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Antimicrobial susceptibility and serotyping of *Listeria monocytogenes* isolated from ready-to-eat seafood and food processing environments in Korea

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Abstract We examined antimicrobial susceptibility and serotypes of 33 *L. monocytogenes* isolates collected from ready-to-eat seafood and food processing environments. The isolated strains belonged to the 1/2b (73%), 4b (15%), and 1/2a (12%) serotypes; 11 of the obtained environmental swab samples belonged to the 1/2b serogroup. Antimicrobial resistance to benzyl penicillin (100%), clindamycin (100%), oxacillin (100%), ampicillin (97%), and tetracycline (18%) was detected, and 27/33 isolates (82%) showed resistance to four antibiotics and 6/33 (18%) were resistant to five. Total typing by automated repetitive sequence-based PCR revealed that the 33 isolates grouped into four distinct clusters with significantly correlated serotypes. These findings provide important information about the safety of ready-to-eat seafood and suggest that control measures should be adopted in order to mitigate the risk to humans posed by *L. monocytogenes* contaminated seafood.

Keywords: *Listeria monocytogenes*, seafoods, characterization, antimicrobial susceptibility, rep-PCR

Introduction

Listeria spp. are Gram-positive facultative bacteria found in a variety of foods and environments. Of the eight species in this genus, *L. monocytogenes* and *L. ivanovii* are considered virulent. In humans, *L. monocytogenes* is a foodborne pathogen of public health concern due to the high mortality rate associated with infection (1), as well as its widespread distribution in nature and ability to survive under various environmental conditions, including at refrigerated temperatures (2). The latter increases the risk of *Listeria* infection from contaminated ready-to-eat food products (3). Cross-contamination and recontamination in processing plants are common source of *L. monocytogenes* in ready-to-eat food products including sea food (4). Environmental contamination can originate from many sources such as raw and packaging materials and personnel, and can include both transient and persistent strains (5).

Over 98% of clinical isolates from human listeriosis belong to one of four *L. monocytogenes* serotypes (4b, 1/2a, 1/2b, and 1/2c), while other serotypes (especially 4a) are mostly found in foods and animals and are rarely responsible for human *L. monocytognes* infection (6). Interestingly, although 1/2a is the serotype most frequently isolated from food, 4b causes the majority of human epidemics (2). It is therefore likely that virulence depends on serotype designation. A multiplex PCR assay for species- and virulence-specific detection of *L. monocytogenes* has been developed (6,7).

Antimicrobial resistance particularly multidrug resistance in microorganisms has become a major global public health problem in recent decades. The excessive and inappropriate use of antimicrobial agents is responsible for the emergence of resistant bacterial strains (8). *Listeria* infection usually requires antimicrobial treatment with penicillin G or ampicillin, either alone or in combination with an aminoglycoside. However, little is known about *L. monocytogenes* antimicrobial resistance, especially for isolates of non-human origin (9). Serotyping, antibiotic susceptibility testing and automated repetitive sequence-based rep-PCR used to assess the contamination of foods and food processing environments (7,8,10). The present study was carried out in order to characterize *L. monocytogenes* serotypes, antimicrobial resistance patterns, and genetic diversity in ready-to-eat seafood and food processing environments in relation to the phenotype and genotype of isolates.

Materials and Methods

Sample collection A total of 33 of *L. monocytogenes* isolates were recovered from ready-to-eat seafoods and food processing environments

in markets across Korea. Environmental samples were obtained using a sponge sampling kit (3M Microbiology Products, St. Paul, MN, USA) from an area of approximately 10 × 10 cm^2. All food and swab tube samples were transported to the laboratory in an ice box. Samples were analyzed immediately.

Isolation and identification of *L. monocytogenes* A portion of each seafood sample (25 g) was homogenized in 225 mL *Listeria* enrichment broth (Merck, Darmstadt, Germany) and incubated at 30°C for 24±2 h, followed by inoculation in Fraser broth (Biomériux, Marcy L'Etoile, France) in 0.1 mL of *Listeria* enrichment broth for 24±2 h at 37°C. The culture broth was streaked on to PALCAM agar with PALCAM *Listeria* selective-supplement (Merck). Fifth typical colonies were selected on tryptic soy agar (Difco Laboratories, Detroit, MI, USA) and analyzed by Gram staining; the hemolysis, the catalase, motility, and CAMP tests; and by biochemical characterization with Vitek 2 (Biomériux). A 10 mL volume of each environmental swab was incubated with 90 mL *Listeria* enrichment broth at 30°C for 24±2 h and isolates were obtained as described above.

Serotyping Serotyping was carried out using the *Listeria* Antisera Set (Denka Seiken Co., Tokyo, Japan), according to the manufacturer's instructions. O (O-I/II, O-V/VI, O-I, OII,O-VI, OVII, O-VIII, and Y O-IX) and H (H-A, H-AB, H-C, and H-D) antigens were identified according to the serotyping scheme. The following *L. monocytogenes* strains (American Type Culture Collection (ATCC), Manassas, VA, USA) were used as standards: ATCC 51772 (1/2a), ATCC 51780 (1/2b), ATCC 19112 (1/2c), and ATCC 19115 (4b).

Antimicrobial susceptibility test Antibiotic resistance was tested by the disc-diffusion method of the Clinical and Laboratory Standards Institute (11). Isolates grown on TSA were transferred to Mueller-Hinton broth (Difco Laboratories) with turbidity adjusted to 0.5 McFarland, then inoculated three times (while rotating by 60° each time) using a sterile cotton swab on to Mueller-Hinton Agar (Difco Laboratories) supplemented with 5% sheep blood. Antibiotic discs were placed on the surface of each plate (four antibiotics per plate) using a disc dispenser (Oxoid USA, Columbia, MD, USA). After incubating at 37°C for 24 h, the diameter of growth inhibition zone surrounding each disc was measured and interpreted according to CLSI guidelines. For antimicrobials for which *Listeria* and *Staphylococcus* criteria were unavailable (cefoxitin, benzylpenicillin, streptomycin, and vancomycin), previously defined breakpoints were used (12). The following antibiotics were tested: ampicillin (10 μg), cefoxitin (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), erythromycin (15 μg), gentamycin (10 μg), kanamycin (30 μg), oxacillin (1 μg), benzyl penicillin (10 U), rifampicin (5 μg), streptomycin (10 μg), tetracycline (30 μg), trimethoprim/sulfamethoxazole (25 μg), and vancomycin (30 μg). *Staphylococcus aureus* (ATCC 25923) was used as a control strain.

Automated repetitive-sequence-based PCR (DiversiLab) microbial typing test DNA was extracted from all samples using the Ultra Clean microbial DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA concentration was measured using a Nano drop instrument (Thermo Fisher Scientific, Waltham, MA, USA) and adjusted to 35 ng. PCR amplification was carried out using the DiversiLab *Listeria* kit (Bacterial BarCodes, Houston, TX, USA) on a Tgradient system (Biometra, Goettingen, Germany). Reaction conditions were as follows: 94°C for 3 min, 35 cycles of 94°C for 30 s, 50°C for 30 s, and 70°C for 90 s, and 70°C for 3 min. Rep-PCR products were analyzed using a Labchip on a Model 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and fingerprint profiles were compared using Divers iLab v.3.4 software, which uses the Pearson correlation coefficient to evaluate distance matrices. Relatedness was determined via cluster analysis according to the manufacturer's guidelines.

Results and Discussion

***L. monocytogenes* serotypes** Of the 33 isolates recovered from ready-to-eat seafoods and food processing environments, three serotypes—i.e., 1/2b (24/33, 73%), 4b (5/33, 15%), and 1/2a (4/33, 12%)—were identified (Table 1). All three serotypes were exited in all tested samples. The 1/2a and 1/2b serotypes were predominant in seafoods from Japan, whereas three serotypes were present in seafoods from Italy (13,14). Serotype 4b is the most frequently associated with listeriosis through ingestion of contaminated food (7). *L. monocytogenes* isolates from food and listeriosis patients included serotypes 1/2a, 1/2b, and 4b (15). The presence of these serotypes in food products is a risk to public health, given that they are associated with foodborne outbreaks and sporadic cases of human listeriosis (16). Contamination of raw seafoods and cross-contamination of end products may occur during post-processing or inapposite storage, during which time the pathogen can continue to grow. Korean regulations have zero tolerance for ready-to-eat foods because of their ability to support the growth of microorganisms; in

Table 1. Serotype distribution of *L. monocytogenes* isolates

Source	No. of isolates	*L. monocytogenes* serotype			
		1/2a	1/2b	1/2c	4b
Ready-to-eat food	22	4	13	0	5
Environment	11	0	11	0	0
Total (%)	33 (100.0)	4 (12%)	24 (73%)	0 (0%)	5 (15%)

Table 2. Antimicrobial resistance of *L. monocytogenes* strains isolated from ready-to-eat seafood and food processing environments

Antimicrobial	Disc potency	No. of isolates (%)		
		S[1)]	I	R
Ampicillin	10 μg	0	1 (3)	32 (97)
Benzylpenicillin	10 U	0	0	33 (100)
Cefoxitin	30 μg	33 (100)	0	0
Chloramphenicol	30 μg	30 (91)	3 (9)	0
Ciprofloxacin	5 μg	30 (91)	3 (9)	0
Clindamycin	2 μg	0	0	33 (100)
Erythromycin	15 μg	33 (100)	0	0
Gentamicin	10 μg	33 (100)	0	0
Kanamycin	30 μg	33 (100)	0	0
Oxacillin	1 μg	0	0	33 (100)
Rifampicin	5 μg	33 (100)	0	0
Streptomyin	10 μg	33 (100)	0	0
Tetracycline	30 μg	26 (79)	1 (3)	6 (18)
Trimethoprim/ Sulfamethoxazole	25 μg	33 (100)	0	0
Vancomycin	30 μg	33 (100)	0	0

[1)]S, susceptible; I, intermediate; R, resistant

particular, there is a high risk of *L. monocytogenes* contamination unless strict hygienic control and preventive measures are needed.

Antimicrobial susceptibility of *L. monocytogenes* Antimicrobial susceptibility of the 33 *L. monocytogenes* isolates as determined by the disc diffusion assay is shown in Table 2. The highest antibiotic resistance was to benzyl penicillin (100%), clindamycin (100%), oxacillin (100%), ampicillin (97%), and tetracycline (18%), whereas the lowest resistance was to cefoxitin, erythromycin, gentamicin, kanamycin, rifampicin, streptomyin, trimethoprim/sulfamethoxazole, and vancomycin. Tetracycline-resistant isolates were detected in samples from food processing environments. *L. monocytogenes* isolates from raw crabs were more resistant to tetracycline than those from environmental samples, but processing plants also harbored resistant isolates (1). Chloramphenicol and ciprofloxacin resistance was detected in isolates from ready-to-eat seafoods (3%) and food processing environments (6%). There are reports of frequent isolation of ciprofloxacin-, tetracycline-, and streptomycin-resistant *L. monocytogenes* strains from food or other sources (17). *L. monocytogenes* isolated from ready-to-eat foods were resistant to ampicillin, penicillin, tetracycline, and trimethoprim/sulfamethoxazole (16). Antibiotic resistance of microorganisms is a major public health issue (8), and the high prevalence of antibiotic-resistant *L. monocytogenes* requires continued surveillance.

Antibiotic resistance profiles revealed that all isolates were multidrug-resistant; five multidrug-resistant isolates were detected in environment samples, including serotype 1/2b (Table 3). Benzyl penicillin is the first-choice antibiotic used for listeriosis treatment, while ampicillin, rifampicin, or penicillin plus gentamicin are used to

Table 3. Multidrug resistance in *L. monocytogenes* isolates from ready-to-eat seafood and food processing environments

No. of drugs	Resistance patterns[1)]	Source		Total (%)
		Ready-to-eat seafood (%)	Food processing environment (%)	
4	OX,DA2, P, AMP	27 (82%)	0 (0%)	27(82)
5	TE, OX, DA2, P, AMP	0	6 (18)	6 (18)
Total				33 (100)

[1)]AMP, ampicillin; DA2, clindamycin; OX, oxacillin; P, benzyl penicillin; TE, tetracycline

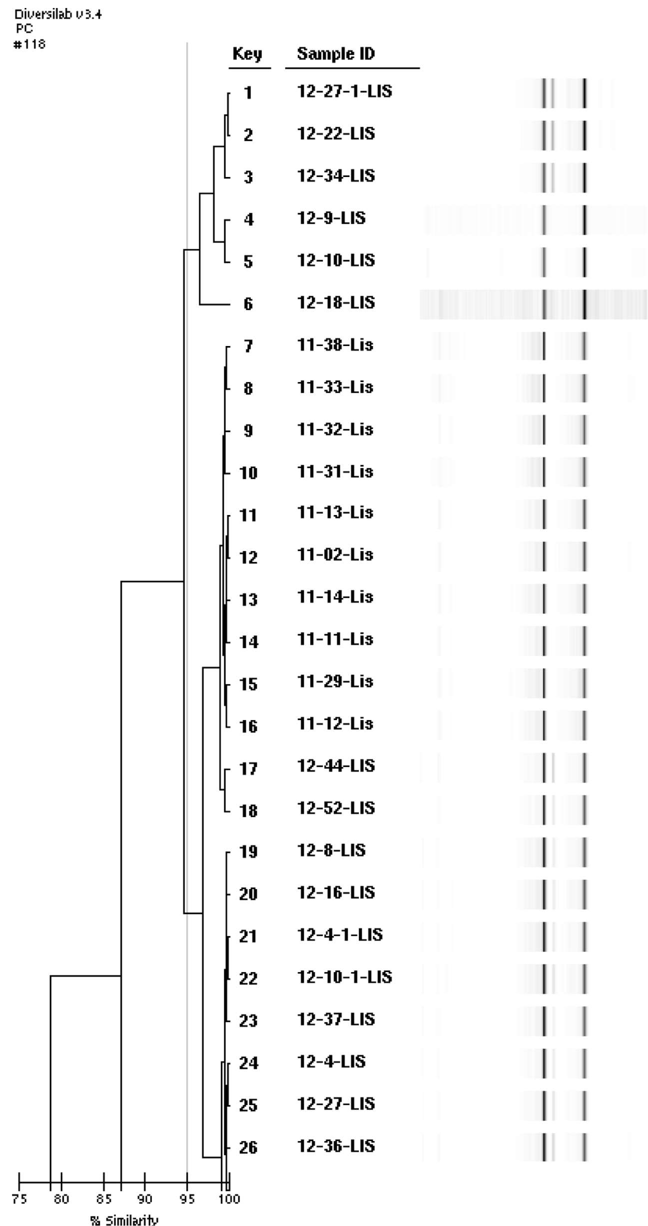

Fig. 1. Rep-PCR-generated dendrogram of the 33 *L. monocytogenes* isolates. A similarity index cutoff of 95% (gray vertical line) was used to establish relatedness.

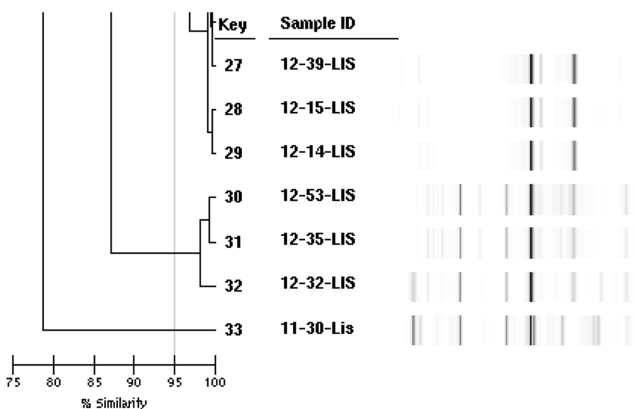

Fig. 1. Continued

treat most manifestations of listeriosis (18,19). Antibiotic-resistant strains can be transmitted to humans through consumption of contaminated food products and resistant bacteria (20). These data provide insight into antibiotic resistance of *L. monocytogenes* strains isolated from seafood and food processing environments that can benefit epidemiological and public health studies of listeriosis.

Automated repetitive-sequence-based PCR (DiversiLab) microbial typing test Typing by automated rep-PCR revealed genetic diversity among the 33 *L. monocytogenes* isolates (Fig. 1): four distinct clusters were detected at 95% similarity. Cluster I was predominant and included serotype 4b-positive isolates (e.g., the key 6 strain was 1/2b). Cluster II included serotype 1/2b-positive isolates (e.g., the key 19 strain was 1/2a). Cluster III included serotype 1/2a-positive isolates, and Cluster IV mainly comprised serotype 1/2b. When rep-PCR fingerprints of the resistance to antibiotics isolates were compared, there was no apparent original grouping. Serotype 1/2b strains had had similarity scores of more than 95% (Table 4). This could serve as an alternative tool for analyzing genetic relatedness among 1/2a strains, which are prevalent in ready-to-eat seafood and food processing environments (21). The automated rep-PCR method could not discriminate between serotypes 1/2b and 4b, but may be useful for discriminating between 1/2a and other serotypes and for tracking isolates within the 1/2a serotype. Our observation that 4b, 1/2b, and 1/2a serotypes are highly related is consistent with previous study (21) of ready-to-eat sea foods and food processing environments. Pathogens can change their phenotypic and genotypic characteristics in response to variations in environmental conditions (14). Therefore, food products should be continually monitored for the presence of novel strains. The results of this study provide evidence that *L. monocytogenes* in ready-to-eat seafoods consumed without further heat processing is a risk for consumers. Continued surveillance of the prevalence of *L. monocytogenes* and the emergence of antibiotic-resistant strains is necessary for quality control of ready-to-eat foods. In addition, the judicious use of antimicrobials is important to limit the emergence of resistant strains.

Table 4. Rep-PCR results for 33 *L. monocytogenes* isolates

Cluster	Sample ID	Sources	Serotype
I	12-27-1-LIS	Food	4b
	12-22-LIS	Food	4b
	12-34-LIS	Food	4b
	12-9-LIS	Food	4b
	12-10-LIS	Food	4b
	12-18-LIS	Food	1/2b
II	11-38-LIS	Environment	1/2b
	11-33-LIS	Environment	1/2b
	11-32-LIS	Environment	1/2b
	11-31-LIS	Environment	1/2b
	11-13-LIS	Environment	1/2b
	11-02-LIS	Environment	1/2b
	11-14-LIS	Environment	1/2b
	11-11-LIS	Environment	1/2b
	11-29-LIS	Environment	1/2b
	11-12-LIS	Environment	1/2b
	12-44-LIS	Food	1/2b
	12-52-LIS	Food	1/2b
	12-8-LIS	Food	1/2a
	12-16-LIS	Food	1/2b
	12-4-1-LIS	Food	1/2b
	12-10-1-LIS	Food	1/2b
	12-37-LIS	Food	1/2b
	12-4-LIS	Food	1/2b
	12-27-LIS	Food	1/2b
	12-36-LIS	Food	1/2b
	12-39-LIS	Food	1/2b
	12-15-LIS	Food	1/2b
	12-14-LIS	Food	1/2b
III	12-53-LIS	Food	1/2a
	12-35-LIS	Food	1/2a
	12-32-LIS	Food	1/2a
IV	11-30-LIS	Environment	1/2b

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