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PEER-REVIEWED ARTICLE

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Characterization and Antimicrobial Resistance of *Listeria monocytogenes* Isolated from Food-related Environments

ABSTRACT

The purpose of this study was to determine the diversity and antimicrobial resistance of *Listeria monocytogenes* strains isolated from food-related environments in the United States. Nineteen unrelated strains of *L. monocytogenes* were recovered from approximately 1300 food and food processing environmental samples collected from 2007 to 2011 as part of the U.S. Food and Drug Administration pathogen surveillance program. The *L. monocytogenes* environmental isolates were characterized by serotyping, subtyping, and identification of antimicrobial resistance determinants. The serovars of *L. monocytogenes* were 1/2a, 4b, and 1/2b. PFGE using *Asc*I digested total DNA showed genetic diversity; there were 10 PFGE pulse-types and 5 PFGE groups. All strains except one strain were susceptible to ampicillin, erythromycin, vancomycin, ciprofloxacin, and chloramphenicol but resistant to extended-spectrum cephalosporins (ESC). The environmental strains were predominantly resistant to streptomycin and tetracycline. No strain was resistant to 3 or more antimicrobial classes. All tetracycline-resistant strains were serotype 1/2a, and only *tetM* was amplified from the chromosomal DNA. This study, which reports the genetic diversity and antimicrobial resistance of foodborne *L. monocytogenes*, may be useful in food safety control programs to reduce the risk of transmission of *L. monocytogenes* to food products.

INTRODUCTION

Listeria monocytogenes, a Gram-positive, facultatively intracellular foodborne bacterial pathogen that causes human listeriosis *(6, 13)*, is widely distributed in the natural environment and foods. *L. monocytogenes* has been recognized as a major human foodborne pathogen ever since a large *Listeria* outbreak occurred in 1983 in the United States (U.S.) from improperly pasteurized milk *(13)*. The hospitalization (91.0%) and mortality (19.5%) rates due to listeriosis are estimated to be the highest among those caused by foodborne pathogens in the U.S.

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(4, 18). Higher mortality rates are typically associated with immunocompromised persons, the elderly, pregnant women, and neonates *(9)*.

Of the recognized *Listeria* species, only *L. monocytgenes* is associated with human listeriosis outbreaks, with serotype 4b being the most commonly implicated serotype *(2)*. Although serotypes 1/2a and 1/2b are more frequently isolated from food products, serotype 4b is more frequently isolated from clinical specimens *(8)*. Hence, serotyping and subtyping of *L. monocytogenes* isolates are epidemiologically important steps in identification and classification of this pathogen during human listeriosis outbreaks as well as in routine regulatory surveillance.

L. monocytogenes in food products is believed to be derived from the food processing environment despite a lack of direct evidence linking a specific foodborne *L. monocytogenes* strain to the food processing plant environment *(7, 19)*. *Listeria* species that persist in the food processing environment may develop resistance to chemicals used in cleaning and sanitation *(3, 11, 14)*. Therefore, persistent monitoring of this pathogen in food-related environments is important to prevent or minimize contamination of the final food product. In addition, microbiological environmental monitoring provides useful information for food safety control programs such as Hazard Analysis Critical Control Point (HACCP) and good manufacturing practices (GMPs) *(5)*.

The National Antimicrobial Resistance Monitoring System (NARMS) has monitored the antimicrobial resistance of all of the major foodborne pathogens, except *L. monocytogenes*, since 1996. *L. monocytogenes* is associated with high hospitalization and mortality rates, and antimicrobial resistance appears to be increasing *(4, 15, 18)*. The contamination of food products by *L. monocytogenes* is a major concern to regulatory agencies and the food industry, both of which seek to minimize consumer exposure to this organism. Therefore, the purpose of this study was to genotypically and phenotypically characterize food-related environmental *L. monocytogenes* isolates and determine their antimicrobial resistance.

MATERIALS AND METHODS

Isolation and identification of *Listeria* spp. strains

The samples were collected from food processing facilities in the U.S. during 2007 to 2011 by the Pacific Regional Laboratory-Southwest of the FDA (PRL, Irvine, CA), using FDA guidance. The guidance is available at http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/FoodProcessingHACCP/ucm073110.htm#app4. Nineteen *L. monocytogenes* strains were recovered, identified, and serotyped as described in the U.S. Food and Drug Administration Bacteriological Analytical Manual (BAM) and in previous studies *(1, 10)*.

Bacterial cultivation and pulsed-field gel electrophoresis (PFGE)

L. monocytogenes isolates were cultured in BHI broth (Difco Laboratories, Detroit, MI). Turbidity measurements and PFGE analysis for subtyping were based on the CDC standard protocol (http://www.cdc.gov/pulsenet/protocols/pulsenet_listeria_protocol%20.pdf) as modified for a previous study *(1)*. The restriction enzymes *Asc*I and *Apa*I were used to digest the DNA plugs.

Antimicrobial susceptibility assays and MIC determination

Antimicrobial susceptibility was determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (http://www.microbiolab-bg.com/CLSI.pdf). Broth microdilution assays were used to determine the minimum inhibitory concentration (MIC) of each antimicrobial: ampicillin, cephalothin, cefoxitin, ceftriaxone, cefepime, gentamicin, kanamycin, streptomycin, tetracycline, erythromycin, vancomycin, rifampicin, ciprofloxacin, sulfamethoxazole-trimethoprim, and chloramphenicol. Antibiotic diffusion disks (BD, Franklin Lakes, NJ) were used to confirm antimicrobial resistance of all strains. *Staphylococcus aureus* (ATCC 25923) and *L. monocytogenes* EGD-e were used as reference strains *(1)*.

Detection of genes involved in antimicrobial resistance

The polymerase chain reaction (PCR) was used to detect genes conferring aminoglycoside, ß-lactam, or tetracycline resistance. Primers used in this study are shown in *Table 1*. Genomic DNA was extracted from resistant strains by use of the Qiagen DNeasy Blood and Tissue kit (Qiagen, Valencia, CA), or plasmid DNA was extracted by a previously described method *(12)*. Amplification reactions were done with the *Taq* PCR Master Mix Kit (Qiagen) and 400 nM primers *(Table 1)* by use of an Applied Biosystem Veriti™ 96 well thermal cycler (Life Technologies: Grand Island, NY, USA). PCR for *tetM* was performed under the following conditions: initially incubated at 95°C for 10 min, then subjected to 35 cycles of 95°C for 15 sec, 56°C for 15 sec, and 72°C for 30 sec, with a final extension at 72°C for 5 min. PCR conditions for other antimicrobial-resistance genes were published previously *(15)*.

RESULTS AND DISCUSSION

Phenotypic and genotypic diversity of *L. monocytogenes* isolates

Nineteen *L. monocytogenes* strains were recovered from food-processing environments in the U.S.: nine, six, and four strains of serotypes 1/2a, 4b, and 1/2b, respectively.

L. monocytogenes isolates were grouped into 10 pulse-types by dendrogram analysis of the PFGE banding patterns from *Asc*I restriction enzyme digestion of total DNA, using a threshold of > 90% genetic similarity among the strains within each pulse-

Table 1. Primer sequences used in the study

	Primer Squences (5'-3')			
Gene	**Forward**	**Reverse**	**Size (bp)**	**Reference**
aad6	AGAAGATGTAATAATATAG	CTGTAATCACTGTTCCCGCCT	978	*(15)*
*dfr*D	AGAGTAATCGGCAAGGATAACG	AATGGGCAATTTCACAATCC	199	*(15)*
tet(K)	CGATAGGAACAGCAGTATGG	TTAGCCCACCAGAAAACAAACC	614	*(15)*
tet(L)	CCACCTGCGAGTACAAACTGG	TCGGCAGTACTTAGCTGGTGA	739	*(15)*
tet(M)	CATTCACATCGAAGTGCCGC	ACACCGAGCAGGGATTTCTC	463	This study
tet(S)	ATCAAGATATTAAGGAC	TTCTCTATGTGGTAATC	589	*(15)*

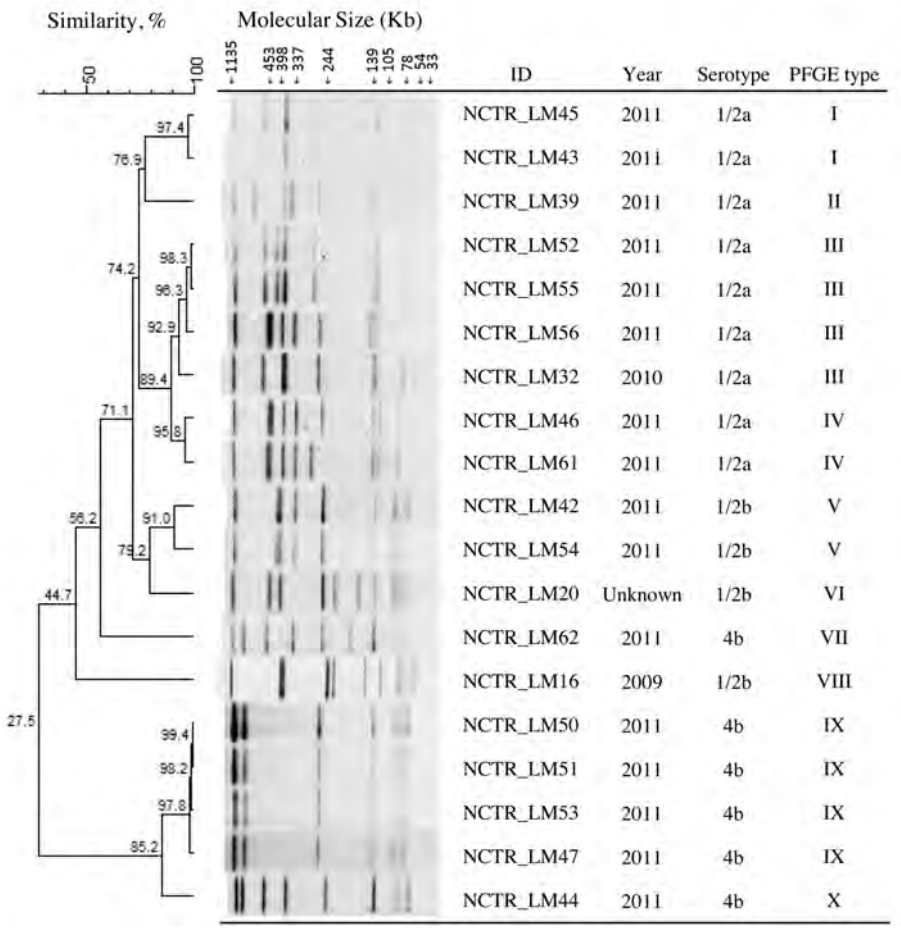

Figure 1. Analysis of PFGE profiles by *Asc*I-digestion and descriptions of *L. monocytogenes* isolated from food-related environments. A total of 19 *L. monocytogenes* environmental isolates were classified by more than 90% similarity of PFGE band pattern among the strains. Edemiological data show detection year, serotype and PFGE (pulse) type.

type *(Fig. 1)*. The PFGE band patterns of *Asc*I-digested DNA were well resolved, but the PFGE patterns obtained with *Apa*I showed low discriminatory power with only a few pulse-types (data not shown). The pulse-types I, III, and IV all belonged to serotype 1/2a, while the V and IX groups were all serotype 1/2b and 4b, respectively. There seemed to be a correlation between serotype and PFGE pulse-type *(Fig. 1)*.

Antimicrobial susceptibility of *L. monocytogenes* isolates

All strains except NCTR_LM50 were resistant to the extended spectrum cephalosporins (cefoxitin, ceftriaxone, and cefepime >32 μg/mL), while all strains were susceptible to ampicillin, cephalothin, gentamicin, kanamycin, erythromycin, vancomycin, rifampin, ciprofloxacin, sulfamethoxazole-trimethoprim, and chloramphenicol *(Table*

Table 2. MICs of antimicrobials for *L. monocytogenes* environmental isolates

ID	Antimicrobial (mg/L)[1]													
	AMP	CF	FOX	CRO	FEP	GEN	KAN	STR	TET	VAN	RIF	CIP	SXT	CHL
NCTR_LM16	1	8	32	> 32	32	2	4	16	1	1	< 0.12	0.5	< 0.25	2
NCTR_LM20	1	8	> 32	> 32	> 32	8	8	32	1	1	< 0.12	0.25	< 0.25	2
NCTR_LM32	1	16	> 32	> 32	> 32	1	2	16	1	1	< 0.12	0.5	0.5	2
NCTR_LM39	2	16	> 32	> 32	> 32	1	4	16	1	1	< 0.12	0.5	0.5	2
NCTR_LM42	1	8	32	> 32	> 32	4	16	32	< 0.5	1	< 0.12	0.5	0.5	4
NCTR_LM43	2	16	> 32	> 32	> 32	2	4	32	64	0.5	< 0.12	0.5	< 0.25	2
NCTR_LM44	4	16	> 32	> 32	> 32	2	8	32	64	1	< 0.12	0.5	0.5	2
NCTR_LM45	2	16	> 32	> 32	> 32	2	4	32	64	1	< 0.12	0.5	< 0.25	2
NCTR_LM46	2	16	> 32	> 32	> 32	1	2	16	1	1	< 0.12	0.5	< 0.25	2
NCTR_LM47	4	16	> 32	> 32	> 32	2	8	32	1	1	< 0.12	0.5	0.5	< 0.5
NCTR_LM50	< 0.5	1	8	8	4	4	16	16	1	2	< 0.12	0.25	0.5	4
NCTR_LM51	4	16	> 32	> 32	> 32	2	8	16	2	1	< 0.12	1	0.5	2
NCTR_LM52	1	16	> 32	> 32	> 32	2	8	16	64	1	< 0.12	1	< 0.25	2
NCTR_LM53	4	16	> 32	> 32	> 32	2	16	16	4	1	0.25	0.5	0.5	2
NCTR_LM54	1	8	> 32	> 32	> 32	4	16	16	2	1	< 0.12	0.5	< 0.25	4
NCTR_LM55	2	16	> 32	> 32	> 32	2	8	16	32	1	< 0.12	0.25	< 0.25	2
NCTR_LM56	1	16	> 32	> 32	> 32	2	8	16	1	1	< 0.12	0.5	< 0.25	2
NCTR_LM61	2	16	> 32	> 32	> 32	2	8	32	64	1	< 0.12	0.5	< 0.25	2
NCTR_LM62	1	8	> 32	> 32	> 32	2	16	32	1	1	< 0.12	1	0.5	2
S. aureus	< 0.5	< 0.5	2	4	2	2	16	4	< 0.5	2	< 0.12	0.5	2	8

[1]AMP, ampicillin; CF, cephalothin; FOX, cefoxitin; CRO, ceftriaxone; FEP, cefepime; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; TET, tetracycline; VAN, vancomycin; RIF, rifampin; CIP, ciprofloxacin; SXT, sulfamethoxazole–trimethoprim; CHL, chloramphenicol. All strains were resistant to nalidixic acid (>32 μg/mL) and susceptible to erythromycin (< 0.12 μg/mL).

2). Eight and six strains were resistant to streptomycin and tetracycline, respectively *(Table 2)*. No strain was resistant to 3 or more antimicrobial classes except for cephalosprins *(Table 2)*. All of the environmental isolates were susceptible to the first-choice antibiotics ampicillin and gentamicin in the treatment of listeriosis. Of the antimicrobial resistance determinants *(Table 1)*, only the *tetM* gene was amplified from the total genomic DNA of strains NCTR_LM43, NCTR_LM45, NCTR_LM52, NCTR_LM55, and NCTR_LM61, all of which were resistant to tetracycline *(Table 2)*. We suggest that *tetM* may be a major determinant of tetracycline resistance in *L. monocytogenes*, as shown in a previous study *(21)*. Interestingly, all tetracycline-resistant strains in this study were serotype 1/2a. In our previous study, more serotype 1/2a strains from the environment (five of 19 strains, 26.3%) than from food products (one of 35 strains, 2.9%) were resistant to tetracycline *(1)*. The higher resistance to tetracycline seen in food environmental strains may be caused by exposure to a sanitizer, such as benzalkonium chloride (BC), which increased the MIC of tetracycline in BC sensitive *L. monocytogenes* strains *(17)*. Strains of *L. monocytogenes* repeatedly exposed to sanitizers or disinfectants in the food processing environment may show increased resistance to tetracycline. Further study may be needed for determining the adaptation or acquisition of tetracycline resistance in *L. monocytogenes* isolates by using a model exposed repeatedly to a sanitizer or disinfectant.

Fewer studies have investigated the distribution of antimicrobial resistance of food processing environment strains of *L. monocytogenes* than of strains isolated directly from food products *(16)*. Data from food-related environmental isolates may be useful for comparative

studies with the genetic and serological characterization of food isolates to determine the origin of contamination with *L. monocytogenes (7, 19, 20)*. Furthermore, surveillance of antibiotic resistance in *L. monocytogenes* strains from food processing environments provides needed information about the development and spread of resistance in the food supply. Because of the severity of listerosis, timely monitoring of the development and spread of antibiotic resistance is important, since resistance may decrease treatment efficacy.

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