Antimicrobial Susceptibility and Multilocus Sequence Typing of *Listeria monocytogenes* Isolated Over 11 Years from Food, Humans, and the Environment in Italy

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Abstract

Due to the increasing number of studies reporting the detection of antimicrobial-resistant isolates of *Listeria monocytogenes*, we sought to determine the antimicrobial susceptibility of *L. monocytogenes* isolates collected in Italy and find potential correlations to their serotypes and multilocus sequence types (MLST). The antimicrobial susceptibility of 317 *L. monocytogenes* isolates collected from food, humans, and the environment from 1998 to 2009 was assessed by minimum inhibitory concentration (MIC). Serotyping and MLST was also performed on all isolates. Potential correlations among antimicrobial resistance profiles, serotyping, and MLST were statistically evaluated. Twenty-four percent of *L. monocytogenes* isolates were resistant to oxacillin, 28.7% intermediate to clindamycin, and 24.3% to ciprofloxacin. The majority of isolates with elevated MIC to oxacillin was of environmental origin and belonged to serotype 4b/4e and ST2. Isolates with intermediate MIC values to clindamycin and ciprofloxacin were mostly of food and human origin and belonged to serotype 4b/4e and ST9. Regarding the time frame of isolate collection, comparing the last 3 years (2007–2009) to previous years (1998–2006), an increase was observed in the percentage of resistant and intermediate isolates per year. This trend strongly suggests the need for increasing attention on the prevalence of antimicrobial resistance in *L. monocytogenes* in Italy. To predict future resistance trends, the monitoring of clinical intermediate resistance might represent a useful tool especially for antibiotics associated to multiple-step mechanisms of acquired resistance. A specific focus should be addressed to antimicrobial-resistant isolates of serotype 4b, repeatedly associated with food-borne outbreaks.

Keywords: *Listeria monocytogenes*, antimicrobial susceptibility, serotyping, multilocus sequence typing

Introduction

IN THE PAST DECADES, *Listeria monocytogenes* has been implicated in several outbreaks of food-borne illness in humans with a statistically significant increasing trend of listeriosis since 2008 (Schlech *et al.*, 1983; EFSA and ECDC, 2015). While it has been established that food-borne transmission constitutes the main acquisition route of listeriosis (Farber and Peterkin, 1991; Swaminathan and Gerner-Smidt, 2007), most healthy humans are not significantly affected by the intake of small numbers of *L. monocytogenes* in foods (Pouillot *et al.*, 2015). However, the risk of developing clinical listeriosis is higher for people with existing chronic illness, suppression of the immune system, pregnancy, or extreme youth or older age (younger than the age of 1 year or

older than 60 years) (Swaminathan and Gerner-Smidt, 2007). For these vulnerable persons, the mortality rate remains high (~16%) (Scallan *et al.*, 2011). Patients affected by listeriosis are usually treated with aminopenicillin or benzylpenicillin in conjunction with an aminoglycoside. Vulnerable and immunocompromised patients at risk of listeriosis might follow an empiric treatment with broad spectrum antibiotics such as piperacillin/tazobactam or carbapenems (Thønnings *et al.*, 2016).

Listeria spp. has been generally reported susceptible to almost all antimicrobials, except for cephalosporins, fosfomycin, and macrolides, against which *Listeria* spp. has been described as naturally resistant or with reduced susceptibility (Charpentier and Courvalin, 1999; Troxler *et al.*, 2000). However, reports of *L. monocytogenes* resistant to a wide

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TABLE 1. CHARACTERISTICS OF THE 317 *LISTERIA MONOCYTOGENES* ISOLATES

Sequence type	No. of isolates (%)	Serotype						Lineage			Origin		
		No. of isolates						No. of isolates			No. of isolates		
		1/2a	1/2c	4b/4e	1/2b	3a	3b	3c	4c	4d/4e	I	II	III
ST9	80 (25.2)	—	79	—	—	—	—	1	—	—	1	79	—
ST121	54 (17.0)	51	—	—	—	3	—	—	—	—	—	54	—
ST2	21 (6.6)	—	—	20	—	—	—	—	1	—	21	—	—
ST3	16 (5.0)	—	—	—	15	—	1	—	—	—	16	—	—
ST8	14 (4.4)	14	—	—	—	—	—	—	—	—	—	14	—
ST199	9 (2.8)	9	—	—	—	—	—	—	—	—	—	9	—
ST6	8 (2.5)	—	—	7	—	—	—	—	—	—	7	—	—
ST18	8 (2.5)	8	—	—	—	—	—	—	1	—	—	8	—
ST1	7 (2.2)	—	—	7	—	—	—	—	—	—	7	—	—
ST14	7 (2.2)	7	—	—	—	—	—	—	—	—	—	7	—
ST4	5 (1.6)	—	—	5	—	—	—	—	—	—	5	—	—
ST29	5 (1.6)	5	—	—	—	—	—	—	—	—	—	5	—
ST37	5 (1.6)	1	—	—	—	4	—	—	—	—	—	5	—
ST155	5 (1.6)	5	—	—	—	—	—	—	—	—	—	5	—
ST236	5 (1.6)	5	—	—	—	—	—	—	—	—	—	5	—
ST398	5 (1.6)	5	—	—	—	—	—	—	—	—	—	5	—
ST7	4 (1.3)	4	—	—	—	—	—	—	—	—	—	4	—
ST59	4 (1.3)	—	—	—	4	—	—	—	—	—	4	—	—
ST224	4 (1.3)	—	—	—	4	—	—	—	—	—	4	—	—
ST429	4 (1.3)	—	—	1	3	—	—	—	—	—	4	—	—
ST517	4 (1.3)	—	—	—	4	—	—	—	—	—	4	—	—
ST38	3 (0.9)	3	—	—	—	—	—	—	—	—	—	3	—
ST101	3 (0.9)	3	—	—	—	—	—	—	—	—	—	3	—
ST120	3 (0.9)	3	—	—	—	—	—	—	—	—	—	3	—
ST5	2 (0.6)	—	—	—	1	—	1	—	—	—	2	—	—
ST11	2 (0.6)	2	—	—	—	—	—	—	—	—	—	2	—
ST31	2 (0.6)	2	—	—	—	—	—	—	—	—	—	2	—
ST32	2 (0.6)	—	—	2	—	—	—	—	—	—	—	2	—
ST515	2 (0.6)	—	—	2	—	—	—	—	—	—	—	2	—
ST575	2 (0.6)	—	2	—	—	—	—	—	—	—	—	2	—
ST10	1 (0.3)	—	—	1	—	—	—	—	—	—	—	—	—
ST21	1 (0.3)	1	—	—	—	—	—	—	—	—	—	—	—
ST26	1 (0.3)	1	—	—	—	—	—	—	—	—	—	—	—
ST68	1 (0.3)	—	—	—	1	—	—	—	—	—	—	—	—
ST91	1 (0.3)	1	—	—	—	—	—	—	—	—	—	—	—
ST175	1 (0.3)	—	—	—	1	—	—	—	—	—	—	—	—
ST177	1 (0.3)	1	—	—	—	—	—	—	—	—	—	—	—
ST181	1 (0.3)	—	—	—	1	—	—	—	—	—	—	—	—
ST184	1 (0.3)	—	—	—	—	—	—	—	—	—	—	—	—
ST188	1 (0.3)	—	—	—	1	—	—	—	—	—	—	—	—

(continued)

TABLE 1. (CONTINUED)

Sequence type	No. of isolates (%)	Serotype					Lineage				Origin		
		No. of isolates					No. of isolates				No. of isolates		
		1/2a	1/2c	4b/4e	1/2b	3a	3b	3c	4c	4d/4e	I	II	III
ST191	1 (0.3)	—	—	—	1	—	—	—	—	—	1	—	—
ST209	1 (0.3)	—	1	—	—	—	—	—	—	—	—	1	—
ST325	1 (0.3)	1	—	—	—	—	—	—	—	—	—	—	—
ST425	1 (0.3)	1	—	—	—	—	—	—	—	—	—	—	—
ST426	1 (0.3)	—	—	—	1	—	—	—	—	—	1	—	—
ST428	1 (0.3)	—	—	—	—	—	—	—	—	—	—	1	—
ST520	1 (0.3)	—	—	1	—	—	—	—	—	—	—	—	—
ST570	1 (0.3)	1	—	—	—	—	—	—	—	—	—	—	—
ST579	1 (0.3)	1	—	—	—	—	—	—	—	—	—	—	—
ST580	1 (0.3)	—	1	—	—	—	—	—	—	—	—	—	—
ST581	1 (0.3)	—	—	—	1	—	—	—	—	—	1	—	—
Unknown	1 (0.3)	—	1	—	—	—	—	—	—	—	—	—	—
Total, n (%)	317	136 (43)	84 (26)	46 (15)	38 (12)	7 (2)	3 (1)	1 (0.3)	1 (0.3)	1 (0.3)	89 (28)	227 (72)	1 (0.3)
											239 (75)	35 (11)	43 (14)

ST, sequence type.

range of antimicrobial agents have been published. Since the first report of a multiresistant clinical isolate in France in 1988, antimicrobial-resistant isolates collected from humans, food, plants, and the environment have been described worldwide (Charpentier *et al.*, 1995; Srinivansan *et al.*, 2005; Granier *et al.*, 2011; Ruiz-Bolivar *et al.*, 2011; Bertsch *et al.*, 2014; Moreno *et al.*, 2014; de Vasconcelos Byrne *et al.*, 2016; and Li *et al.*, 2016). In humans, resistance of *L. monocytogenes* against tetracycline and ciprofloxacin has been described (Morvan *et al.*, 2010). In food, high prevalence of oxacillin and clindamycin resistance was described recently among isolates from meat and fish production chains (Walsh *et al.*, 2001; Lyon *et al.*, 2008; Chen *et al.*, 2010; Gómez *et al.*, 2014). Similarly, significant percentages of resistance against ampicillin, penicillin G, and tetracycline were reported in *L. monocytogenes* isolated from meat, fish, and dairy production chains (Aras and Ardic, 2015; Jamali *et al.*, 2015; Kevenk and Gulel, 2016).

The emergence and spread of antimicrobial-resistant *Listeria* spp. has been attributed to the overuse of antibiotics in disease treatments and growth promotion in domestic live-stock (Charpentier *et al.*, 1995; Walsh *et al.*, 2001).

In the present study, the antimicrobial susceptibility of 317 *L. monocytogenes* isolates collected in a 11-year period (1998–2009) from humans, food, and environmental surfaces was assessed along with serotype and multilocus sequence type (MLST) to: (1) evaluate the occurrence of antimicrobial resistance and intermediate resistance in *L. monocytogenes* over time; (2) correlate antimicrobial resistance phenotype with origin, serotype, and sequence type.

Materials and Methods

L. monocytogenes isolates

A total of 317 *L. monocytogenes* isolates recovered between 1998 and 2009 from food ($n=239$, including 162 meat products, 38 dairy products, 17 seafood, 22 ready-to-eat products), humans ($n=43$), and environment ($n=35$, including 17 food-processing environment, 6 animal feces, 4 soil, 4 water, and 4 plants) were analyzed in this study.

All the isolates were previously identified using confirmation tests: Gram stain, motility at 25°C in motility agar, catalase test, oxidase test, fermentation of xylose and rhamnose, hemolysis test on sheep blood agar plates, CAMP test against *Staphylococcus aureus* and *Rhodococcus equi*, and the API *Listeria*® (BioMérieux, Marcy l'Etoile, France).

Serotyping

Serotyping was carried out using commercial specific antisera (Denka Seiken, Tokyo, Japan) against the serovars 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4c, 4d/4e, and 4b/4e, following the manufacturer's instructions (Ueda *et al.*, 2002).

Multilocus sequence typing

Genomic DNA was extracted using GenomicPrep® cell and tissue isolation kit (Amersham, Piscataway, NJ) following the manufacturer's instructions. DNA concentrations were determined at a wavelength of 260 nm using the spectrophotometer DU640 (Beckman, Fullerton, CA). The final concentration of DNA was adjusted to 10 ng/mL with RNase, DNase, and Protease-free water (Sigma-Aldrich, Milan, Italy).

Seven DNA sequences belonging to the *abcZ*, *bglA*, *cat*, *dapE*, *dat*, *ldh*, and *lhkA* genes were amplified by polymerase chain reaction (PCR) and sequenced. All genes were amplified using previously published primers (Salcedo *et al.*, 2003), except for the *ldh* gene, for which the primers suggested from a modified MLST scheme (www.pasteur.fr/mlst) were used (Ragon *et al.*, 2008). PCRs were carried out in 25 μ L reaction volumes containing 10 pmol of each primer, 0.2 mM of each dNTP's, 1 U HotMaster Taq (Eppendorf, Milan, Italy), and 2.5 mL of 10X HotMaster Taq Buffer (Eppendorf). The reaction conditions were an initial denaturation at 94°C for 2 min, followed by 25 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min. The PCR products were purified using Montage® PCR filter units (Millipore, Milan, Italy). Sequence reactions were carried out using BigDye 3.1 Ready Reaction Mix (Thermo Fisher Scientific, Milan, Italy) according to the manufacturer's instructions. The sequenced products were separated with a 3130 Genetic Analyzer (Thermo Fisher Scientific). Sequences were imported and assembled with the BioNumerics 7.1 software (Applied Maths, Belgium). Alleles and sequence type (ST) were assigned by submitting the DNA sequences to the Listeria MLST database at the Pasteur Institute in France (www.pasteur.fr/mlst). A unweighted pair group method with arithmetic mean (UPGMA) tree was built using MLST profiles of the selected isolates using BioNumerics 7.6 software (Applied Maths). The visualization and the annotation of the phylogenetic tree were performed using the web-based tool Interactive Tree of Life. Strains were grouped into clonal complexes, defined as groups of profiles differing by no more than one gene from at least one other profile of the group (Feil, 2004).

Antimicrobial susceptibility testing

Isolates were subjected to minimum inhibitory concentration (MIC) using Sensititre® plates GPN4F and GPALL1F (Trek Diagnostic Systems, Cleveland, OH) specific for Gram-positive bacteria and including a wide variety of antibiotics at a full range of MIC: erythromycin (0.25–4 μ g/mL), clindamycin (0.5–2 μ g/mL), quinupristin/dalfopristin (0.5–4 μ g/mL), streptomycin (1000 μ g/mL), vancomycin (1–32 μ g/mL), tetracycline (2–16 μ g/mL) ampicillin (0.12–8 μ g/mL), gentamycin (2–16 μ g/mL), rifampin (0.5–4 μ g/mL), trimethoprim/sulfamethoxazole (0.5/9.5–4/76 μ g/mL), chloramphenicol (2–16 μ g/mL), oxacillin +2% NaCl (0.25–8 μ g/mL), penicillin G (0.06–8 μ g/mL), and ciprofloxacin (1–2 μ g/mL). The MIC against *L. monocytogenes* isolates was assessed following the broth microdilution method reported in Clinical and Laboratory Standards Institute (CLSI) M45-A3 document accordingly CLSI breakpoints were used for MIC categorization (CLSI, 2016). In brief, isolated colonies were suspended in sterile demineralized water to achieve a 0.5 McFarland. From the bacterial suspension, 10 μ L were transferred to 90 μ L Mueller–Hinton Broth with lysed horse blood (Trek Diagnostic Systems) and inoculated onto the MIC plates, which were incubated 24 h at 35°C. MIC values were manually recorded. *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as quality control strains. Each experiment was performed in duplicate.

As CLSI breakpoints for *L. monocytogenes* only include ampicillin, penicillin, and trimethoprim/sulfamethoxazole, and *L. monocytogenes* belongs to the Bacillales order, CLSI

breakpoints for *Bacillus* were used for the following antimicrobials: erythromycin, clindamycin, vancomycin, tetracycline, gentamicin, rifampin, chloramphenicol, and ciprofloxacin (document CLSI M45-A3) (CLSI, 2016; Yan *et al.*, 2016). CLSI breakpoints for *Staphylococci*, *Enterococci*, and *Corynebacteri* were used for oxacillin, streptomycin, and quinupristin/dalfopristin, respectively (CLSI M45-A3, CLSI VET01-S2) (CLSI, 2013, 2016).

Statistical analysis

Chi-square test was performed using StatView 5.0 software (SAS 155 Institute Inc., USA) to compare nominal variables between serotypes, lineages, and sequence types.

Results

Serotype, lineage, sequence type

Serotyping of the 317 *L. monocytogenes* strains identified the following serotypes: 1/2a (43%; $n=136$), 1/2c (26%; $n=84$), 4b/4e (15%; $n=46$), 1/2b (12%; $n=38$), 3a (2%; $n=7$), 3b (1%; $n=3$), 3c, 4c, and 4d/4e (0.32%; $n=1$).

In the population of *L. monocytogenes*, there are three lineages: the lineage I includes isolates of serotype 4b/4e, 1/2b, 3b, 4d/4e, and 3c, the lineage II isolates of serotype 1/2a, 1/2c, and 3a, and the lineage III isolates of serotype 4a and 4c. Of the 317 strains, 72% (227/317) belonged to the lineage II, 28% (89/317) to the lineage I, and 0.31% (1/317) to the lineage III.

MLST analysis of the 317 strains identified 52 different sequence types. The most representative STs were ST9 (25%; $n=80$), ST121 (17%; $n=54$), ST2 (7%; $n=21$), ST3 (5%; $n=16$), and ST8 (4%; $n=14$). The results of MLST analysis, serotyping and Lineage distribution of the 317 isolates, and their source are summarized in Table 1.

Antimicrobial susceptibility

The MIC distribution of the 317 isolates is reported in Table 2. Using the breakpoints given, all isolates were susceptible to erythromycin, streptomycin, vancomycin, ampicillin, gentamicin, rifampin, trimethoprim/sulfamethoxazole, chloramphenicol, and penicillin. From all isolates tested, 54% (170/317) were intermediate or resistant to certain antimicrobials. In detail, of the 317 isolates tested, 91 (29%) were intermediate to clindamycin, 77 (24%) to ciprofloxacin, and 2 (1%) to quinupristin/dalfopristin; 76 (24%) were resistant to oxacillin, 4 (1%) to tetracycline, and 2 (1%) to ciprofloxacin. Among all intermediate or resistant isolates, 59% (101/170) were intermediate or resistant to only one antimicrobial, 33% (56/170) to two antimicrobials, and 8% (13/170) to three antimicrobials (Table 3).

In relationship to the origin, the percentage of oxacillin-resistant isolates was significantly higher in the environment in comparison to food and humans (46%, 19%, and 35%, respectively) ($p<0.05$) (Table 4), while the percentages of both clindamycin and ciprofloxacin intermediate strains were significantly higher in human isolates in comparison to food and the environment (60%, 23%, and 29%, respectively, for clindamycin intermediate isolates and 70%, 19%, and 3%, respectively, for ciprofloxacin intermediate isolates) ($p<0.001$) (Table 4).

TABLE 2. MINIMUM INHIBITORY CONCENTRATION DISTRIBUTION OF THE 317 *LISTERIA MONOCYTOGENES* STRAINS

MIC ($\mu\text{g/mL}$)	0.06	0.12	0.25	0.5	1	2	4	8	16	32	1000
Antimicrobials											
Erythromycin			316	1							
Clindamycin				226	76	15					
Quinupristin/dalfopristin				90	225	2					
Streptomycin											317
Vancomycin					315	2					
Tetracycline						313			4		
Ampicillin		250	51	16							
Gentamicin						317					
Rifampin				317							
Trimethoprim/sulfamethoxazole				317							
Chloramphenicol						59	240	18			
Oxacillin			1		20	220	71	5			
Penicillin	86	127	85	19							
Ciprofloxacin					238	77	2*				

Black vertical lines indicate breakpoints for resistance. Gray vertical lines indicate breakpoints for intermediate. Gray shading indicates that the isolates were not tested for susceptibility to those concentrations of a given antimicrobial agent. Asterisked number indicates the number of isolates exhibiting MIC values equal to or higher than concentration of the test range.

MIC, minimum inhibitory concentration.

In relationship to the lineage, the percentage of oxacillin-resistant strains was significantly higher in isolates of lineage I (58/89; 65%) compared with lineage II (18/227; 8%) ($p < 0.001$) (Table 5) (Fig. 1). No significant differences were found between the percentages of clindamycin and ciprofloxacin intermediate strains belonging to the two lineages (Fig. 1) (Table 5).

In relationship to the serotype, the percentages of oxacillin-resistant as well as clindamycin and ciprofloxacin intermediate strains were significantly higher in isolates of serotype 4b/4e (72%, 35%, and 33%, respectively) ($p < 0.05$) (Table 6).

Isolates were collected in a broad time period ranging from 1998 to 2009. Comparing the last 3 years (2007–2009) to previous years (1998–2006), a clear positive trend of increase was observed in the percentage of resistant and intermediate

isolates per year. In particular, the percentage of oxacillin-resistant and clindamycin intermediate strains increased from 22% to 28% and from 26% to 35%, respectively, and the percentage of ciprofloxacin intermediate strains increased from 21% to 34% (Table 7). The increase was statistically significant only for ciprofloxacin intermediate strains ($p < 0.05$).

Discussion

The *Listeria* genus was described as susceptible to antibiotics active against Gram-positive bacteria, including ampicillin or penicillin (combined with aminoglycosides), trimethoprim (alone or combined with sulfamethoxazole), tetracyclines, erythromycin, and gentamicin (Rota *et al.*, 1996; Teuber, 1999; Yücel *et al.*, 2005). High susceptibility of isolates to ampicillin (100%), penicillin (100%), tetracyclines (99%), gentamicin (100%), erythromycin (100%), and trimethoprim-sulfamethoxazole (100%) were observed in the present study (Table 1). Similar results were also previously reported (Conter *et al.*, 2009; Yan *et al.*, 2010; Barbosa *et al.*, 2013; Wang *et al.*, 2013; Madeo *et al.*, 2015).

Few isolates showed resistance to tetracycline (4/317; 1.3%) and ciprofloxacin (2/317; 0.6%), whereas a consistent number of isolates showed resistance to oxacillin and intermediate resistance to clindamycin or ciprofloxacin (24%, 28.7%, and 24.3%, respectively). Also, intermediate resistance to quinupristin/dalfopristin was observed in 2 (0.6%) *L. monocytogenes* isolated from water and plant (environmental source).

In our study, resistance to oxacillin was 24% for *L. monocytogenes* isolated from selected sources. Oxacillin resistance has been already associated to *L. monocytogenes* with even higher percentages in research studies from Brazil, United States, and Poland (Davis and Jackson, 2009; Wiczorek *et al.*, 2012; Camargo *et al.*, 2015). In *Staphylococcus* infections, oxacillin susceptibility test is used as a marker for methicillin-resistant *Staphylococcus aureus*. This resistance is associated with the *mecA* gene coding for an alternative

TABLE 3. ANTIMICROBIAL RESISTANCE PATTERNS OF *LISTERIA MONOCYTOGENES* STRAINS

Resistance patterns	No. of isolates
OXA	45
CLI	35
CIP	20
TET	1
CLI-CIP	35
CLI-CIP	1
OXA-CIP	10
OXA-CIP	1
CLI-OXA	7
OXA-TET	1
TET-CIP	1
CLI-OXA-CIP	10
CLI-SYN-OXA	2
CLI-TET-CIP	1
TOTAL	170

Underlined names represent intermediate susceptibility.

CIP, ciprofloxacin; CLI, clindamycin; OXA, oxacillin; SYN, quinupristin/dalfopristin; TET, tetracycline.

TABLE 4. ANTIMICROBIAL RESISTANCE AND ORIGIN OF THE 317 *LISTERIA MONOCYTOGENES* STRAINS

Origin	Antimicrobial agent											
	Oxacillin			Tetracycline			Ciprofloxacin			Clindamycin		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Food	194 (81)	—	45 (19)	235 (98)	—	4 (2)	191 (80)	46 (19)	2 (1)	184 (77)	55 (23)	—
Environment	19 (54)	—	16 (46)	35 (100)	—	—	34 (97)	1 (3)	—	25 (71)	10 (29)	—
Humans	28 (65)	—	15 (35)	43 (100)	—	—	13 (30)	30 (70)	—	17 (40)	26 (60)	—
Total	241 (76)	—	76 (24)	313 (99)	—	4 (1)	238 (75)	77 (24)	2 (1)	226 (71)	91 (29)	—
										239 (100)	—	—
										33 (94)	2 (6)	—
										43 (100)	—	—
										315 (99)	2 (1)	—

I, number (%) of intermediate strains; R, number (%) of resistant strains; S, number (%) of sensitive strains.

TABLE 5. ANTIMICROBIAL RESISTANCE AND LINEAGE OF THE 317 *LISTERIA MONOCYTOGENES* STRAINS

Lineage	Antimicrobial agent											
	Oxacillin			Tetracycline			Ciprofloxacin			Clindamycin		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
I	31 (35)	—	58 (65)	88 (99)	—	1 (1)	67 (75)	21 (24)	1 (1)	65 (73)	24 (27)	—
II	211 (92)	—	18 (8)	224 (99)	—	3 (1)	170 (75)	56 (25)	1 (0.4)	160 (70)	67 (30)	—
III	1 (100)	—	—	1 (100)	—	—	1 (100)	—	—	1 (100)	—	—
Total	241 (76)	—	76 (24)	313 (99)	—	4 (1)	238 (75)	77 (24)	2 (1)	226 (71)	91 (29)	—
										88 (99)	1 (1)	—
										226 (99.6)	1 (0.4)	—
										1 (100)	—	—
										315 (99)	2 (1)	—

I, number (%) of intermediate strains; R, number (%) of resistant strains; S, number (%) of sensitive strains.

FIG. 1. Unweighted pair group method with arithmetic mean tree based on multilocus sequence type profiles. Rings are as follows: strain ID (1), source (2), serotype (3), sequence type (4), oxacillin susceptibility (5), ciprofloxacin susceptibility (6), and clindamycin susceptibility (7).

TABLE 6. ANTIMICROBIAL RESISTANCE AND SEROTYPE OF THE 317 *LISTERIA MONOCYTOGENES* STRAINS

Serotype	Antimicrobial agent											
	Oxacillin			Tetracycline			Ciprofloxacin			Clindamycin		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
1/2a	120 (88)	—	16 (12)	136 (100)	—	—	99 (73)	37 (27)	—	97 (71)	39 (29)	—
1/2c	83 (99)	—	1 (1)	81 (96)	—	3 (4)	65 (77)	18 (21)	1 (1)	56 (67)	28 (33)	—
4b/4e	13 (28)	—	33 (72)	46 (100)	—	—	31 (67)	15 (33)	—	30 (65)	16 (35)	—
1/2b	14 (37)	—	24 (63)	37 (97)	—	1 (3)	32 (84)	6 (16)	—	31 (82)	7 (18)	—
3a	6 (86)	—	1 (14)	7 (100)	—	—	6 (86)	1 (14)	—	7 (100)	—	—
3b	2 (67)	—	1 (33)	3 (100)	—	—	2 (67)	—	1 (33)	3 (100)	—	—
3c	1 (100)	—	—	1 (100)	—	—	1 (100)	—	—	—	1 (100)	—
4c	1 (100)	—	—	1 (100)	—	—	1 (100)	—	—	1 (100)	—	—
4d/4e	1 (100)	—	—	1 (100)	—	—	1 (100)	—	—	1 (100)	—	—
Total	241 (76)	—	76 (24)	313 (99)	—	4 (1)	238 (75)	77 (24)	2 (1)	226 (71)	91 (29)	—
										315 (99)	2 (1)	—
												136 (43)
												84 (26)
												46 (15)
												38 (12)
												7 (2)
												3 (1)
												1 (0.3)
												1 (0.3)
												1 (0.3)
												317

I, number (%) of intermediate strains; R, number (%) of resistant strains; S, number (%) of sensitive strains.

penicillin-binding protein namely PBP2A with low-binding affinity to β -lactam antibiotics (Reichmann and Pinho, 2017). Further studies need to be performed to verify whether similar molecular mechanisms of oxacillin resistance are true also for *Listeria*.

In the present study >20% of *L. monocytogenes* isolates showed intermediate MIC values for clindamycin and ciprofloxacin. These intermediate values are particularly interesting. Acquired resistance to both antimicrobials is a multistep process linked to the subsequent acquisition of multiple point mutations in core genes. In particular, clindamycin resistance was associated with methylation and mutations in the 23S rRNA (Kehrenberg *et al.*, 2005; Poehlsgaard *et al.*, 2005; Kulczycka-Mierzejewska *et al.*, 2018). Ciprofloxacin resistance was associated to mutations in the quinolone resistance determining region of *gyrA*, overexpression of efflux pump gene *lde* and mutation in the *fepR* gene, and regulator of the efflux pump protein gene *fepA* in *L. monocytogenes* (Lampidis *et al.*, 2002; Godreuil *et al.*, 2003; Moreno *et al.*, 2014; Jiang *et al.*, 2018; Wilson *et al.*, 2018).

Intermediate MIC values suggest a probable future shift to resistance phenotypes in case of acquisition of additional mutations. Significant percentages of clindamycin and ciprofloxacin resistance have been already described worldwide (Davis and Jackson, 2009; Wieczorek *et al.*, 2012; Camargo *et al.*, 2015; Noll *et al.*, 2017).

An interesting statistical correlation has been found between resistance and lineages/serotypes. In particular, oxacillin resistance was significantly more frequently identified in lineage I and in particular in serotype 4b/4e. Similarly, the percentages of isolates with intermediate clindamycin or ciprofloxacin MIC values were significantly higher in serotype 4b/4e. A correlation between some *L. monocytogenes* serotypes and antimicrobial resistance has been previously reported although no explanation for these observations has been proposed yet. For instance, Barbosa *et al.* (2013) found that resistance to erythromycin was correlated with the serotype 4b, whereas no correlation was observed between ciprofloxacin resistance and *L. monocytogenes* serotypes (Safdar and Armstrong, 2003). On the contrary, other authors found that serotype 1/2a isolates were more frequently resistant to ciprofloxacin compared to serotype 4b (Safdar and Armstrong, 2003; Kovacevic *et al.*, 2013). Besides, the association of antimicrobial resistance or reduced susceptibility to serotype 4b is of concern, since strains belonging to this serotype were associated to outbreaks (Althaus *et al.*, 2017; Buchanan *et al.*, 2017; Burall *et al.*, 2017).

The two most frequently identified STs among intermediate and resistant isolates to clindamycin, ciprofloxacin, and oxacillin were ST9 and ST2, respectively. These two STs have been found in different food categories. The high level of exposure might be the reason of the high contribution to listeriosis cases of these STs (Henri *et al.*, 2016).

Along with single antimicrobial resistance or reduced susceptibility, 8% of resistant isolates showed resistance to three or more antimicrobials and therefore fall within the definition of multidrug-resistant isolates (Table 3) (Magiorakos *et al.*, 2012). Results from other studies reported multidrug resistance in *L. monocytogenes* isolated from food and animals to chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin, and rifampin (Charpentier *et al.*, 1995; Walsh *et al.*, 2001; Srinivasan *et al.*, 2005). However,

TABLE 7. ANTIMICROBIAL RESISTANCE OF THE 317 *LISTERIA MONOCYTOGENES* STRAINS OVER TIME

Time period	Oxacillin			Clindamycin			Ciprofloxacin			Total
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	
1998–2006	177 (78)	—	51 (22)	168 (74)	60 (26)	—	180 (79)	47 (21)	1 (0.4)	228
2007–2009	64 (72)	—	25 (28)	58 (65)	31 (35)	—	58 (65)	30 (34)	1 (1)	89

I, number (%) of intermediate strains; R, number (%) of resistant strains; S, number (%) of sensitive strains.

Godreuil *et al.* (2003) reported that only five of 488 clinical isolates of *L. monocytogenes* were susceptible to chloramphenicol and vancomycin (Walsh *et al.*, 2001).

Although *L. monocytogenes* was noted to be relatively susceptible to a wide range of antimicrobials as few as 20 years ago, a number of more recent reports suggests that the rate of antimicrobial resistance in *L. monocytogenes* is increasing from 1% to 10% up to 50% (Vela *et al.*, 2001; Walsh *et al.*, 2001; Godreuil *et al.*, 2003; Barbosa *et al.*, 2013; Gómez *et al.*, 2014; Thønnings *et al.*, 2016; Arslan *et al.*, 2019). Accordingly, in the present study, a median increase per year of the percentage of antimicrobial resistant or reduced susceptible isolates to oxacillin, clindamycin, and ciprofloxacin was observed in the period 2007–2009 in comparison to previous years (1998–2006). These results are consistent to a previous Italian study based on a large antimicrobial susceptibility survey conducted in 1995. In this study, all 148 isolates were susceptible to β -lactams, lincosamides, and tetracycline (Aureli *et al.*, 2003). Conversely, in a more recent study performed in France on 4816 clinical *L. monocytogenes* strains isolated between 1926 and 2007, although low, resistance to specific antimicrobials (tetracyclines and fluoroquinolones) was found to be emerging (Morvan *et al.*, 2010).

In France, a 10-year period study, mostly overlapping the years in the present work (1996–2006) isolated only 4 resistant isolates among the 202 food and environmental samples analyzed (Granier *et al.*, 2011). This is in contrast with the observation of the present study, in which 170 isolates were identified resistant over 317 isolates analyzed. Although it must be said that in the present dataset, isolates of human origin were also included, their relative few number (43) does not significantly bias the comparison.

In comparison to previous studies, results suggest significant different trends of antimicrobial-resistant *L. monocytogenes* from food and the environment in European neighboring countries in the first decade of the century. In addition, in line with the concept of epidemiological cutoff values, evaluation of clinical intermediate resistance in *L. monocytogenes* might represent a useful tool to predict future occurrence of resistant strains against antibiotics associated with a multistep acquisition mechanism. If confirmed on additional *L. monocytogenes* isolates collected in more recent years, the Italian increase of resistant and intermediate *L. monocytogenes* isolates to oxacillin, clindamycin, and ciprofloxacin is a matter of concern. This observation is particularly relevant especially in case of antimicrobial-resistant isolates of serotype 4b, which is repeatedly associated with food-borne outbreaks.

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