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Research Note

Molecular Typing Reveals Frequent Clustering among Human Isolates of *Listeria monocytogenes* in Italy

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ABSTRACT

In Italy, the annual incidence of reported cases of listeriosis amounts in recent years (2004 to 2006) to 0.8 case per million inhabitants. Our study is a subtyping analysis by serotyping, ribotyping, and pulsed-field gel electrophoresis analysis of 44 human isolates from apparently sporadic cases of infection in the Lombardy region and in the Province of Florence, Italy, in the years 1996 to 2007. Based on the results of the different subtyping methods, 10 occasions were detected when strains of *L. monocytogenes* with the same subtype were isolated from more than one listeriosis case. A total of 28 (66.7%) of 44 isolates were attributed to molecular subtype clusters. Our data support the use of sensitive molecular approaches to identify and trace *L. monocytogenes* isolates responsible for foodborne outbreaks of human listeriosis.

Listeria monocytogenes is the etiological agent of listeriosis, a foodborne disease affecting humans and a variety of vertebrates *(23).* Listeriosis occurs primarily in immunocompromised individuals, causing septicemia and central nervous system infections, and in pregnant women who may suffer from preterm delivery, miscarriage, or stillbirth *(21).* Healthy adults may show afebrile gastroenteritis after ingesting a large number of *L. monocytogenes* cells *(16).*

Most industrialized countries, including those within the European Union, have an annual incidence of listeriosis between 2 and 10 reported cases per million people per year *(22).* However, listeriosis has one of the highest case fatality rates (approximately 20%) among foodborne diseases *(21).*

In Italy, notification of listeriosis cases has been mandatory since 1993, but transmission of data to the national level is done quarterly. A second syndrome-based surveillance system is in place, which covers infections of the central nervous system. A national reference laboratory, which receives strains and epidemiological and clinical information on a voluntary basis, is also present. However, the incidence of reported cases by the Ministry of Health is lower than most European Union countries, amounting in recent years (2004 to 2006) to 0.8 case per million inhabitants annually *(10).* This low incidence must be considered with care because of poor sensitivity of the passive surveillance systems and the diagnostic difficulties posed by *L. monocytogenes* infections, the long incubation period (7 to 60 days), the typical occurrence in specific risk groups (pregnant women, newborns, and persons who are immunocompromised), and the association to different clinical presentations *(22).* Indeed, a recent report shows an incidence of 1.3 cases per 1,000,000 inhabitants during a 1-year (2002 to 2003) period of laboratory-based surveillance *(5).* Moreover, a reinforcement of the surveillance system put in place in 2006, in the Lombardy region, combining collaborative epidemiological investigations, use of subtyping methods for cluster identification, and application of the capture-recapture method to estimate incidence, has given an estimated annual incidence equal to 6.6 per million inhabitants *(17).*

Throughout Europe and the United States, the majority of cases of listeriosis reported to public health authorities is apparently not linked to a common source and is therefore defined as ''sporadic'' *(20).* 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 are scattered over a wide geographic area *(18, 19).* Therefore, use of effective subtyping methods, including serotyping as well as different DNA-based subtyping methods—ribotyping, pulsed-field gel electrophoresis (PFGE), PCR, restriction fragment length polymorphism (RFLP) analysis, and multilocus sequence typing—is essential for

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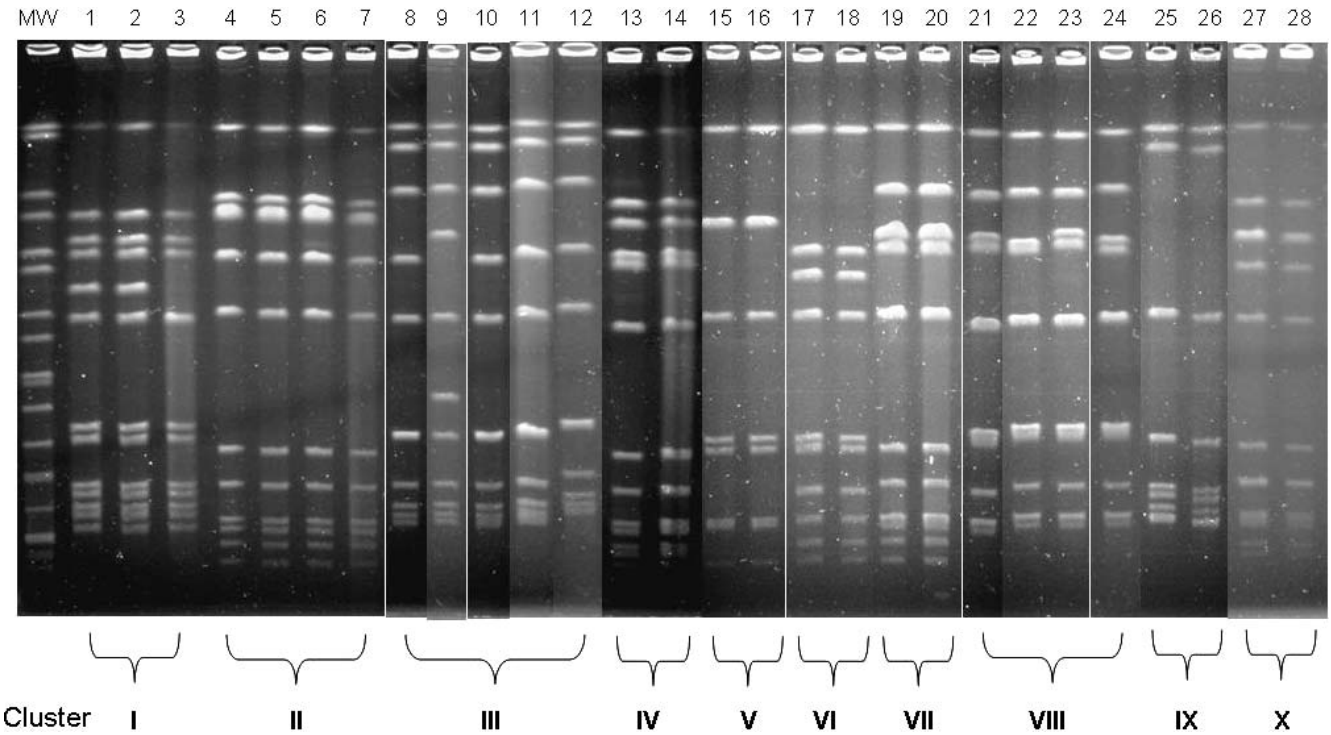

FIGURE 1. *PFGE typing after digestion by* Asc*I of clustered strains of* Listeria monocytogenes *isolated in Lombardy and Tuscany, Italy. Lane MW,* Xba*I-digested DNA of* Salmonella enterica *serotype Braenderup H9812; lanes 1 through 28, isolates belonging to clusters I to X (strain designation, place and time of isolation, and phenotypic and genetic properties are shown in the Table 1).*

the epidemiological investigations of *L. monocytogenes (12, 18, 19, 24).*

Our study is a retrospective subtyping analysis of 32 human isolates identified from apparently sporadic cases of infection in the Lombardy region and in the Province of Florence, Italy, in the years 2006 to 2007. Twelve further isolates from human cases occurring in the same geographic areas since 1996 were available for the study. The objective was to detect clustering of isolates by using serotyping and DNA-based subtyping methods, and to evaluate contribution of the molecular subtyping approach to identification of possible common source outbreaks.

MATERIALS AND METHODS

Bacterial isolates. Between January 2006 and July 2007, 24 human isolates from cases of listeriosis were collected from the laboratories of diagnostic microbiology of the Lombardy region, Italy. Eight further isolates were recovered in the same period from the Careggi Hospital of Florence, Tuscany, Italy. Twelve isolates of *L. monocytogenes* from human sources identified by the same laboratories during the previous years were also collected. These geographical areas were chosen based on 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.

The strains were isolated from blood or from cerebrospinal fluid, mostly from immunocompromised or elderly patients. Only three strains were from maternal-fetal 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.

The International Life Sciences Institute North America *L. monocytogenes* outbreak and diversity sets were used as reference strains *(4).*

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

Ribotyping. Isolates were characterized by automated ribotyping by using the RiboPrinter (DuPont Qualicon, Inc., Wilmington, DE), as described elsewhere *(1). Eco*RI was selected as the restriction enzyme. The automatic classification into ribotypes performed by the RiboPrinter software was the first criterion to cluster the strains into ribotypes. However, some strains underwent manual classification after visual inspection of the profile; a single, clear band was considered difference enough to distinguish ribotypes.

PFGE analysis. PFGE was performed according to the PulseNet protocol with enzymes *Asc*I and *Apa*I *(7).* Bacterial cultures were embedded in agarose, lysed, washed, digested with the restriction enzyme, and electrophoresed on a Chef Mapper XA (Bio-Rad Laboratories, Hercules, CA) at 6 V/cm for 22 h, with switch times of 4 to 40.01 s. *Xba*I-digested DNA of *Salmonella enterica* serotype Braenderup H9812 was used as size reference standard *(7).* PFGE patterns were compared by means of the Dice coefficient by using the fingerprinting Molecular Analyst software (Bio-Rad Laboratories). The results of the clustering analysis were confirmed by visual comparison of the PFGE profiles. In a first round of typing, *Asc*I was used. Two profiles obtained by *Asc*I were classified as ''indistinguishable'' when the DNA fragment patterns matched each other completely, ''closely related'' if they differed by one to three bands, or ''unrelated'' if they differed by more than three bands. Numbers were used to designate the *Asc*I profiles. Closely related patterns were assigned an additional cap-

TABLE 1. *Characteristics of clustered isolates of* Listeria monocytogenes *in Italy*

Lane	Isolate	Isolation place	Isolation time	Serotype	Ribotype	DUP-ID	*Asc*I PFGE type	Lineage	Cluster
1	LMOH-MI-1	Lodi, Lombardy	6 July 2006	1/2b	309 S2	1034	1A	I	I
2	LMOH-MI-7	Brescia, Lombardy	11 May 2006	1/2b	309 S2	1034	1A	I	
3	LMOH-MI-11	Milan, Lombardy	20 October 2006	1/2b	356 S3	1042	1B	I	
4	LMOH-MI-3	Bergamo, Lombardy	2 August 2006	1/2a	204 S5	1045	2	II	II
5	LMOH-MI-19	Bergamo, Lombardy	21 January 2007	1/2a	204 S5	1045	2	II	
6	LMOH-MI-21	Bergamo, Lombardy	21 February 2007	1/2a	204 S5	1045	2	II	
7	LMOH-MI-25	Lodi, Lombardy	30 April 2007	1/2a	204 S5	1045	2	II	
8	LMOH-MI-13	Milan, Lombardy	17 November 2006	4b	423 S6	1044	3	I	III
9	LMOH-MI-20	Milan, Lombardy	19 January 2007	4b	423 S6	1044	3	I	
10	LMOH-FL-21	Florence, Tuscany	26 January 2007	4b	423 S6	1044	3	I	
11	LMOH-FL-31	Florence, Tuscany	26 January 2007	4b	423 S6	1044	3	I	
12	LMOH-MI-27	Milan, Lombardy	8 June 2007	4b	423 S6	1044	3	I	
13	LMOH-MI-16	Milan, Lombardy	12 January 1996	1/2a	303 S5	18603	4	II	IV
14	LMOH-FL-26	Florence, Tuscany	27 July 2005	1/2a	303 S5	18603	4	II	
15	LMOH-MI-17	Bergamo, Lombardy	11 December 2006	1/2a	303 S1	18613	5	II	V
16	LMOH-MI-18	Bergamo, Lombardy	8 December 2006	1/2a	303 S1	18613	5	II	
17	LMOH-FL-28	Florence, Tuscany	27 July 2006	1/2a	347 S1	18598	7	II	VI
18	LMOH-FL-20	Florence, Tuscany	2 August 2006	1/2a	347 S1	18598	7	II	
19	LMOH-FL-24	Florence, Tuscany	19 March 2005	1/2a	303 S5	18603	8	II	VII
20	LMOH-FL-25	Florence, Tuscany	19 March 2005	1/2a	303 S5	18603	8	II	
21	LMOH-FL-18	Florence, Tuscany	25 July 2005	4b	355 S4	1038	6B	I	VIII
22	LMOH-FL-19	Florence, Tuscany	27 July 2005	4b	355 S4	1038	6A	I	
23	LMOH-MI-4	Como, Lombardy	26 June 2007	4b	355 S4	1038	6A	I	
24	LMOH-MI-28	Milan, Lombardy	8 June 2007	4b	355 S4	1038	6A	I	
25	LMOH-MI-9	Brescia, Lombardy	19 July 2006	4b	356 S1	18624	22	I	IX
26	LMOH-MI-14	Milan, Lombardy	16 November 2006	4b	356 S1	18624	22	I	
27	LMOH-MI-23	Lodi, Lombardy	11 October 2000	1/2a	202 S1	19171	23	II	X
28	LMOH-MI-26	Milan, Lombardy	30 April 2007	1/2a	202 S1	19171	23	II	

ital letter. Indistinguishable or closely related strains were subsequently cleaved with *Apa*I.

PCR-RFLP of virulence genes. For all isolates, the *L. monocytogenes* virulence genes encoding listeriolysin O (*hly*), actA (*actA*), and internalin A (*inlA*) were PCR amplified, and *hly* and *inl*A amplicons screened for allelic polymorphisms by PCR-RFLP according to Wiedmann et al. *(25).* PCR products were digested separately by using *Hha*I and *Hpa*II for *hly,* and *Apo*I, *Hin*fI, and *Hha*I for *inlA.* For *actA,* PCR amplification of the proline-rich region, with primers PR5 and PR3 was performed according to Inoue et al. *(9).* Two alleles, characterized by the presence of one proline-rich unit (518-bp amplification product) or two (623-bp amplification product), were classified as *actA* type II and *actA* type I, respectively. Different combinations of allelic polymorphisms were assigned arbitrary small letters.

Genetic lineage analysis. Isolates were assigned to lineages I and II based on serotype, DuPont identification pattern number (DUP-ID), and PCR-RFLP of virulence genes according to Wiedmann et al. *(25).*

Simpson's index of discrimination (SID). The suitability of serotyping, ribotyping, PFGE, and PCR-RFLP for differentiation of *L. monocytogenes* human isolates included in this study was determined by using SID, as described by Hunter and Gaston *(8).*

RESULTS

Serotyping. A total of 20 (46%) clinical isolates were classified as serotype 1/2a, 19 (43%) as serotype 4b, and 5 (11%) as serotype 1/2b (Table 1).

Ribotyping. A total of 17 different ribotypes, characterized by eight or nine bands with a molecular mass ranging between 2.1 and 48 kbp, were identified among the 44 human strains. The similarity between these profiles ranged between 49 and 92%. Overall, 5 ribotypes were associated to single strains, whereas 12 ribotypes were shared by two to six isolates (Table 1).

Fourteen different DUP-IDs were assigned to the isolates tested (Table 1). Among the identified DUP-IDs, 1034, 1038, 1042, 1044, and 1052 are classified in lineage I, whereas DUP-IDs 1045 and 1047 are classified in lineage II *(25).*

PFGE. Twenty-six *Asc*I pulsotypes were recognized among the 44 human isolates. Unique *Asc*I pulsotypes were demonstrated by 16 isolates, whereas eight indistinguishable and two closely related pulsotypes were shared by 2 to 5 isolates (Fig. 1 and Table 1). *Apa*I profiles of the strains with indistinguishable *Asc*I profiles showed no difference in number or position of bands in seven out of eight cases, whereas in one case, the *Apa*I profile of one isolate differed by two bands. Closely related isolates by *Asc*I differed by one to three bands by *Apa*I as well.

PCR-RFLP. PCR-RFLP of virulence genes *actA, hly,* and *inlA* identified nine different combinations of restriction patterns. Except for one pattern associated to a single strain, all PCR-RFLP patterns were shared between 2 and 11 strains.

SID. The SID values of PFGE, *Eco*RI ribotyping, and PCR-RFLP for the clinical isolates of this study were 0.972, 0.941, and 0.852, respectively.

Clustering of *L. monocytogenes* isolates. Combining the results of PFGE and ribotyping, 10 different clusters were identified. PFGE and ribotyping yielded consistent results except for two cases, (i) cluster I, which included two isolates with indistinguishable PFGE patterns and ribotypes, and one isolate with a closely related PFGE pattern and a different ribotype, and (ii) two additional clusters, IV and VII, consisting of isolates with unrelated PFGE patterns, but identical ribotype (Table 1). Overall, a total of 28 (66.7%) of 44 isolates were attributed to molecular subtype clusters. The number of infected people in each cluster ranged from two to a maximum of five cases. Seven clusters included isolates from the Lombardy region or, alternatively, from the Province of Florence, whereas three clusters contained isolates from the two different geographic areas. With the exception of clusters IV, VIII, and X, the strains of *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 14 months. Cluster IV grouped together isolates identified in the years 1996 and 2005, cluster VIII in the years 2005 and 2007, and cluster X in the years 2000 and 2007 (Table 1).

DISCUSSION

This article describes phenotypic and genetic profiles of isolates of *L. monocytogenes* obtained from two different geographic areas of Italy, which show relatively high incidence rates of listeriosis (in the years 2005 and 2006, the incidence of notifiable cases was 1.4 and 4.5 per million inhabitants in Lombardy and Tuscany, respectively).

Twenty-three and 21 human isolates tested in this study belonged to lineages I and II, respectively, in contrast to most previous literature reports in which lineage I strains were much more common among human listeriosis cases than were lineage II strains *(24, 25).* However, a recent study conducted in Italy shows, accordingly, that 44% of the strains isolated from listeriosis cases in 2002 to 2003 in Italy belonged to serotype 1/2a, typically associated with lineage II *(5).* Consistently, some European countries, such as Finland, report that serotype 1/2a is apparently replacing serotype 4b as the serotype recovered most frequently from human infections *(13).*

Retrospective subtyping analysis of the 44 human *L. monocytogenes* isolates from two different geographic of Italy reveals that a large proportion of listeriosis cases can be grouped into subtype clusters, some or all of which could represent common source outbreaks. Hence, although most human listeriosis infections in Italy are being generally considered to represent sporadic cases, our results suggest that such clusters could be more common than previously assumed *(3, 5, 6).*

The SID values of PFGE, *Eco*RI ribotyping, and PCR-RFLP for the clinical isolates of this study were 0.972, 0.941, and 0.852, respectively, confirming the highest discriminatory ability of PFGE. Therefore, although ribotyping provides an automated, highly discriminatory, and quick (approximately 8 h of run time) tool able to detect putative single-source disease clusters, in public health investigations, PFGE typically provides higher sensitivity for identifying differences in molecular subtypes.

The subtype clusters reported here include both single-region and multiregional clusters, according to previous reports documenting that human listeriosis clusters can be geographically widely distributed *(2, 14).* Specifically, cluster I, including human isolates from Lombardy, proved retrospectively 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 in an Italian plant *(1).* Although no specific epidemiologic investigations were conducted to confirm this cluster, our findings indicate that aggressive foodborne disease-surveillance efforts would allow for a timely detection of a possible foodborne outbreak, which could in turn minimize the health and economic impact of listeriosis.

Unlikely did the eight cluster grouped strains separate by a few days to months as to their time of isolation; three clusters (IV, VIII and X) included strains isolated over intervals of 9, 2, and 7 years, respectively. A first obvious consideration about the occurrence only by chance of two indistinguishable pheno- and genotypes cannot be ignored, even though, based on the large heterogeneity of circulating pulsotypes, a coincidental episode should be unlikely. Alternative hypothesis about these long-lasting clusters should consider 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 *(11, 15).*

This study has some limits. Because of the restricted geographical source of *L. monocytogenes* strains, a generalization of results is questionable. Moreover, an overestimate of clustering could arise from the inclusion of closely related strains. However, according with the considerations of Sauders et al. *(18),* for the purpose of detecting a possible outbreak, the use of more stringent criteria of inclusion, i.e., completely identical banding pattern, could miss some clusters.

From a public health point of view, a more sensitive, subtyping-based first step that is followed by a more discriminative, epidemiologically based second step appears to be the more effective approach to detection of a foodborne outbreak of listeriosis, considering the peculiar characteristics of the causal organism, most food vehicles, and the susceptible people and the consequent atypical appearance of epidemic events within a community. Although further and more extensive epidemiological studies are needed, our observations are consistent with the hypothesis that a considerable number of listeriosis cases might occur in clusters, many of which could represent single-source outbreaks liable to go undetected without the combined use of molecular subtyping methods and more aggressive and timely epidemiological investigations.

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