health due to the ubiquitous nature of this organism, its attitude to persistently colonize food processing plants and its ability to multiply in foods at refrigeration temperature. The epidemiological picture is often intricate because of the increasingly wider scale of commercialization of many food products and the evolving food habits associated to demographic, social and economic changes. Finally, further difficulties arise from the protracted period of incubation of listeriosis and the possible space-time scattered distribution of cases (7, 18, 23, 24). PFGE typically provides high sensitivity for identification of differences in molecular subtypes. In our experience, overall, 11 clusters were identified within 54 human isolates from two different geographic areas of Italy based upon serotyping and \textit{AscI} pulsotypes and clustering confirmation by \textit{ApaI}. In previous studies \textit{AscI–PFGE} has provided evidence to accurately cluster epidemiologically
related isolates and separate those from different outbreaks, whereas the more discriminative Apal
PFGE as a primary approach led in some instances to an inaccurate separation of epidemiologically
related isolates (15). Consequently, a large proportion of the human listeriosis cases under
investigation could be grouped into molecular subtype clusters, some of which could represent
common source outbreaks. Furthermore, the geographic scale of these clusters appeared in some
cases to be limited to a single region but in others multiregional, according to previous reports
documenting that human listeriosis clusters can be geographically widely distributed (18, 23, 24).
Two clusters, IV and X, included strains isolated through an interval of time of approximately eight
and seven years, respectively. The occurrence only by chance of two indistinguishable subtypes
should be first considered as a possible explanation. However, alternative hypotheses considering
the year-long persistence of *L. monocytogenes* colonization in food processing plants, the delayed
shelf-lives of some food products and the combined effects of these two events should not be
aprioristically discarded (18). In any case, additional information apart from molecular subtyping
data should be gathered before drawing more reliable epidemiological inferences. A previous report
on human listeriosis cases from eight different regions of Italy by Gianfranceschi *et al.* (12) had
also identified isolates with identical restriction patterns from different geographic sources and
interpreted this last finding as an evidence of no correlation between them. However, such a
conservative interpretation appears to be questionable in the light of the frequent nationwide and,
often, worldwide dissemination of foodborne pathogens, including *L. monocytogenes*.
Food vehicles had not been identified for any case of listeriosis, but cluster I, including four human
isolates from Lombardy, retrospectively proved to contain isolates indistinguishable by serotyping
and DNA-based methods from *L. monocytogenes* strains DUP-1034 previously detected on the
rinds of Taleggio cheeses produced by an Italian plant (5, 22).
Detection of EC markers in both clustered and sporadic isolates is an intriguing finding. These
clones, indeed, have been associated to major nationwide and international foodborne outbreaks,
involving both US and some European countries (17). It seems reasonable to assume that previously
identified epidemic clones will likely be involved in future foodborne events, maybe because of their enhanced transmissibility, virulence and/or persistence. Therefore, the ability of EC identification by a rapid and reliable method to provide useful clues to surveillance systems and Public Health policies warrants further consideration. However, only three out of the eleven clusters of strains proved to possess EC I, II and III markers, proving that these markers can be associated to several different subtypes. Detection of ECII marker in three clustered and two unrelated strains by AscI PFGE warrants consideration, because strains of serotype 4b belonging to this clonal group have been associated to multistate outbreaks in 1998-1999 and 2002 in US, but never in Europe unlike the cosmopolitan clone ECI (4). ECII strains belonging to cluster IX appear to be very similar in their AscI PFGE pattern to the 1998 outbreak clone (3). This finding confirms also the specificity of detection of the ECII genetic marker by the multiplex PCR protocol of Chen and Knabel (2). On the other hand, our results confirm the observation about heterogeneity of serotype 4b strains within the ECII clonal group published by Franciosa et al. (10). Indeed, this report that describes distribution of selected ECI and ECII markers among L. monocytogenes 4b strains isolated in Italy from human, food and environmental sources discloses a higher heterogeneity among strains exhibiting at least one of the three ECII markers SF7, 1365-66 and SF18 than among those strains carrying ECI markers.

Attempts to improve discrimination of virulent from avirulent strains have been made since many years, although current understanding of genomic of L. monocytogenes does not allow for a straightforward differentiation. Internalin genes inlC and inlJ, involved in the passage through the intestinal barrier and the post-intestinal stages of infection, performed well in discriminating strains able or not to cause mouse mortality via the intraperitoneal route (20). Though this does not automatically relate to ability to produce disease in humans via the oral route, detection of inlC and inlJ genes in all human isolates under study is a preliminary interesting finding that should be completed by the comparison with food and environmental isolates from the same geographic context.
This study has some limits. Because of the limited geographical source of \textit{L. monocytogenes} strains a generalization of results is questionable and an overestimate of clustering has to be considered. Moreover, lack of epidemiological information about possible food vehicles or at risk food exposures makes it impossible to compare human and food isolates and track their transmission chains.

The Scientific Panel on Biological Hazards of the European Food Safety Authority (EFSA) recently recommended that efforts to reduce risks to human health should focus on risk reduction practices both during the production process of ready-to-eat foods (RTE) and at home by consumers \(9\). The report recommended also to further investigate listeriosis cases and generate and analyse data on foods where \textit{L. monocytogenes} is most commonly being found. Additional areas for attention may include changes to food formulation, such as the salt or other preservative contents \(13\).

The recent increase of listeriosis cases in Europe emphasizes the need for enhanced surveillance at the EU level to better estimate the burden of disease and the prevalence of this bacterium in the food chain. The results obtained in this study, despite the previously described limits, may provide a reading key that would be helpful in reinterpreting epidemiology of \textit{L. monocytogenes} in Italy.

Complementing conventional and molecular epidemiology in both foodborne events investigations and surveillance systems is an essential requisite in prevention and control of human listeriosis.

\textit{Acknowledgments}

We are sincerely grateful to Dr. Martin Wiedmann, Cornell University, Ithaca, New York, US, for providing us the diversity and outbreak strain sets of \textit{L. monocytogenes}.

We thank also the personnel of the Hospital microbiological laboratories of Lombardy for facilitating collection of isolates and providing clinical information.

This work has been supported by a fund from the Italian University Minister (MIUR): Programmi di ricerca di interesse nazionale (PRIN), year 2005.
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Multistate outbreak of *Listeria monocytogenes* infection linked to delicatessen turkey meat.


Legend to Figures

Figure 1
Agarose gel electrophoresis of EC markers detected in representative human isolates of *L. monocytogenes*.

Lanes (agarose gels A and B): MW (molecular weight): 1 Kbp DNA ladder (Promega); lanes 2-9, PCR products from human isolates analyzed in this study; lanes 10, 11 and 12, positive control strains: ECI, strain FSL J1-110; ECIII, strain FSL R2-499; ECII, strain FSL N1-225 (International Life Sciences Institute, North America *L. monocytogenes* Strain Collection, Fugett *et al.*, 2006). Genetic markers and molecular sizes in bp corresponding to the amplified fragments are indicated on the right.

Figure 2
*Ascl* PFGE profiles of the ECII and ECIII strains.

Lanes: 1-3, profile 19 detected in the three ECII isolates included in cluster IX; 4 and 5, profiles 12 and 21 detected in the ECII sporadic isolates; 6-7, profile 7A, and 8, profile 7B detected in the three ECIII isolates included in cluster VI. Molecular sizes in Kbp corresponding to *XbaI* –digested *Salmonella* Braenderup H9812 DNA are indicated on the left.

Figure 3
Agarose gel electrophoresis of *inlA*, *inlC* and *inlJ* amplicons obtained from representative human isolates of *L. monocytogenes*.

Lanes: MW (molecular weight): 1 Kbp DNA ladder (Promega); lanes 1-6, PCR products from human isolates analyzed in this study. Molecular sizes in bp corresponding to the amplified fragments are indicated on the right.
Table 1. Characteristics of cluster-associated strains of *L. monocytogenes* from human source

<table>
<thead>
<tr>
<th>cluster</th>
<th>no. of strains</th>
<th>place of isolation (region)</th>
<th>time of isolation first case</th>
<th>time of isolation last case</th>
<th>serotype</th>
<th>pulstype AscI</th>
<th>EC marker</th>
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<td>I</td>
<td>4</td>
<td>Lombardy</td>
<td>May 11, 2006</td>
<td>February 6, 2007</td>
<td>1/2b</td>
<td>1A (3), 1B (1)</td>
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<td>II</td>
<td>5</td>
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<td>August 2, 2006</td>
<td>1/2a</td>
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<tr>
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<td>6</td>
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<td>November 2, 2007</td>
<td>4b</td>
<td>3</td>
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<td>2</td>
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<td>3</td>
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<td>7A (2), 7B (1)</td>
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<td>September 4, 2007</td>
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Table 2. Characteristics of non cluster-associated strains of *L. monocytogenes* from human source

<table>
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<tr>
<th>place of isolation (region)</th>
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<th>pulsortype</th>
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<th>EC marker</th>
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