Letter to the Editor

Antimicrobial Resistance, Virulence Profile, and Molecular Characterization of *Listeria monocytogenes* Isolated from Ready-to-eat Food in China, 2013-2014

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We aimed to investigate the potential pathogenic profile and antibiotic resistance of *Listeria monocytogenes* isolated from ready-to-eat food in China. Antimicrobial resistance was determined by broth microdilution following the Clinical and Laboratory Standards Institute protocol. Molecular serotyping, virulence, and resistance genes were identified using PCR. Multi-locus sequence typing was performed on resistant strains. A total of 11.53% (113/980) isolates were resistant, from which 82.3% (93/113) harbored all the virulence genes tested. The resistant strains were subtyped into 18 sequence types (STs), from which ST2, ST5, ST8, and ST9 were involved in listeriosis. This study indicated that several *L. monocytogenes* isolates from ready-to-eat foods in China have pathogenic potential and are resistant to antibiotics, including antibiotics used as medicines by humans for listeriosis treatment.

Listeria monocytogenes is a Gram-positive bacterium that can survive through tough conditions, including low pH, high concentration of salt, low temperature, and even grow in a refrigerator at 4 °C. *L. monocytogenes*is considered as one of the most important food-borne pathogens that can be a public threat to human health[1]. The bacteriumis responsible for listeriosis in humans, including meningitis, fetal loss, sepsis, and febrile gastroenteritis. The fatality rate of listeriosis can be as high as 30%, and it primarily affects the elderly, neonates, and immunocompromised individuals[2]. *L. monocytogenes* can grow in a wide variety of potential reservoirs and sources within food-processing plants and contaminated ready-to-eat foods[3]. Because of its high pathogenicity, food contamination surveillance of *L. monocytogenes* has been regularly implemented around the world. The regular way to treat listeriosis is generally antibiotics such as penicillin G or ampicillin combined with or without an aminoglycoside treatment. Although rifampin, vancomycin, linezolid, and carbapenems have been proposed as possible alternatives, trimethoprim is generally used in cases of intolerance to penicillin G or ampicillin. Although the antimicrobial resistance of *L. monocytogenes* is not as severe as other food-borne pathogens such as *Staphylococcus aureus*, it has been recently gradually growing. Therefore, it is important to acquire the profile of the antimicrobial susceptibility and resistant genes of *L. Monocytogenes* during surveillance and to monitor the use of antibiotics.

Not all *L. monocytogenes* isolates have an equal pathogenicity[4]. Therefore, it is important to investigate the virulence potential of *L. Monocytogenes*, resistant isolates. The virulence factors of *L. monocytogenes* include internalins (encoded by *inlA*, *inlC*, and *inlJ*), listeriolysin O (LLO encoded by hly), actin (*actA*), phosphatidylinositol-phospholipase C (PI-PLC encoded by *plcA*), iap (invasion associated protein encoded by *iap*), and virulence regulator (encoded by *prfA*)[5].

The molecular characterization of *L. monocytogenes* was proved to be important in epidemiological investigations, in source tracking food-processing plants, and in determining evolutionary relationships. As the first approach of subtyping used to identify outbreak-associated serotypes, traditional serotyping is still used. The molecular serotyping of *L. monocytogenes*, based on PCR, has been ubiquitously used as the primary serotyping method[6]. Multi-locus sequence typing (MLST) is a highly discriminatory sequence-based method for subtyping *L. monocytogenes.* Compared with pulse-field gel electrophoresis, MLST was found to be more discriminative for phylogenetic analyses, because it relies on all sequence differences in the

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amplified portion of the gene[6].

Here, 980 *L. monocytogenes* isolates were obtained from different ready-to-eat food products through food surveillance from 23 provinces or cities in China, and antimicrobial susceptibility was performed. The resistant strains were characterized by MLST. Resistant and virulence gene profiles were also performed for characterizing the resistance mechanism and pathogenicity of resistant *L. monocytogenes* strains.

All *L. monocytogenes* isolates were tested for their susceptibility to eight antibiotics commonly used in veterinary and human therapy, including ampicillin (AMP), trimethoprim-sulfamethoxazole (TMP-SMZ), chloramphenicol (CHL), tetracycline (TET), gentamicin (GEN), ciprofloxacin (CIP), vancomycin (VAN), and erythromycin (ERY). Tests were performed following the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Because CLSI breakpoints for *L. monocytogenes* only include AMP and TMP-SMZ and *L. monocytogenes* belongs to the *Bacillus* genus, CLSI breakpoints for *Bacillus* were also used for the other six antibiotics. All antibiotics were purchased from Sigma-Aldrich, Germany.

Genomic DNAs of the resistant isolates were extracted using QIAGEN DNeasy® Blood & Tissue kit (Qiagen, Germany). PCR amplification was conducted with QIAGEN HotStar Tag KIT (Qiagen, Germany). The tetracycline resistance genes (*tetM*, *tetL*, *tetS*, *tetK*, and *int-Tn*), ampicillin resistance gene (*ampC*), erythromycin resistance genes (*ermA*, *ermB*, *ermC*, *ermTR*, and *mefA*), TMP-SMZ resistance gene (*dfrD*), chloramphenicol resistance genes (*cmlA* and *cat*), gentamycin resistance gene (*aadB*), and ciprofloxacin resistance gene (*lde*) were detected using PCR. Virulence genes *inlA*, *inlC*, *inlJ, hly*, *actA*, *plcA*, *iap* and the virulence regulation gene *prfA* were identified using PCR for all resistant *L. monocytogenes* strains. Molecular serotyping was performed on resistant isolates by multiplex PCR. This method distributed the *L. monocytogenes* strains into five serovar groupings: I.1 (1/2a, 3a), I.2 (1/2c, 3c), II.1 (1/2b, 3b, 7), II.2 (4b, 4d, 4e), and III (4a, 4c). All primers used in this study are presented in Table S1 (see the www. besjournal.com).

For MLST, the primers and PCR condition used for amplification of the seven housekeeping genes and sequencing are available on http://bigsdb.web.pasteur.fr/listeria/primers_used.html. The DNA fragments were purified using the standardized Ethanol-NaAc-EDTA method. All PCR products were sequenced in each direction with BigDye fluorescent terminators on an ABI 3500 xl sequencer (Applied Bio Systems, USA). For each resistant strain, the allele combination at the 7 loci defines an allelic profile or sequence type (ST). Minimum spanning tree analysis was used to infer relationships among the isolates and was performed with BioNumerics 7.1 (Applied Maths, Belgium). Antimicrobial susceptibilities of all *L. monocytogenes* isolates were tested against eight different categories of antibiotics using the minimum inhibitory concentration (MIC) distribution method, following the CLSI protocol. The MIC distribution values of each antibiotic [in which the growth of 50% (MIC_{50}) or 90% (MIC_{90}) of the isolates is inhibited] are presented in Table 1. From all isolates tested, 11.53% (113/980) were resistant to certain antibiotics, as determined by the CLSI criteria. The identification of AMP (3) and TMP-SMZ (27) resistant isolates indicates a potential risk when treating listeriosis with these two antibiotics. However, resistance genes *ampC* and *dfrD* associated with AMP and TMP-SMZ were not detected, which means that the mechanisms under AMP or TMP-SMZ resistance were not correlated with these two genes. A high TET resistance rate of *L. monocytogenes* has already been reported[7]. In our research, we found that more than half of the resistant isolates were resistant to TET (56.64%, 64/113), and 79.69% of TET-resistant isolates harbored *tetM* (Table S2 see the www. besjournal.com). A high TET resistance rate indicated abuse of TET in the animal farm. All *L. monocytogenes* isolates tested were susceptible to VAN. As for ERY-resistant isolates, *ermB* (23.08%, 3/15) and *mefA* (84.62%, 11/13) genes were detected, while the other 3 ERY-associated resistant genes (*ermA*, *ermC*, *and ermTR*) were not. ERY resistance in *L. monocytogenes* isolates may indicate a potential problem in the clinical implementation of ERY in treating certain infection.

In total, 18 antibiotic susceptibility profiles are presented in Table S3 (see the www. besjournal.com). A total of 88.47% (867/980) of all the isolates tested were susceptible to all eight antibiotics. From all resistant isolates, 68.14% (77/113) of *L. monocytogenes* isolates were resistant to only one antibiotic. Thirty isolates were resistant to two antibiotics. The number of isolates resistant

Table 1. MIC Distribution of 980 *L. monocytogenes* Isolates

Antibiotic	Year (n_{2014} = 780, n_{2013} = 200)	Number of Isolates with MIC (μg/mL) of														μg/mL		Resistant Proportion/% (Number of Isolates)
		256	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	MIC_{50}	MIC_{90}	
AMP	2014										5	55	629	91		0.125	0.125	0.00 (0)
	2013							3	4	7	44	115	24	2	1	0.25	0.5	1.50 (3)
TMP (TMP/SMZ)	2014				4	4	8	11	2	3	64	644	40			0.25	0.5	3.46 (27)
	2013								2	2	1	6	4	24	161	0.03125	0.0625	0.00 (0)
TET	2014	1	6	32	9	4	1	6	138	567	16					1	2	6.92 (52)
	2013			2	9	1	1	3	8	30	84	62				0.5	2	6.00 (12)
CHL	2014		3		3	1	10	559	204							8	8	0.77 (6)
	2013			1		2	131	62	2	2						8	8	0.50 (1)
VAN	2014							1	1	762	14	2				1	1	0.00 (0)
	2013							2	3	12	181	2				0.5	0.5	0.00 (0)
GEN	2014							1	3	75	391	270	40			0.5	1	0.00 (0)
	2013					4	46	71	47	18	8	2	2	2		4	8	2.00 (4)
ERY	2014					10	1	1	3	12	45	643	64			0.25	0.25	1.41 (11)
	2013						2	1	4	2	31	134	24	2		0.25	0.5	1.00 (2)
CIP	2014							13	115	536	113	3				1	2	1.67 (13)
	2013						7	22	47	91	32		1			1	4	14.50 (29)

to three, four, and five antibiotics was two, three, and one, respectively. The number of isolates resistant to three and more antibiotics were six (5.3%, 6/113), which indicates that the status of multidrug resistance of *L. monocytogenes* was at a low level.

We investigated the virulence potential of *L. monocytogenes* isolates through PCR identification of virulence genes. Virulence genes *inlA*, *inlC*, *inlJ*, *hly*, *actA*, *plcA*, and *iap*; and the virulence regulation gene *prfA* were identified using PCR for all resistant *L. monocytogenes* strains. It has already been reported that most *L. monocytogenes* possess all the virulence genes mentioned above[5]. Similar to previous reports, in our study, 82.3% (93/113) of the resistant strains harbored all the virulence genes. *hly* (100%, 113/113) was found to be positive in all the isolates of our study (Table 2). *Hly* encoded protein listeriolysin O (LLO). LLO allows bacteria to live intracellularly, where they are protected from extracellular immune system factors[8]. This may indicate that 82.3% of the resistant strains have the higher potential to escape the immune system and lead to listeriosis.

Molecular serotyping and MLST were conducted on each one of the resistant isolates. One hundred and thirteen resistant isolates were divided into three serogroups using the molecular serotyping method. A total of 83.19% (94/113) of them belonged to 1/2a, 3a, which were the dominant serogroup in this study. The serogroup 1/2b, 3b, 7 was observed at 15.93% of the resistant isolates. Only one resistant isolate belonged to the 4a, 4c group (Table S4 see the www. besjournal.com).

Here, 113 resistant *L. monocytogenes* isolates were divided into 18 STs (Figure 1), which all have previously been established in the MLST database. Among those STs found in resistant strains, ST2 has already been reported to cause an outbreak in Italy[9]. We identified five ST2 isolates, from which four belonged to the 1/2b, 3b, 7 serogroups and one belonged to the 1/2a, 3a serogroup. The food origins of these isolates were chicken, vegetable, and pork; and they were resistant to AMP, TMP-SMZ, CIP, and TET (Table S5 see the www.besjournal.com). More attention must be paid to the pathogenicity and epidemiology of these five isolates. ST5 (four strains), ST8 (16 strains), and ST9 (18 strains), which were found in our research, are known to cause listeriosis in humans[10]. ST9 was related to four multidrug-resistant isolates in our study. Further studies are warranted to elucidate the relationship between ST9 and multidrug resistance. Similar to previous studies, lineage II (82.30%, 93/113) consists of most of the strains isolated from food in China and they all belonged to the 1/2a, 3a serogroup[4,10]. Most ST155, ST121, and ST705 were found to be related to TET resistance, and the food origins were mostly meat, which indicate that they may have originated from the animal farm and abuse of TET.

In conclusion, the resistant isolates of *L. monocytogenes* from ready-to-eat food in China have the potential to cause listeriosis and can be more dangerous because of their resistance to antibiotics used for treating listeriosis. The ST2, ST5, ST8, and ST9, which caused maternal fetal infections or outbreaks in other countries, were also detected in those resistant

Table 2. Virulence Gene Detection in Antibiotic-resistant *L. monocytogenes* Isolates

Virulence Gene	Number of Positive Isolates	Percentage
inlA	112	99.12%
inlC	111	98.23%
inlJ	96	84.96%
InlA+inlC+inlJ	96	84.96%
plcA	111	98.23%
prfA	111	98.23%
actA	94	83.19%
hly	113	100.00%
Iap	112	99.12%

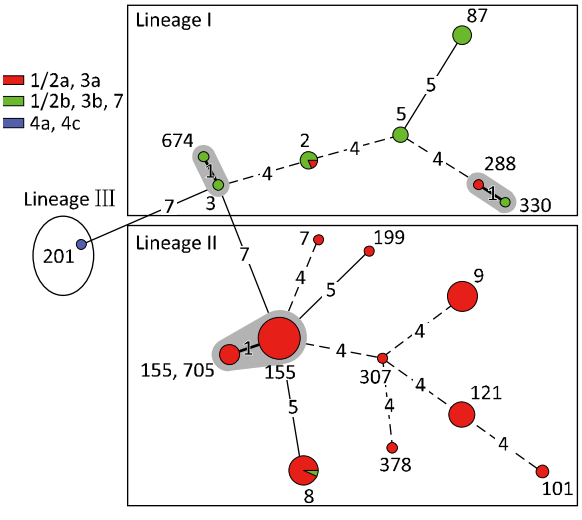

Figure 1. The minimum spanning tree of the 18 STs from resistant *L. monocytogenes*. Each circle corresponds to a sequence type. The shadow zones in grey correspond to different clonal complexes. The size of the circle is proportional to the number of isolates, and the color within the cycles represents the serotypes of the isolates.

isolates. It is necessary to strengthen the surveillance of clinical listeriosis and its antimicrobial profiles to prevent the emergence and outbreaks of human *L. monocytogenes* infections in China.

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