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UNIVERSITÀ DEGLI STUDI DI TORINO

A five year surveillance report on PFGE types of *Listeria monocytogenes* isolated in Italy from food and food related environments

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Abstract

Listeria monocytogenes can cause severe invasive disease in humans and has been isolated from a variety of foods. This study aimed to investigate type diversity and distribution across sources by subtyping via PFGE a set of 300 *L. monocytogenes* isolates collected in Italy from foods over a five year period (from 2003 to 2007).

The most frequent serotypes were 1/2a (45%), 1/2c (22%), and 4b/4e (16%); 5% of the isolates were untypeable by conventional serotyping. Significant associations were observed between serotype 1/2a with dairy (O.R. = 13.9) and 1/2c with meat (O.R. = 33.3). All isolates were typeable, generating 164 combined PFGE profiles. Of these, 121 were unique, being displayed by only one isolate. The other 43 profiles grouped the remaining isolates and were shared between two (N = 22), three (N = 10), four (N = 3) and five isolates (N = 4). The remaining 4 profiles were shared between 7, 14, 17 and 46 isolates, respectively. Some profiles (N = 7) were retrieved in samples collected in different years, indicating persistency in foods and processing plants. This research may pose the ground for designing a broad typing database which could ease the understanding of *L. monocytogenes* diversity and could be used for facilitating epidemiological investigations for the identification of listeriosis outbreaks. Data show how large subtype databases may facilitate the identification of common and source-specific types. More comprehensive databases may be needed to fully understand *L. monocytogenes* diversity and to provide useful data to be considered in epidemiological investigations.

1. Introduction

Listeria monocytogenes may cause severe invasive disease in humans. In Europe, listeriosis is predominantly an infection of immuno-compromised individuals amongst the older sections of the population and in association with pregnancy (EFSA-Q, 2007). In Italy, it has mainly occurred as sporadic cases with a reported incidence of 0.1 cases per 100,000 people in 2007 and similar to incidences reported in 2005 (EFSA, 2007). In particular, in the Piedmont region, area where this research was performed, there were 18 notified cases reported from 2003 to 2006 (Ministero del Lavoro, 2006).

Listeriosis is almost exclusively a food-borne disease, and *L. monocytogenes* has been isolated from a variety of raw and ready-to-eat (RTE) products (EFSA, 2007). Most food-borne listeriosis cases are believed to be caused by RTE products contaminated during and after processing as the bacterium is able to survive at low pH (down to 4.4), low aw values (0.83) and at refrigeration temperatures (Tompkin, 2002).

Human listeriosis outbreaks are often difficult to detect, since cases associated with a single outbreak may be geographically and temporally dispersed (Graves et al., 2005 and Mead et al., 2005). Therefore, the use of molecular subtyping methods is often critical for the initial detection of human listeriosis outbreaks (Swaminathan et al., 2001 and Olsen et al., 2005). Molecular subtyping methods can also be used to characterize *L. monocytogenes* isolates from food and food processing environments allowing the identification of persistent contamination, possibly related to adaptation to specific production niches (Vogel et al., 2001 and Autio et al., 2002).

Currently, many microbial subtyping methods are available: pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), and

ribotyping (Hyytiä-Trees et al., 2007). PFGE is considered the gold standard technique by the Centers for Disease Control and Prevention (CDC) to successfully track *L. monocytogenes* (Graves et al., 2005 and Gerner-Smidt et al., 2006). In fact, exchange of bacterial PFGE patterns through PulseNet, a national network coordinated by the CDC, has been useful in identifying and controlling listeriosis outbreaks, emphasizing the importance of PFGE for *L. monocytogenes* surveillance in U.S. (Graves et al., 2005 and Gottlieb et al., 2006).

Such a system still needs to be implemented in Europe, where subtyping of isolates is performed on a voluntary basis in each Member State and thus the availability and representativeness of subtyping data differ greatly across Europe (EFSA Scientific Report, 2009).

Considering the high importance of an updated European Typing database in order to understand *L. monocytogenes* type diversity and distribution in time and across sources, the present study is aimed at subtyping via PFGE *L. monocytogenes* collected during official control sampling programs (N = 300) in a five year period.

2. Materials and methods

2.1. Samples

A panel of 311 isolates from different sources were analysed: (i) 300 *L. monocytogenes* isolates from 184 samples collected from 2003 to 2007 gathered during official controls aimed at detecting *L. monocytogenes* in food business operations in the Piedmont–Liguria regions (Italy). Isolates were collected from 72 producers/retailers (Table 1); (ii) 5 isolates from human clinical cases (collected in 2004–2005 and matching in time/space the collected food samples) kindly provided by two Laboratories annexed to Hospitals located in the study area and (iii) 6 reference strains comprising 2 isolates belonging to the most represented serotypes for each of the three lineages, selected from the Diversity Subset of the International Life Science Institute (Fugett et al., 2006). In particular, 1/2a (FSL J2-054) and 1/2c (FSL J1-094) were chosen for Lineage II, 1/2b (FSL J2-064) and 4b (FSL C1-122) for Lineage I, and 4a (FSL J1-168) and 4c (FSL W1-110) for Lineage III.

The AFNOR NF V08/055 (1997) procedure was used for *L. monocytogenes* detection. Briefly, a ten gram sample was enriched in two subsequent steps and then plated onto specified selective agar media and incubated at 37 °C for 24–48 h. *Listeria monocytogenes* identification was based on colony morphology and results of conventional tests: gram-staining, catalase test, beta-haemolysis, carbohydrate utilisation and Camp test. Positive colonies were further confirmed by API-*Listeria* (BioMerieux) and pfrA-PCR (D'Agostino et al., 2004). All the isolates gave the expected amplification product.

Table 1 shows the distribution of the isolates according to source and year of isolation.

2.2. Serotyping

Antisera against somatic (O) and flagellar (H) antigens were used according to manufacturer instructions (Denka Seiken, Tokyo, Japan), with minor modifications: for determination of H antigens, isolates were passed four times at 25 °C through semi-solid BHI (agar 0.2% w⁄v). No further test was performed to discriminate isolates typed as 4b/4e.

A serotyping PCR (Doumith et al., 2004) was applied to isolates that were untypeable with conventional serotyping and to those clustering with others belonging to different genetic lineages.

2.3. Statistical analysis

Analysis was performed using EPIINFO (v6). Yates $\chi 2$ test was applied to assess the association of different *L. monocytogenes* serotypes with sources of isolation. Significant associations were revealed with $p < 0.05$. The measure of the association was evaluated through the Odds Ratio (O.R.) and the related 95% Exact Confidence Intervals (95% C.I.). Out of 311 isolates, only 265 were considered for statistical analysis: isolates retrieved from fish and food preparations were not considered due to the limited sample size, as well as serotypes with low frequencies (i.e. 3a, 3b, 3c, 4a, 4c), human and reference strains.

2.4. PFGE

Listeria monocytogenes genomic DNA was prepared as described by Graves and Swaminathan (2001). DNA cleavage was achieved by AscI and ApaI restriction (New England Biolabs, Beverly, MA). Genomic fingerprints were firstly compared by visual analysis and then by BioNumerics software (v2.0, Applied Maths, Kortrijk, Belgium); similarity was determined by the Dice coefficient using an optimization and a band position tolerance values of 1.5% for both enzymes. Restriction profiles were combined and analysed using the UPGMA algorithm. PFGE types were considered identical when the patterns were indistinguishable.

3. Results and discussion

The most frequent serotypes were 1/2a (45%), 1/2c (22%), 4b/4e (16%), 1/2b (6%) and 3a (3%). Serotypes 3b, 3c, 4a, 4c and 4d were observed in 3% of the isolates, whereas the others (5%) were untypeable with conventional serotyping. Regarding the latter group of isolates (N = 16), serotyping PCR allowed the identification of the following serogroups: 1/2b–3b (N = 7), 4a–4c (N = 5), 1/2c–3c (N = 2) and 4b–4d–4e (N = 2).

Statistical analyses confirmed the association reported previously. In particular, serotype 1/2a was associated with isolates from dairy and dairy production environments (Yates $\chi 2 = 85.6$ $p < 0.01$; O.R. = 13.9; 95% C.I.: 7.4–26.1); 1/2c with isolates from meat and related environments (Yates $\chi 2 = 61.8$ $p < 0.01$; O.R. = 33.3; 95% C.I: 10.2–169.5) (Gianfranceschi et al., 2003). However it should also be noted that the most common serotypes (1/2a, 1/2c and 4b) could be isolated from different food industries and they can therefore be considered ubiquitous (Autio et al., 2002).

In the present study, in 120 food samples it was possible to retrieve only one colony for further subtyping. In the remaining 64 samples multiple colonies (from 2 to 7) were isolated and typed. In 21 out of 64 (33%) all isolated colonies were indistinguishable, in 38 PFGE revealed two genetic patterns (59%) and in the remaining 5 (8%) three or more genotypic patterns were identified. Differences in PFGE profile obtained from colonies of the same samples ranged from values greater than 80% (68% of the isolates) to values lower than 50% (12% of the isolates). Recently, Döpfer et al. (2008) underlined the significance of characterizing multiple isolates per sample and suggested the analysis of ten and six isolates for fecal and

soil samples for *L. monocytogenes*, respectively. However, no specific information for food samples is currently available. The presented results evidenced that three colonies per samples were suitable for revealing isolate variability within the same sample, allowing the detection of very low similarity values. Therefore this research may represent a starting point for the planning of future sampling aimed at the characterization of *L. monocytogenes* population in food and related environments.

Considering the year distribution of food samples, only 6% of the isolates were retrieved in 2006–2007. This disparity, compared to 2003–2005, can be attributed to a reduction in frequency and number of official controls specifically aimed at the detection of the pathogen. Nonetheless, those samples were analysed with PFGE as they could still provide information on isolate persistency.

All isolates were typeable, generating 164 combined PFGE profiles, divided into 14 clusters (Fig. 1). As described by Ward et al. (2004), *L. monocytogenes* isolates can be divided into III lineages: Lineage I (serotypes 1/2b, 4b, 3b), Lineage II (1/2a, 1/2c, 3a) and Lineage III (4a, 4c, some 4b). In the present study, isolates belonging to Lineage I were grouped in 7 clusters (VI–VIII and X–XII) and those belonging to Lineage II into 6 (I–V, IX). The two lineages were not completely separated on the dendrogram (Fig. 1), as already observed by other authors (Neves et al., 2008 and Chou & Wang, 2006; Revazishvili et al., 2004).

All 4c and some 4b/4e isolates were grouped in a heterogeneous cluster (XIII), possibly representing Lineage III (Ward et al., 2004). Cluster XIII also contained 1/2a (N = 2) and 1/2b (N = 1) isolates, suggesting that, as already observed for Lineages I and II, PFGE may overlap isolates of different genetic lineages (Garrido et al., 2008 and Revazishvili et al., 2004). Finally, cluster XIV and one outlier isolate were highly divergent from all other isolates, indicating that even isolates collected from a limited geographic area could show a very high PFGE profile variability.

In this study, 8 out of 11 unserotypeable isolates with the conventional method were assigned by PCR to the same serotype of their neighbours in the dendrogram, indicating correct grouping by PFGE. The remaining untypeable isolates were gathered with different serotypes, supporting the hypothesis of a horizontal gene transfer or point mutations resulting in phenotypic shifts (Nadon et al., 2001).

PCR was also applied to some isolates (N = 11) that clustered with others belonging to serotypes of different lineages or in cluster XIV. For eight of these isolates, PCR results agreed with conventional serotyping, supporting the hypothesis of horizontal gene transfer, speculated already by Nadon et al. (2001) who observed atypical ribotype–serotype combinations. However, the remaining isolates evidenced conventional serotyping misidentifications, highlighted by PFGE clustering. The disagreement may be due to isolates low antigenic expression in culture (Okwumabua et al., 2005) or antigenic switch without nucleic acid modifications (Revazishvili et al., 2004). However, in two isolates the disagreement was due to lab errors and resolved when conventional serotyping was performed again. These findings highlight that phenotypic typing methods, such as serotyping, may be subjective and give result difficult to interpret. For these reasons, molecular subtyping methods should always be coupled with serotyping for the characterization of *L. monocytogenes*.

Among the 164 combined profiles, 121 were unique, being displayed by only one isolate and were not assigned ID number. The others 43 (P1–P43) grouped the remaining 190 isolates. Table 2 shows the distribution of PFGE types across years and sample typologies. Of the 43 profiles, 22 (51%) were shared between two isolates, 10 (23%) among three isolates, and 3 (7%) and 4 (9%) among four and five isolates, respectively. The remaining 4 profiles (9%) were shared between 7, 14, 17 and 46 isolates respectively (Fig 1).

In seven profiles (P2, P3, P5, P6, P9, P14, P38) two different serotypes belonging to the same genetic lineage were recovered. Apparently, only P36 was shared between isolates of different lineages, 4b/4e and 4c. In particular, some other authors showed that the same PFGE profiles could also be shared between different serotypes and lineages (Clark et al., 2010 and Gianfranceschi et al., 2007). However, when serotyping PCR was performed, the isolates were both confirmed as 4b/4d/4e.

Some profiles were retrieved in samples collected in different years, indicating persistency in foods and processing plants, in agreement to literature (Vogel et al., 2001). In the current study, persistent profiles were observed in dairy and meat plants. In dairy plants, P2, P4 (persisting two years in five and two plants, respectively) and P3 (persisting three years in 15 plants), were retrieved from producers not sharing the same suppliers and located far apart from each other (from 37 up to 150 km). However, they shared the same processing conditions since they produced Gorgonzola PDO (Protected Denomination of Origin). This may suggest the presence of Gorgonzola-adapted types (Carminati et al., 2004, Manfreda et al., 2005 and Lomonaco et al., 2009). Indeed, profiles retrieved from Gorgonzola cheese production sites never matched those recovered from other foods. Interestingly, the consumption of Gorgonzola was recently implicated in human listeriosis in Italy (Gianfranceschi et al., 2007). Persistent profiles were also evidenced in meat (P13, P20, P22), as reported in literature (Thevenot et al., 2006). In particular, P13 was found in different meat products/preparations in the same area but from different producers, all sharing the same supplier, which may be the putative source of the contamination. Also P9 was retrieved over time (from 2003 to 2005) and from different products (fish and food preparations) collected from the same producer.

The presence of the same profile in different manufacturers in the same and in different years may indicate that the isolate is persistent and spread in the study area. However, when considering the epidemiological implications it should be considered that the presented data do not refer to producers followed over time, thus it is not possible to detect persistency within plant. Conversely, 12 different profiles (P5, P6, P18, P19, P20, P36, and six unique) were detected in one single sampling of the same producer of frankfurter. This may suggest that multiple contamination had occurred or that the sanification procedures were not effective, allowing the access and the establishment of different isolates over time.

Other profiles (P6, P14, P16, P25, P31, P32), grouped isolates retrieved from meat/meat producing environment with those from dairy/dairy plants. P16 was found in different years, indicating that some *L. monocytogenes* subtypes may be ubiquitous, adapting and persisting in different environments. Noteworthy, persistent contaminating isolates have already been shown to cause human listeriosis outbreaks (Lyytikainen et al., 2000). In the present study, no profiles were shared between human and food isolates. It should be stated that the absence of matching may be due to the low number of clinical cases analysed and/or may imply that larger databases of profiles of food isolates are needed. In fact, analyzed food samples may not represent what is usually eaten in the region and conclusive epidemiological evidences are always critical to highlight epidemiological connections (Wiedmann, 2002). Presented data represent the most recent attempt in Italy to explore PFGE type diversity over an extended time frame and from different sources and emphasize the need of a national subtyping database. Expanding the current European EnterNet database, implementing the subtyping of isolates in all the Member States, would be useful in further improving the value of PFGE-typing. Efforts should also be made to include PFGE patterns of food and animal isolates (i.e. from EnterVet participating laboratories) as these information are critical to fully understand *L. monocytogenes* diversity and to provide data useful for epidemiological investigations.

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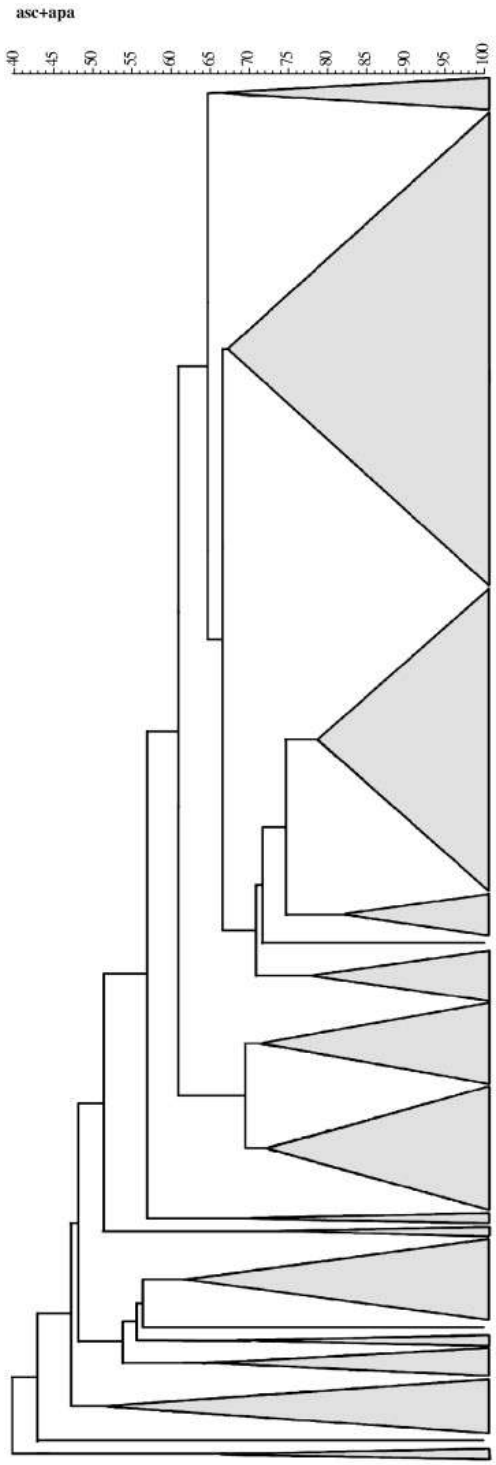
Table 1. Distribution of food and environmental samples across source and year of isolation (M, meat; ME, meat production environment; D, dairy; DE, dairy production environment; Fh, fish; FP, food preparation and FPE, food preparation environment).

Source	Number of samples per year					No. and type of plants
	2003	2004	2005	2006	2007	
M	61	41	9	3	/	Producers (26), retailers (19)
ME	3	29	2	/	/	
D	13	21	34	/	6	Producers (11), retailers (2)
DE	1	22	23	1	10	
Fh	1	6	5	/	/	Producers (3), retailers (6)
FP	5	3	/	/	/	Producers (3), retailers (2)
FPE	/	/	1	/	/	
	84	122	74	4	16	

Table 2. Distribution of pulsotypes across year of isolation and sources (M, meat; ME, meat environment; D, dairy; DE, dairy environment; Fh, fish; FP, food preparation and FPE, food preparation environment). Samples collected in 2006 are not reported because they originated PFGE unique types.

	2003				2004							2005					2007	
	M	ME	D	FP	M	ME	D	DE	Fh	FP	FPE	M	ME	D	DE	Fh	D	DE
P1, P27														■				
P2														■	■		■	■
P3							■	■						■	■		■	■
P4							■	■							■			
P5, P7, P10, P15, P18, P19, P29, P36	■																	
P6	■		■															
P8					■					■								
P9				■					■							■		
P11							■	■										
P12												■	■					
P13	■				■	■						■						
P14	■		■															
P16	■	■												■				
P17					■	■												
P20	■				■													
P21	■	■																
P22	■											■						
P23									■	■								
P24, P33			■															
P25					■		■											
P26												■						
P28				■														
P30									■									
P31					■		■											
P32					■		■	■										
P34			■					■										
P35																■		
P37, P38, P42, P43					■													
P39, P40, P41						■												

Fig. 1. Dendrogram showing UPGMA clustering by Dice coefficient of the combined AscI–ApaI profiles. The figure shows the clustering according to lineages: Lineage I (underlined), Lineage II (bold) and Lineage III (grey marked). The reference strains are reported below the cluster they were grouped into or next to the horizontal bars (if not clustered). Human isolates are listed with the % of similarity with the nearest food isolate. Results of PCR serotyping are reported for isolates untypeable with the conventional method and for those with discordant results between PCR and conventional serotyping (both indicated by *).



Cluster	Similarity (%)	Serotype (N° of isolates)	Profiles (N° of isolates)
I	66	1/2a (8) Human isolate (66%)	P1(2)
II 1/2c	67	1/2a (98), 3a (8), 1/2c (2), 1/2a-3a (1)*	P2(17), P3(46), P4(5), P5(3), P6(2), P7(2), P8(2), P9(7), P10(2), P11(2)
III	79	1/2c (64), 3c (2), 1/2a (3), 4b-4d-4e (1)*	P12(2), P13(14), P14(2), P15(2), P16(5), P17(3), P18(4), P19(2), P20(3), P21(3), P22(5)
IV 1/2a	82	1/2a (9), 1/2c (1)	P23(2), P24(4)
V	78	1/2a (12)	P25(2), P26(2)
VI 1/2b	71	1/2b (15), 3b (3), 4b/4e (1) Human isolate (74.5%)	P27(3), P28(2), P29(2), P30(2)
VII 4b	72	4b/4e (24), 1/2b (1), 4d (1), 1/2b-3b (1)*, 4b-4d-4e (2)* Human isolate (97.6%) Human isolate (75.2%) Human isolate (89.5%)	P31(3), P32(5), P33(2), P34(3), P35(2), P36(2)
VIII	68	1/2b (2), 1/2a(1)	/
IX	74	1/2c-3c (2)*, 4b-4d-4e (1)*	P37(2)
X 4a	62	4b/4e (10), 4a/4c (3)* 1/2b-3b* (5), 4b-4d-4e (1)*	P38(3), P39(3), P40(3)
XI	66	4b/4e (1), 1/2b-3b (2)*	/
XII	64	4b/4e (7)	P41(4)
XIII 4c	51	4b/4e (5), 4c (4), 1/2b (1), 4a/4c (1)*, 1/2a (2)	P42(2), P43(2)
XIV	64	1/2a (2), 4b/4e (1)	/