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Antimicrobial resistance profiles of *Listeria monocytogenes* isolated from chicken meat in Fukuoka, Japan

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ABSTRACT

In this study, the antimicrobial resistance profiles of *L. monocytogenes* isolated from chicken meat in Fukuoka in 2017 were compared with the isolates of 2012. A total of 85 and 50 chicken meat samples, including different body parts, were collected from different supermarkets in Fukuoka in 2012 and 2017, respectively. Detection, isolation, identification, and characterization of *L. monocytogenes* were performed according to the conventional methods. Forty-five among 85 samples (53%) were positive for *L. monocytogenes* in 2012, while 12 among 50 samples in 2017 (24%) tested positive. One hundred fifty-three and 29 *L. monocytogenes* strains were isolated in 2012 and 2017, respectively. The serotypes of isolates in 2012 were 1/2a (21.5%), 1/2b (73.9%), 1/2c (1.5%), and 4b/4e (3.1%). In contrast, the 2017 isolates showed 1/2a (48.3%) and 1/2b (51.7%) serotypes. While all isolates in 2012 were positive for *hlyA* (listeriolysin O) in the PCR assay with *hlyA* primer set 7, only 17 *hlyA* positive isolates were seen in 2017. Moreover, 75 isolates with different ribotypes in 2012 and 29 isolates in 2017, respectively, were tested for antimicrobial susceptibility by broth microdilution for 18 different antimicrobial agents. Most of the 2012 and 2017 isolates displayed antimicrobial susceptibility. However, among the 2012 and 2017 isolates, 98.7% and 100% of the isolates were resistant to cefoxitin, 57.3% and 95.7% to fosfomycin, 72.0% and 82.6% to oxacillin, 8.0% and 17.4% to clindamycin, respectively. In addition, 2.7% of the isolates in 2012 were resistant to flomoxef and 4.3% of the isolates in 2017 to linezolid. Multidrug resistance (MDR) to 3 or more antimicrobials was observed in 35/75 (46.7%) isolates of 2012 and 19/23 (82.6%) in 2017. Detection of antimicrobial resistance (AMR) genes by PCR showed that the resistant isolates of 2012 were positive for *mecA* (96.3%) and *ermC* (83.3%), whereas the resistant isolates in 2017 screened positive for *mecA* (94.7%) and *mefA* (25.0%). Other *cfxA*, *ermA*, *ermB*, *fosA*, *fosB*, and *fosC* genes were absent in the PCR assay for any of the isolates. This study investigated for the first time the change in the *L. monocytogenes* contamination of chicken meat and antibiotic resistance of the isolated *L. monocytogenes* strains in Fukuoka, Japan, in the course of 5 years. Although the contamination rate of *L. monocytogenes* in 2017 was found to be lower than that in 2012, AMR of the isolates in 2017 was higher.

1. Introduction

Listeria monocytogenes is a Gram-positive, facultative intracellular food borne pathogen that can cause listeriosis in humans and animals (Karadal and Yildirim, 2014). It is widely prevalent in nature and responsible for several outbreaks of listeriosis linked to the consumption of contaminated ready to eat meat, poultry, dairy, fish, and vegetable products (Meloni, 2015). It includes 13 serotypes such as 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e, and 7. Among them, serotypes 1/2a, 1/2b, and 4b are frequently isolated from food as well as patients. Moreover, 95% of listeriosis cases in humans have been caused by these 3 serotypes (Kramarenko et al., 2013). Serotype 4a has been detected mostly in foods and animals though it is rarely responsible for human listeriosis (Liu et al., 2006).

Listeriosis can cause either non-invasive febrile gastroenteritis with influenza-like symptoms in healthy people or serious invasive illness with meningitis, encephalitis, and septicemia in pregnant women, newborns, and immunocompromised and elderly people, with a mortality of 20–40% (Farber and Peterkin, 1991; Khan et al., 2013; Mead et al., 1999; Swartz, 2002). While the outbreak of listeriosis has

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occurred frequently in the United States and European countries (Hasegawa et al., 2013), only one food-borne outbreak has been reported in Japan, caused by consumption of contaminated cheese in 2001 (Makino et al., 2005).

Antimicrobial resistance pathogens are recognized as a global public health threat and found in people, animals, foods, and environment including water, soil, and air (Doyle et al., 2013). Antimicrobial-resistant *L. monocytogenes* has been frequently detected since a multidrug resistant strain was found in France in 1988 (Poyart-Salmeron et al., 1990). Antimicrobial agents have been used for many purposes such as prevention, treatment, and control of infectious diseases in both animals and humans as well as growth enhancement in food-producing animals in most of the countries including Japan (Phillips et al., 2004; Tamura, 2016). Their extensive usage has increased antimicrobial resistance in bacterial pathogens and the proliferation of resistant pathogens through the food chain, leading to an increased public health problem in terms of morbidity, mortality, and cost of treatments (Gyles, 2008).

The emergence and dissemination of AMR in *L. monocytogenes* is one of the serious problems for the treatment of infectious diseases. Moreover, the prevalence of *L. monocytogenes* in raw meat and its derived products was higher than that in any other foods such as cultured milk products, processed cheese, preserved fish, raw seafood, vegetables, fruits, and dry/semi-dry fermented sausage (Swaminathan and Gerner-Smidt, 2007). It was reported that *L. monocytogenes* was most likely isolated from chicken meat than pork or beef in Japan (Ochiai et al., 2010). Very few studies have been done on the antimicrobial resistance of *L. monocytogenes* in Japan (Okada et al., 2011) and there are no reports regarding the changes in contamination rate and antibiotic resistance of the bacterium. Therefore, in this study, *L. monocytogenes* was isolated from chicken meat samples in 2012 and 2017, and the isolates were characterized. The antimicrobial resistance profiles of the isolates were compared.

2. Materials and methods

2.1. Sample collection

A total of 135 raw chicken meat samples including various parts (breast, minced and chopped meats, wing, thigh, skin, bone, cartilage, fat, gizzard, liver, heart and tail) were analyzed in 2012 (85 samples) and in 2017 (50 samples). Samples were collected randomly from 9 different supermarkets in Fukuoka with an average of 5 samples/supermarket in 2012 and 3 samples/supermarket in 2017 from March to December. All the samples were transported in ice boxes to the Laboratory of Food Hygienic Chemistry, Kyushu University within 3 h after sampling and analyzed immediately.

2.2. Detection and isolation of L. monocytogenes

All the samples were examined for the presence of *L. monocytogenes* according to the guidelines of International Standards Organization (ISO, 1996). A total 25 g sample was aseptically homogenized with 225 ml Half-Fraser broth (SR0166E, Oxoid) in a stomacher bag for 30 s and incubated at 30 °C for 24 h as primary enrichment. For secondary enrichment culture, 0.1 ml from the primary enrichment culture was added to 10 ml Fraser broth (SR0156E, Oxoid) and incubated at 37 °C for 48 h. After incubation, a loopful of both enrichment cultures was streaked on CHROMagar™ Listeria (CHROMagar, Paris, France) and Listeria selective agar (Oxford formulation, SR0140E, Oxoid), and incubated at 37 °C for 24–48 h. As presumptive colonies of *L. monocytogenes*, blue colonies with white halos on CHROMagar and brown colonies with black halos on Oxford agar were isolated. For purification, these suspected colonies from each plate were streaked on trypticase soy agar supplemented with 0.6% yeast extract (TSAYE) and incubated at 37 °C for 18–24 h. The colonies from TSAYE were confirmed as *L. monocytogenes* by biochemical tests; Gram staining, catalase, oxidase, β-hemolysis, Christine Atkins Munch-Peterson (CAMP) and carbohydrate fermentation (L-Rhamnose, D-Xylose) tests. A stock culture of *L. monocytogenes* strain, serotype 1/2b (Honjoh et al., 2008) was used as a reference strain for biochemical tests and *Staphylococcus aureus* ATCC 25923 was used for the CAMP test.

2.3. DNA extraction

L. monocytogenes isolates were cultured overnight with shaking in 5 ml trypticase soy broth (TSB, Becton, Dickinson and Co., USA) to extract the genomic DNA. The genomic DNA was extracted from 1 ml of culture by using the DNeasy Tissue kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Briefly, the cells were collected from the culture by centrifugation at 6000 ×*g* for 10 min and discarding the flow-through. The cell pellet was suspended in 180 µl buffer ATL, after which 20 µl Proteinase K (Qiagen, Tokyo, Japan) was added and mixed thoroughly by vortexing. The mixture was incubated at 56 °C for 1–3 h. After incubation, it was mixed with 200 µl buffer AL (lysis buffer), followed by addition of 200 µl of 99% ethanol and vortexing. The mixture was pipetted and placed into a DNeasy mini spin column with a 2 ml collection tube, and centrifuged at 6000 ×*g* for 1 min to discard the ethanol. Then the spin column was placed in a new 2 ml collection tube. The samples were washed with 500 µl buffer AW 1 and centrifuged at 6000 ×*g* for 1 min. The spin column was placed in a new 2 ml collection tube and washed with 500 µl buffer AW 2. After centrifugation at 20,000 ×*g* for 3 min, the spin column was transferred to a new 1.5 ml microcentrifuge tube and the DNA was eluted by adding 20–100 µl buffer AE (elution buffer) to the centre of the membrane in the spin column. Then it was incubated at room temperature for 1 min and centrifuged at 6000 ×*g* for 1 min to yield the genomic DNA.

Alternatively, for some isolates, the genomic DNA was extracted by the boiling method (Honjoh et al., 2008). Briefly, the cells were collected from 1 ml of the culture by centrifugation at 8000 ×*g* for 5 min. The cells were suspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), boiled for 10 min, and cooled in ice for 1 min. The suspension was centrifuged at 8000 ×*g* for 5 min. The supernatant was used as genomic DNA template solution.

The quantity and quality of the DNA extract were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and only good quality DNA was used (ratio of absorbance at 260 nm and 280 nm of the DNA samples < 1.8) for PCR.

2.4. Detection of hlyA gene of L. monocytogenes isolates by PCR

The primer set targeting *hlyA* gene of *L. monocytogenes (hlyA*-F; 5′-AAATCATCGACGGCAACCT-3′ and *hlyA*-R; 5′-GGACGATGTGAAAT GAGC-3′), which was reported as *hlyA* primer set 7 designed by Liu et al. (2012), was used in this experiment. The PCR amplification was carried out in a total volume of 10 µl reaction mixture containing 5 µl 2× KOD FX Neo buffer (Toyobo, Japan), 2 µl dNTPs (2 mM), 0.2 µl each of *hlyA*-F and *hlyA*-R primers (0.3 µM), 1 µl template DNA (50 ng), 0.2 µl KOD FX Neo enzyme (1 U) (Toyobo, Japan) and 1.4 µl distilled water. The PCR conditions were: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s and extension at 68 °C for 15 s, and finally extension at 68 °C for 10 min. The reaction mixture was stored at 4 °C until electrophoresis. PCR products were confirmed by agarose gel electrophoresis using 1.5% agarose gel stained with Midori Green Advance DNA stain (Nippon Genetics Europe GmbH, Germany) and visualized on WSE-

6100H LuminoGraph I (ATTO, Tokyo, Japan) with a 100 bp DNA marker (Toyobo, Japan) to determine the amplicon size.

2.5. Serotyping of L. monocytogenes isolates

The *L. monocytogenes* isolates were serotyped using commercially available *Listeria* antisera (Denka Seiken Co., Tokyo, Japan) based on the manufacturer's instructions. For O-antigen serotyping, each bacterial isolate was cultured overnight on BHI agar at 37 °C. After incubation, some colonies were picked and suspended in 0.2% NaCl solution. Bacterial suspension was mixed with I/II, V/VI, I, IV, VI, VII, VIII, and IX antisera on a glass slide. Samples with aggregation, when observed visually, were recorded as positive. For H-antigen serotyping, each bacterial isolate was inoculated on BHI agar with filter paper and incubated at 25 °C for 4 days to increase motility. Some colonies were picked and incubated overnight in BHI broth at 30 °C. The culture was suspended in physiological salt solution containing 1% formaldehyde. The formalin-fixed cell suspension (0.5 ml) was mixed well with 2 drops antiserum solution (HA, HAB, HC and HD) in sterile test tubes and incubated in a water bath at 51 °C for 1 h. Samples visually observed with agglutination were recorded as positive.

2.6. Antimicrobial susceptibility tests

For antimicrobial susceptibility tests, 75 isolates were selected from 153 strains isolated in 2012 to contain different ribotypes (data not shown). Together with 29 *L. monocytogenes* isolates in 2017, 104 isolates were tested for antimicrobial susceptibility by the broth microdilution method (according to the manufacturer's instructions) and CLSI (2012). Bacterial isolates were grown on BHI agar at 37 °C for 24 h. Single colonies were suspended in 0.9% NaCl and adjusted to attain an OD_{600nm} of 0.1 (approximately 10^8 CFU/ml). Bacterial suspension (25 µl) was diluted with 12 ml Mueller Hinton broth to yield a cell suspension with 5×10^4 CFU/ml. Within 15 min after inoculum preparation, 100 µl bacterial suspension was added to each of 96-well dry plate, DP 32 (Eiken Chemical Co., Tokyo, Japan) including the following different concentrations of 18 antimicrobial agents; oxacillin (0.12–4 µg/ml), ampicillin (0.12–16 µg/ml), cefazolin (0.5–16 µg/ml), cefmetazole (1–32 µg/ml), flomoxef (0.5–16 µg/ml), Imipenem (0.25–8 µg/ml), gentamycin (0.25–8 µg/ml), arbekacin (0.25–8 µg/ml), minocycline (2–8 µg/ml), cefoxitin (4–16 µg/ml), erythromycin (0.12–4 µg/ml), clindamycin (0.06–2 µg/ml), vancomycin (0.5–16 µg/ml), teicoplanin (0.5–16 µg/ml), linezolid (0.25–8 µg/ml), fosfomycin (32–128 µg/ml), sulfamethoxazole/trimethoprim (9.5/0.5–38/2 µg/ml) and levofloxacin (0.25–4 µg/ml) and incubated at 37 °C for 16–20 h. The cell suspension was added to a well without any antibiotics for control. After incubation, the changes of culture absorbance were measured with a microplate reader to determine the minimum inhibitory concentration (MIC) for each antimicrobial agent. The MIC for 50% and 90% of the isolates (MIC_{50} and MIC_{90}) were also calculated. Each of the isolates was classified as susceptible, intermediate, or resistant towards the antimicrobials by using breakpoints (CLSI, 2015; Lyon et al., 2008) in Table 4. The antimicrobial susceptibility tests were duplicated, and the same results were obtained for all the isolates.

2.7. Tetracycline susceptibility test

Tetracycline susceptibility tests were performed by the disc diffusion method according to the CLSI (2012). *L. monocytogenes* isolates from BHI agar were suspended with 0.9% NaCl and diluted to attain an OD_{600nm} of 0.1 (approximately 10^8 CFU/ml). Within 15 min after preparation of bacterial suspension, a sterile cotton swab was dipped into the suspension and streaked on Mueller Hinton agar. Then, 30 µg tetracycline disc (BD BBL™ Sensi-Disc™, USA) was placed onto the surface of agar by using sterile forceps. After incubation at 37 °C for 18–24 h, the diameter of the inhibitory zone was measured and compared with zone diameter breakpoints of *Staphylococcus* species (CLSI, 2015) to determine them to be susceptible (≥19 mm), intermediate (15–18 mm), and resistant (≤14 mm). Upon duplication, same results were obtained for all the isolates.

2.8. Detection of genes involved in antimicrobial resistance by PCR

L. monocytogenes isolates were investigated for the oxacillin resistance gene (*mecA*), the cefoxitin resistance gene (*cfxA*), the macrolide resistance genes (*ermA*, *ermB*, *ermC* and *mefA*) and the fosfomycin resistance genes (*fosA*, *fosB*, and *fosC*). Depending upon the phenotypic antimicrobial resistance profiles, oxacillin, cefoxitin, clindamycin and fosfomycin resistance isolates in both of 2012 and 2017 were selected, and cultured in 5 ml TSB at 37 °C for 24 h. The cultures were used to extract the genomic DNA for PCR detection of the genes related to their antimicrobial resistance. Some novel primers were designed based on homologous bacterial sequences using Primer 3 input (version 0.4.0) to detect the resistance genes of *L. monocytogenes* (Table 1), while others were adapted from previous reports. The primer sets for detecting *mecA*, *cfxA*, *ermA*, *ermC*, *fosA*, and *fosB* were designed in this study. The DNA reference sequences of AMR genes were searched from NCBI (www.ncbi.nlm.nih.gov). Genomic DNA solution (0.2 µl of 50 ng DNA) was used in a 10 µl reaction mixture consisting of 1 µl of 10× PCR buffer (Mg^{2+} plus), 1 µl of 2.5 mM dNTPs, 0.2 µl of 1 µM each of the primers, 0.2 µl of 5 U TaKaRa Taq (Takara Bio, Co. Ltd., Japan), and 7.2 µl distilled water. PCR conditions consisted of pre-heating was at 94 °C for 5 min, 30 PCR cycles of denaturation at 98 °C for 10 s, annealing for 30 s, and elongation at 72 °C for 1 min, followed by final elongation 72 °C for 7 min. Annealing temperature for each primer is shown in Table 1. For agarose gel electrophoresis, PCR products were analyzed on 1.5% agarose gel stained with Midori Green Advance DNA stain (Nippon Genetics Europe GmbH, Germany) visualized on WSE-6100H LuminoGraph I (ATTO, Tokyo, Japan).

Key resources table

Resource	Source	Identifier
Antibodies		
Listeria 8 antisera		
Chemical		
ampicillin		
arbekacin		
cefazolin		
cefmetazole		
cefoxitin		
clindamycin		
erythromycin		
ethanol		
flomoxef		
formaldehyde		
formalin		
fosfomycin		
gentamycin		
Imipenem		
levofloxacin		
linezolid		
minocycline		
NaCl		
oxacillin		
sulfamethoxazole		
teicoplanin		
Tetracycline		
trimethoprim		
vancomycin		

Table 1
Primer sequences used for detection of antimicrobial resistance genes.

Gene	Primer sequence (5′-3′)	Product size (bp)	Annealing temperature	Bacterial origin	References
mecA	F: GTGGAAAAGGCGAAGATGGC R: TCAAGTCCTGTTGCTCGTGA	199	59 °C	*L. monocytogenes*	This study[a]
cfxA	F: GCTCAAACAGATAGTTTTAT R: GGCAACATTGTGAGCTG	333	60 °C	*Bacteroides spp.* *Prevotella spp.*	Tran et al., 2013
	F: GTGATGATGGACGGATCGCT R: TGTGGTCGAGCTTTCCTTCC	202	60 °C	*Rhodobacter sphaeroides*	This study[a]
ermA	F: GAGGTGTAATTTCGTAACTGCC R: TTAGCAAACCCGTATTCCAC	249	56 °C	*Staphylococcus aureus*	This study[a]
ermB	F: GAAAAGGTACTCAACCAAATA R: AGTAACGGTACTTAAATTGTTTAC	636	49 °C	*L. monocytogenes*	Morvan et al., 2010
ermC	F: TGAAAGCCATGCGTCTGACA R: AACATCTGTGGTATGGCGGG	202	60 °C	*Staphylococcus aureus*	This study[a]
mefA	F: AGTATCATTAATCACTAGTGC R: TTCTTCTGGTACTAAAAGTGG	345	53 °C	*L. monocytogenes*	Morvan et al., 2010
fosA	F: CAAGTGGAATAAAGGCGCTT R: ACCTACATGAACCTCCAACT	241	56 °C	*Brucella melitensis*	This study*
	F: GCTGCACGCCCGCTGGAATA R: CGACGCCCCCTCGCTTTTGT	217	56 °C	*Staphylococcus aureus*	Chen et al., 2014
fosB	F: TGTGGAGTATGGATAGCGCT R: CATCCCGTTCCCTTCCTTGT	166	59 °C	*Bacillus cereus*	This study[a]
	F: CAGAGATATTTTAGGGGCTGACA R: CTCAATCTATCTTCTAAACTTCCTG	312	48 °C	*Staphylococcus aureus*	Chen et al., 2014
fosC	F: GGGTTACATGCCCTTGCTCA R: AACCCGCACAACGACAGATG	354	49 °C	*Staphylococcus aureus*	Chen et al., 2014

[a] Designed on the basis of nucleotide sequences from NCBI database (www.ncbi.nlm.nih.gov).

Table 2
Characterization of *L. monocytogenes* isolated from chicken meat in 2012.

No.	Strain name	Source	Serotype	*hlyA*[b]	No.	Strain name	Source	Serotype	*hlyA*[b]
1	2-HO[a]	Minced	1/2b	+	78	38-FA	Thigh fillet	–	+
2	2-FA[a]	Minced	1/2b	+	79	38-FO[a]	Thigh fillet	–	+
3	2-FO[a]	Minced	1/2b	+	80	40-HA[a]	Thigh	–	+
4	4-HA[a]	Thigh	–	+	81	40-HO	Thigh	–	+
5	4-FA[a]	Thigh	1/2b	+	82	40-FA	Thigh	–	+
6	4-FO[a]	Thigh	1/2b	+	83	40-FO	Thigh	–	+
7	5-HA[a]	Minced	1/2b	+	84	47-HA[a]	Thigh	1/2b	+
8	5-HO[a]	Minced	1/2b	+	85	47-HO[a]	Thigh	1/2b	+
9	5-FA	Minced	1/2b	+	86	48-HA[a]	Breast	1/2b	+
10	5-FO	Minced	–	+	87	48-HO	Breast	1/2b	+
11	6-HA[a]	Breast	1/2b	+	88	48-FA[a]	Breast	1/2b	+
12	6-HO	Breast	–	+	89	48-FO	Breast	1/2b	+
13	6-FA	Breast	1/2b	+	90	50-FA[a]	Minced	1/2a	+
14	6-FO[a]	Breast	1/2b	+	91	50-FO	Minced	1/2a	+
15	8-HA[a]	Fillet	1/2b	+	92	53-HA[a]	Breast	1/2b	+
16	8-HO[a]	Fillet	1/2b	+	93	53-HO	Breast	1/2b	+
17	8-FA[a]	Fillet	–	+	94	53-FA[a]	Breast	1/2b	+
18	8-FO[a]	Fillet	1/2b	+	95	53-FO	Breast	1/2b	+
19	10-HA	Thigh	–	+	96	55-HA[a]	Minced	1/2a	+
20	10-HO	Thigh	–	+	97	55-HO	Minced	1/2a	+
21	10-FA	Thigh	–	+	98	55-FA	Minced	1/2a	+
22	10-FO[a]	Thigh	1/2b	+	99	55-FO	Minced	1/2a	+
23	11-HA	Minced	–	+	100	56-HA[a]	Thigh	1/2b	+
24	11-HO[a]	Minced	1/2a	+	101	56-HO[a]	Thigh	1/2c	+
25	11-FA[a]	Minced	–	+	102	56-FA[a]	Thigh	1/2b	+
26	11-FO[a]	Minced	1/2b	+	103	56-FO	Thigh	–	+
27	12-HA[a]	Thigh	1/2b	+	104	57-HA	Thigh	–	+
28	12-HO[a]	Thigh	1/2b	+	105	57-HO	Thigh	–	+
29	12-FA[a]	Thigh	1/2b	+	106	59-HA	Minced	–	+
30	12-FO	Thigh	1/2b	+	107	59-HO[a]	Minced	1/2a	+
31	18-FA	Thigh	–	+	108	59-FO	Minced	–	+
32	21-HA[a]	Breast	–	+	109	62-HA[a]	Breast	1/2b	+
33	21-HO[a]	Breast	1/2b	+	110	62-HO	Breast	1/2b	+
34	21-FA[a]	Breast	1/2b	+	111	68-HA[a]	Minced	1/2a	+
35	21-FO[a]	Breast	1/2b	+	112	68-HO[a]	Minced	–	+
36	22-HA	Thigh	1/2b	+	113	68-FA[a]	Minced	1/2a	+
37	22-HO[a]	Thigh	1/2b	+	114	68-FO	Minced	–	+

-, Serotype not identified.
[a] Isolates used for antimicrobial susceptibility tests.
[b] Detection by PCR using hlyA primer set 7 (Liu et al., 2012).

Table 3
Characterization of *L. monocytogenes* isolated form chicken meat in 2017.

No.	Strain names	Source	Serotype	*hlyA*[a]
1	S4HO	Broiler	1/2a	–
2	S4FO	Broiler	1/2a	+
3	S8HC	Breast	1/2a	+
4	S8FC	Breast	1/2a	+
5	S8FO	Breast	1/2a	+
6	S15FC	Breast	1/2a	–
7	S15FO	Breast	1/2a	–
8	S16FO	Wing fillet	1/2b	–
9	S18HO	Breast	1/2b	–
10	S24FC	Breast	1/2b	+
11	S24FO	Breast	1/2b	+
12	S25FC	Minced	1/2b	+
13	S31HC	Thigh	1/2a	–
14	S31HO	Thigh	1/2a	+
15	S31FC	Thigh	1/2a	+
16	S31FO	Thigh	1/2a	–
17	S38HC	Thigh	1/2a	+
18	S38HO	Thigh	1/2a	+
19	S38FC	Thigh	1/2a	+
20	S39HC	Wing	1/2b	+
21	S39FC	Wing	1/2b	–
22	S41HC	Skin	1/2b	+
23	S41HO	Skin	1/2b	–
24	S41FC	Skin	1/2b	–
25	S41FO	Skin	1/2b	+
26	S42HC	Breast	1/2b	+
27	S42HO	Breast	1/2b	+
28	S42FC	Breast	1/2b	–
29	S42FO	Breast	1/2b	–

[a] Detection by PCR using hlyA primer set 7 (Liu et al., 2012).

3. Results

3.1. Detection of L. monocytogenes from meats

Among 85 chicken meat samples analyzed in 2012, 45 samples (52.9%) were positive for *L. monocytogenes* and 153 *L. monocytogenes* strains were isolated (Tables 2 and 3). On the other hand, 12 samples (24.0%) were positive for *L. monocytogenes* among 50 chicken meat samples examined in 2017. From these positive 12 samples, 29 strains of *L. monocytogenes* were isolated from the primary and secondary enrichment cultures of CHROMagar and Oxford agar (Table 3).

3.2. Detection of L. monocytogenes targeting hlyA gene by PCR

All the 2012 isolates were positive for *hlyA* gene in the PCR assay designed by Liu et al. (2012). Out of 29 *L. monocytogenes* isolates detected in 2017, 17 isolates (58.6%) were positive by PCR using the respective primer set (Table 3).

3.3. Serotyping of the L. monocytogenes isolates

The serotypes of the isolates in 2012 were 1/2a (21.5%), 1/2b (73.9%), 1/2c (1.5%) and 4b/4e (3.1%) (Table 2). Serotyping results of 29 *L. monocytogenes* isolates in 2017 showed that 15 isolates (51.7%) were serotype 1/2b and 14 isolates (48.3%) were 1/2a (Table 3).

3.4. Antimicrobial susceptibility of the isolates

The MIC values of *L. monocytogenes* isolates in 2012 and 2017 are shown in Table 4. The antimicrobial susceptibility profile of the isolates with different ribotypes in 2012 was clear, however, the isolates in 2017 was calculated with 23 isolates because two or more isolates (S31HO/S31FC/S31FO, S41HC/S41HO/S41FC, and S42HC/S42FC/S42FO) from the same sample have to be counted one strain since they showed the same profile. All the 2012 and 2017 isolates were susceptible to imipenem, gentamycin, arbekacin, minocycline, erythromycin, vancomycin, teicoplanin, sulfamethoxazole-trimethoprim, and levofloxacin. More than 95% of the isolates were susceptible to ampicillin, cefazolin, flomoxef, and linezolid. However, *L. monocytogenes* isolates obtained in 2012 were most resistant to cefoxitin (98.7%), followed by oxacillin (72.0%), fosfomycin (57.3%), clindamycin (8.0%), and flomoxef (2.7%), while the 2017 isolates exhibited resistance to cefoxitin (100%), fosfomycin (95.7%), oxacillin (82.6%), clindamycin (17.4%), and linezolid (4.3%) (Table 5). One of the 2012 isolates, was susceptible to all of the tested antimicrobials and 8 different resistance

Table 4
MIC50 and MIC90 of antimicrobial agents for *L. monocytogenes* isolates in 2012 and 2017.

Antimicrobial agents	Concentrations (μg/ml)	MIC Breakpoints (μg/ml)			MIC50 (μg/ml)		MIC90 (μg/ml)	
		S	I	R	2012	2017	2012	2017
Oxacillin	0.12, 0.25, 0.5, 1, 2, 4	≤2	–	≥4	4	4	4	4
Ampicillin	0.12, 0.25, 0.5, 4, 8, 16	≤4	–	> 16	0.25	0.5	0.5	0.5
Cefazolin	0.5, 1, 2, 4, 8, 16	≤8	16	≥32	2	2	2	2
Cefmetazole	1, 2, 4, 8, 16, 32	≤16	32	≥64	16	16	32	32
Flomoxef	0.5, 1, 2, 4, 8, 16	≤8	–	> 8	8	8	8	8
Imipenem	0.25, 0.5, 1, 2, 4, 8	≤4	–	≥16	0.25	0.25	0.25	0.25
Gentamycin	0.25, 0.5, 1, 2, 4, 8	≤4	8	≥16	0.25	0.25	0.5	0.5
Arbekacin	0.25, 0.5, 1, 2, 4, 8	–	–	≥4	0.5	0.25	1	1
Minocycline	2, 4, 8	≤4	8	≥16	2	2	2	2
Cefoxitin	4, 8, 16	≤4	–	≥8	> 16	> 16	> 16	> 16
Erythromycin	0.12, 0.25, 0.5, 1, 2, 4	≤0.5	1–4	≥8	0.25	0.25	0.25	0.25
Clindamycin	0.06, 0.12, 0.25, 0.5, 1, 2	≤0.5	1–2	≥4	2	2	2	> 2
Vancomycin	0.5, 1, 2, 4, 8, 16	≤2	4–8	≥16	0.5	1	1	1
Teicoplanin	0.5, 1, 2, 4, 8, 16	≤8	16	≥32	0.5	0.5	0.5	0.5
Linezolid	0.25, 0.5, 1, 2, 4, 8	≤4	–	> 4	2	2	2	2
Fosfomycin	32, 64, 128	≤32	–	> 32	128	> 128	> 128	> 128
Sulfamethoxazole-Trimethoprim	9.5/0.5, 19/1, 38/2	≤9.5/0.5	19/1–38/2	76/4	0.5	0.5	0.5	0.5
Levofloxacin	0.25, 0.5, 1, 2, 4	≤2	4	≥8	1	1	1	1

MIC- Minimum Inhibitory Concentration.
S- Susceptible, I- Intermediate, R- Resistance.

Table 5
Antimicrobial susceptibility profiles of *L. monocytogenes* isolates in 2012 and 2017.

Antimicrobial agents	Number of isolates (%)					
	Susceptible		Intermediate		Resistance	
	2012	2017	2012	2017	2012	2017
Oxacillin	21 (28.0)	4 (17.4)	0	0	54 (72.0)	19 (82.6)
Ampicillin	74 (98.7)	23 (100)	1 (1.3)	0	0	0
Cefazolin	74 (98.7)	23 (100)	1 (1.3)	0	0	0
Cefmetazole	59 (78.7)	17 (73.9)	16 (21.3)	6 (26.1)	0	0
Flomoxef	73 (97.3)	23 (100)	0	0	2 (2.7)	0
Imipenem	75 (100)	23 (100)	0	0	0	0
Gentamycin	75 (100)	23 (100)	0	0	0	0
Arbekacin	75 (100)	23 (100)	0	0	0	0
Minocycline	75 (100)	23 (100)	0	0	0	0
Cefoxitin	1 (1.3)	0	0	0	74 (98.7)	23 (100)
Erythromycin	75 (100)	23 (100)	0	0	0	0
Clindamycin	6 (8.0)	0	63 (84.0)	19 (82.6)	6 (8.0)	4 (17.4)
Vancomycin	75 (100)	23 (100)	0	0	0	0
Teicoplanin	75 (100)	23 (100)	0	0	0	0
Linezolid	75 (100)	22 (95.7)	0	0	0	1 (4.3)
Fosfomycin	32 (42.7)	1 (4.3)	0	0	43 (57.3)	22 (95.7)
Sulfamethoxazole-Trimethoprim	75 (100)	23 (100)	0	0	0	0
Levofloxacin	75 (100)	23 (100)	0	0	0	0

patterns were observed (Table 6). All of the 2017 isolates were resistant to at least one antimicrobial and 5 resistance patterns were found (Table 6). Four of the resistance patterns in both the groups were common. These were 1) cefoxitin (CFX); 2) cefoxitin, fosfomycin (CFX, FOM); 3) oxacillin, cefoxitin, fosfomycin (MPIPC, CFX, FOM); and 4) oxacillin, cefoxitin, clindamycin, fosfomycin (MPIPC, CFX, CLDM, FOM).

Tables 5 and 6 display the profiles for the rate of resistance and MDR of *L. monocytogenes* isolates in 2012 and 2017. Among *L. monocytogenes* isolates in 2012, 12.0% were resistant to 1 antimicrobial, 40.0% were resistant to 2 and 3 antimicrobials, and 6.7% were resistant to 4 antimicrobials. On the other hand, isolates in 2017 were resistant to 1 (4.3%), 2 (13.0%), 3 (65.2%), 4 (13.0%) and 5 (4.3%) antimicrobials. The MDR rates of *L. monocytogenes* isolates in case of at least 3 antimicrobials were calculated to be 46.7% (35/75) in 2012 and 82.6% (19/23) in 2017.

For tetracycline susceptibility test, the inhibitory zone diameter of all the isolates in 2012 and 2017 ranged between 24 and 34 mm, indicating that all the isolates in both of the groups were highly susceptible to tetracycline (30 μg).

3.5. Detection of genes involved in antimicrobial resistance by PCR

Since the ratios of antimicrobial resistance of the isolates were high against oxacillin, cefoxitin, clindamycin, and fosfomycin, the presence of the corresponding resistance conferring genes was examined by PCR. As shown in Table 7, 52 (96.3%) out of 54, and 18 (94.7%) out of 19 oxacillin resistance isolates in 2012 and 2017, respectively, were positive for *mecA* by PCR. Among 6 clindamycin resistant isolates in 2012, amplicon was obtained from 5 isolates (83.3%) for *ermC* although its size was not as expected (data not shown). Also, one out of 4 (25.0%) clindamycin resistant isolates in 2017 was positive for *mefA*. Other genes involved in macrolide resistance (*ermA*, *ermB*), cefoxitin resistance (*cfxA*), and fosfomycin resistance (*fosA*, *fosB*, *fosC*), were not detected in either of the 2012 and 2017 isolates by PCR using the primers in Table 1.

4. Discussion

This study has shown a decrease in the prevalence of *L. monocytogenes* infected chicken meat from 52.9% in 2012 to 24% in 2017. The results support a previous report showing the prevalence of *L. monocytogenes* in chicken samples in 1993 and 2006 to be 32% and 24.5%, respectively (Alonso-Hernando et al., 2012). This may be due to the improvement of hygienic practices for processing and handling of meat from farms to markets. The contamination rate of *L. monocytogenes* in chicken in the present study was similar to that recorded in Italy (24.5%) (Pesavento et al., 2010) and China (20%) (Wu et al., 2015). Reports suggest that the contamination rate of *L. monocytogenes* in Japan was similar to those in United States, France, and Canada (Okutani et al., 2004). However, the reported contamination rates of 38.8% poultry products in Iran (Fallah et al., 2012), 37% minced chicken in Japan (Inoue et al., 2000), 37.5% minced meat and 39% meat cuts in Estonia (Kramarenko et al., 2013), and 28.7% chicken meat in Tokyo Metropolitan area (Ochiai et al., 2010) were higher than that observed in 2017 isolates and lower than the rate seen in the 2012 isolates of our study. It seems that the difference in the contamination rate is caused by the different sample sizes, period of sampling, and detection methods (Jang et al., 2006).

The *hlyA* gene encodes listeriolysin O, one of the most important virulence factors of *L. monocytogenes* (Mengaud et al., 1988). In this respect, the detection of *hlyA* gene seems to be sufficient to determine the presence of *L. monocytogenes*. However, spontaneous mutations or deletion in the virulence genes are not uncommon in pathogenic bacteria like *hlyA* in *L. monocytogenes* (Cooray et al., 1994). In this study, *hlyA* was not detected in 12 of the 29 strains in 2017. Usman et al. (2016) also have reported that only 9 isolates harboured *hlyA* gene among 36 isolates of *L. monocytogenes* because of the non-virulent characteristics of some of the *L. monocytogenes* isolates due to the spontaneous mutations. The 2017 isolates not produced the amplicon by PCR using *hlyA* primer set 7 (Liu et al., 2012) seem to have some mutations in the *hlyA* 7 primer regions, though validation by sequencing is required.

L. monocytogenes isolates in 2017 were serotyped into 1/2b (15/29, 51.7%) and 1/2a (14/29, 48.3%). Similar results show that the serotypes 1/2b (14.7%), and 1/2a (14%) were not significantly different in chicken meat in Japan (Ochiai et al., 2010). However, different observations regarding the serotypes 1/2a, followed by 1/2b, 1/2c, and 4b were reported in Korea (Jang et al., 2006), Japan (Shimojima et al., 2016), and China (Wu et al., 2015). In this 2017 study, serotypes 1/2c and 4b/4e were not detected in the isolates. Hasegawa et al. isolated *L. monocytogenes* serotype 1/2b (55%) and 4b (45%) from bovine colostrum in Hokkaido, Japan in 2013 (Hasegawa et al., 2013). In 2016, Shimojima et al. reported that the ratio of serotype 1/2a has risen after

Table 6
Antimicrobial resistance patterns of *L. monocytogenes* isolates in 2012 and 2017.

Resistance pattern	Strain name		Number of isolates	
	2012	2017	2012	2017
None		–	1	0
CFX	87-FO	S39HC	9	1
CFX, FOM	23-HO, 34-HA, 50-FA, 53-HA, 68-HO, 68-FA, 87-HO, 89-FA 8-FA, 10-FO, 12-HO, 29-FA, 56-HO, 71-HA, 76-FA, 93-HA,	S15FC, S24FO, S39FC	8	3
MPIPC, CFX	5-HA, 5-HO, 6-HA, 21-HA, 21-FA, 22-HO, 22-FA, 23-FA, 24-HA, 38-HO, 47-HA, 47-HO, 48-HA, 48-FA, 53-FA, 55-HA, 59-HO, 75-FA, 81-HA, 81-FA, 87-HA, 87-FA	–	22	–
MPIPC, CFX, CLDM	26-FA	–	1	–
MPIPC, CFX, FOM	2-HO, 2-FA, 2-FO, 4-HA, 4-FA, 4-FO, 6-FO, 8-HA, 8-HO, 11-HO, 11-FO, 12-HA, 21-HO, 21-FO, 31-HA, 37-HO, 37-FA, 37-FO, 38-HA, 40-HA, 56-HA, 56-FA, 62-HA, 72-HA, 73-HA, 77-FA, 90-FA	S4FO, S8FC, S8FO, S15FO, S16FO, S18HO, S24FC, S31HC, S31HO/S31FC/S31FO, S38HO, S38FC, S41HC/S41HO/S41FC, S41FO, S42HO, S42HC/S42FC/S42FO	27	15
CFX, CLDM, FOM	12-FA, 30-FO	–	2	–
MPIPC, CFX, CLDM, FOM	11-FA, 37-HA, 38-FO	S4HO, S25FC, S38HC	3	3
MPIPC, FMOX, CFX, FOM	8-FO, 76-HA	–	2	–
MPIPC, CFX, CLDM, LZD, FOM	–	S8HC	–	1
Antimicrobial resistant strains			74	23

CFX- Cefoxitin, FOM- Fosfomycin, MPIPC- Oxacillin, CLDM- Clindamycin, LZD- Linezolid.

2000, though serotype 4b was common earlier than 2000 in Japan too (Shimojima et al., 2016). The most common serotype seems different due to variations in the animal species, and regional and seasonal parameters.

The antimicrobial susceptibility tests for 18 antimicrobials revealed that the *L. monocytogenes* isolates, both in 2012 and 2017, were susceptible to most of the antimicrobial agents except cefoxitin, fosfomycin, oxacillin and clindamycin. The isolates were less resistant to flomoxef (2.67%) in 2012 and linezolid (4.3%) in 2017. This was not surprising because *L. monocytogenes* strains are naturally resistant to cephalosporins, fosfomycin, oxacillin, and macrolides (Gómez et al., 2014; Troxler et al., 2000). These results are in agreement with the previous reports that *L. monocytogenes* from poultry and red meat in Morocco was 100% resistant not only to the 2nd generation cephalosporin; cefoxitin, but also to the 3rd generation cephalosporins; cefotaxime and ceftazidime (Ennaji et al., 2008). *L. monocytogenes* isolates in Japan, France, and Italy are reported to be nearly 100% resistant to oxacillin and fosfomycin (Aureli et al., 2003; Harakeh et al., 2009; Hasegawa et al., 2013), which is consistent with the results of this study in case of fosfomycin, but not oxacillin. It is also reported that β-lactam oxacillin was the most common resistance phenotype and was identified in 100% *L. monocytogenes* from meat products in Spain (Gómez et al., 2014). Also, Conter et al. described that *L. monocytogenes* screened in different food samples in Italy was resistant to oxacillin (96.7%), fosfomycin (78.3%), clindamycin (3.3%), and linezolid (3.3%), similar to the results observed in the present study (Conter et al., 2009). The ratio of clindamycin resistant strains in this study (13.8%) was lower than those in other countries such as 16.6% in Turkey (Aras and Ardic, 2015), 35% in Spain (Gómez et al., 2014), and 46.8% in China (Wu et al., 2015).

The MDR in *L. monocytogenes* isolates in 2017 (82.6%) was higher than that seen in 2012 (46.67%). Likewise, it was reported in Spain in 2012 that MDR of *L. monocytogenes* isolates from chicken meats was higher in 2006 (84%) than that in 1993 (18.6%) (Alonso-Hernando et al., 2012). This may be due to an increase in the antimicrobial usage with time. As a result, *L. monocytogenes* has developed resistance against various antibiotics by acquiring mobile genetic components such as transmissible plasmids and conjugative transposons (Conter et al., 2009; Harakeh et al., 2009). Hasegawa et al. also reported that antimicrobial agents were routinely used for growth promotion in Japan because of the antibiotic resistance in *L. monocytogenes* after acquiring antibiotic resistant genes (Hasegawa et al., 2013). Multidrug resistance rate of *L. monocytogenes* is higher in China (72.3%) (Wang et al., 2013), which is more than the rate found in the 2012 isolates of our study. Similar results have been reported in other countries, where rates of multidrug resistance in the *L. monocytogenes* isolates were 100% in Egypt, 91.7% (raw milk and milk products) and 100% (salad vegetables) in Nigeria, and 80–90% in India (AL-Ashmawy et al., 2014; Ieren et al., 2013; Sharma et al., 2012; Usman et al., 2016). Multidrug resistance rate of *L. monocytogenes* isolates in Japan seems to be lower compared to other countries.

All of the isolates in 2012 and 2017 were susceptible to tetracycline (30 μg/ml) in this investigation. In contrast, tetracycline resistance of *L. monocytogenes* was observed commonly in other countries. However, our study is in agreement with the other Japanese studies that all the *L. monocytogenes* isolates from bovine colostrum were tetracycline susceptible and it inhibited 90% of 48 isolates even at 1 μg/ml (Hasegawa et al., 2013). In addition, *L. monocytogenes* isolates from imported and domestic food items in Japan were susceptible to tetracycline, wherein 2 μg and 4 μg concentrations inhibited 50% and 90% of the total, respectively (Okada et al., 2015). Apparently, due to low usage of tetracycline in Japanese population, *L. monocytogenes* isolates were susceptible to it.

The *mecA* gene encodes a penicillin binding protein mediating the oxacillin resistance. The enzymes such as β-lactamase destroy cephalosporins and *cfxA* gene is responsible for cefoxitin resistance. In some

Table 7
The rates of antimicrobial resistance genes in *L. monocytogenes* by PCR.

Year	No. of total isolates	Oxacillin		Cefoxitin		Macrolides					Fosfomycin		
		No. of isolates (% in resistant isolates)		No. of isolates (% in resistant isolates)		No. of isolates (% in resistant isolates)					No. of isolates (% in resistant isolates)		
		Resistant	*mecA* positive	Resistant	*cfxA* positive	Resistant	*ermA* positive	*ermB* positive	*ermC* positive	*mefA* positive	Resistant	*fosA* positive	*fosB* positive
2012	75	54	52 (96.3)	74	0 (0.0)	6	0 (0.0)	0 (0.0)	5[a] (83.3)	0 (0.0)	43	0 (0.0)	0 (0.0)
2017	23	19	18 (94.7)	23	0 (0.0)	4	0 (0.0)	0 (0.0)	0 (0.0)	1 (25.0)	22	0 (0.0)	0 (0.0)

[a] PCR products were obtained for 5 isolates, but the size of the PCR products was about 550 bp, not identical to the expected size, 202 bp.

cases, multiple alternative mechanisms may provide resistance to one antibiotic e.g. clindamycin. Some bacteria show resistance against clindamycin by modification of either the ribosomal target (*ermA*, *ermB*, *and ermC)* genes, or the efflux pump (*mefA*) gene. Antibiotic inhibitory and degrading enzymes encoded and controlled by *fosA*, *fosB*, and *fosC* genes are involved in the fosfomycin resistance. These genes were therefore selected for PCR detection, depending on the different participating resistance mechanisms. The *mecA* gene involved in oxacillin resistance was detected in 96.3% and 94.7% of the isolates of 2012 and 2017, respectively. For clindamycin resistance, PCR assay tested positive for *ermC* in 83.3% of 2012 isolates; and *mefA* for 25.0% of 2017 isolates. However, other genes involved in clindamycin resistance (*ermA* and *ermB*), cefoxitin resistance (*cfxA*), and fosfomycin resistance (*fosA*, *fosB*, and *fosC*) were not detected by PCR in 100% of the isolates of 2012 and 2017. Some of the primer sequences were designed on the basis of other bacterial species, and not *L. monocytogenes* genome as the genes conferring antimicrobial resistance are lesser understood in *L. monocytogenes*. The primer sequences used in this study seemed unsuitable to detect the *L. monocytogenes* genes involved in antimicrobial resistance. Alternatively, gene mutations or other mechanisms may contribute to the antimicrobial resistance in *L. monocytogenes* (Charvalos et al., 1995; Fanner et al., 1992; Yan and Taylor, 1991).

5. Conclusion

The contamination rate caused by *L. monocytogenes* decreased in chicken meat in 2017 relative to that in 2012 in Japan. However, the multi-antimicrobial resistance of *L. monocytogenes* isolates in 2017 was higher than that of the isolates in 2012. According to our evaluation and investigation, the farmers should reduce the use of antimicrobials in livestock farming for public health welfare. We have succeeded in providing useful information for antimicrobial selection based on their suitability, as well as imparted guidance to avoid particular antibiotics for prevention of infections. Continuation of such progressive studies is much desired to design the new primers specific to *L. monocytogenes* for detecting AMR genes and knowing the AMR pattern of the pathogen in different food sources and geographical locations.

Declaration of Competing Interest

The authors have no conflict to declare.

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