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Research Article

Serotyping and Antibiotic Resistance Profile of *Listeria monocytogenes* Isolated from Organic Chicken Meat [1]

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Abstract

In this study, 240 organic chicken pieces (80 thighs, 80 wings, 80 skinless-breast meat) were analyzed for the presence of *Listeria monocytogenes.* Within the framework of the analysis findings; *L. monocytogenes* was detected in 60 (25%) of all 240 collected samples. In particular, *L. monocytogenes* was detected in 24 (30%) of 80 thigh samples, 20 (25%) of 80 wing samples and 16 (20%) of 80 skinless-breast meat samples. Serotyping distribution of 96 *L. monocytogenes* isolates determined as 71.8% serotype 1/2a, 21.9% serotype 1/2b, 4.2% serotype 4b and 2.1% serotype 1/2c. According to antibiotic resistance profile, 26 isolates (27%) were found to be resistant to ampicillin. The other isolates were found to be resistant to meropenem, tetracycline, sulfamethoxazole/trimethoprim, penicillin G, amoxicillin/clavulanic acid, vancomycin, oxytetracycline, erythromycin and chloramphenicol as 23 (23.9%), 14 (14.5%), 13 (13.5%), 12 (12.5%), 9 (9.3%), 7 (7.2%), 5 (5.2%), 4 (4.1%) and 3 (3.1%) respectively. Multiple antibiotic resistance profiles were determined in 12 of *L. monocytogenes* isolates. The findings of this study are thought to be unique data for serotyping studies that will help in revealing the epidemiology of *L. monocytogenes* in organic poultry meat, enterprises operating in food sector and diagnosis and treatment of listeriosis.

Keywords: *Listeria monocytogenes, Organic chicken, Serotype, mPCR, Antibiotic resistance*

Organik Tavuk Etlerinden İzole Edilen *Listeria monocytogenes* İzolatlarının Serotip ve Antibiyotik Direnç Profilinin Belirlenmesi

Öz

Bu çalışmada, 240 organik tavuk parça eti (80 but, 80 kanat, 80 derisiz-göğüs eti) *Listeria monocytogenes* varlığı yönünden analiz edildi. Analiz bulguları çerçevesinde; toplam 240 örneğin 60'ında (%25) *L. monocytogenes* saptandı. Bulgularının örneklere göre dağılımı incelendiğinde; but örneklerinin 24'ünde (24/80-%30), kanat örneklerinin 20'sında (20/80-%25), derisiz göğüs eti örneklerinin ise 16'sında (16/80-%20) *L. monocytogenes* tespit edildi. Serotip dağılımında ise but örneklerinden elde edilen 40 *L. monocytogenes* izolatının 31'inin *L. monocytogenes* 1/2a, 6'sının *L. monocytogenes* 1/2b, 3'ünün *L. monocytogenes* 1/2c, 1'inin ise *L. monocytogenes* 4b serotipinde olduğu, kanat örneklerinden elde edilen 34 *L. monocytogenes* izolatının 24'ünün *L. monocytogenes* 1/2a, 9'unun *L. monocytogenes* 1/2b, 1'inin ise *L. monocytogenes* 4b serotipi olduğu, derisiz göğüs eti örneklerinden elde edilen 22 *L. monocytogenes* izolatının 14'ünün *L. monocytogenes* 1/2a, 6'sının *L. monocytogenes* 1/2b, 2'sinin ise *L. monocytogenes* 4b serotipi olduğu belirlendi. Antibiyotik direnç profiline bakıldığında; 26 izolat (%27) ampisiline dirençli bulunurken, meropenem, tetrasiklin, sülfametoksazol/trimetoprim, penisilin G, amoksisilin/klavulanik asit, vankomisin, oksitetrasiklin, eritromisin ve kloramfenikole karşı dirençli izolat sayısı sırası ile 23 (%23.9), 14 (%14.5), 13 (%13.5), 12 (%12.5), 9 (%9.3), 7 (%7.2), 5 (%5.2), 4 (%4.1) ve 3 (%3.1) olarak saptandı. *L. monocytogenes* izolatlarının 12'sinde ise çoklu antibiyotik direnç profili belirlendi. Sonuç olarak bu çalışmada tespit edilen bulguların *L. monocytogenes*'in organik kanatlı etlerinde epidemiyolojisini ortaya koyacak serotiplendirme çalışmalarına, gıda sektöründe faaliyet gösteren işletmelere ve listeriyosizin tanı ve tedavisinde özgün veri niteliğinde olacağı düşünülmektedir.

Anahtar sözcükler: *Listeria monocytogenes, Organik tavuk, Serotip, mPCR, Antibiyotik direnç*

INTRODUCTION

The awareness of balanced nutrition, which emerged after the second half of the twentieth century, has led to drastic changes in people's lifestyles and food consumption. However, the increase in chronic diseases in recent years drove people's desire towards eating more reliable and healthier food, and it is observed that people prefer organic

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products in their nutrition [1,2]. "Organic", "biological", "biodynamic" and "agricultural ecological production" is a production system in which animal welfare comes to the fore in a controlled and certified manner with appropriate breeding techniques for the consumer mass demanding of high quality, healthy and risk-free products [3].

Besides the high nutritional value of poultry meat, it is an ideal environment for the development of saprophyte and pathogenic microorganisms as a result of the shredding and possible cross-contamination due to the technological processes that applied. Poultry meat is most commonly contaminated with pathogens such as *Salmonella* spp., *Campylobacte*r spp., *Staphyloccus aureus*, *Escherichia coli*, *Listeria* spp., *Yersinia enterocolitica*, *Aeromonas* spp. and *Clostridium perfringens* [4].

Several studies have focused on the comparison of conventional and organic production techniques in the presence of pathogens in poultry. Control of pathogens cannot be guaranteed due to the fact that poultry in organic breeding is more likely to be released in the open environment by free release, and there are restrictions on antimicrobial agents in feed and therapeutic use [5-7]. However, it is emphasized that contamination in poultry meat produced by both organic and conventional methods occurs through cross-contamination in the slaughter-house and during the processing [8]. The presence and serotype distribution of *L. monocytogenes*, which is the most common cause of foodborne infections among pathogenic microorganisms in poultry meat, is of great importance. *L. monocytogenes* is one of the most emphasized microorganisms due to the occurrence of the sporadic or epidemic character of listeriosis in humans, especially through food of animal origin, and its presence as a common flora in the food production [9]. In various studies, it was reported that contamination with *L. monocytogenes* was mostly observed in slaughter-houses and in the processing of foodstuffs, and that the prevalence of *L. monocytogenes* increased after cutting by 70-100% compared to pre-slaughter [10].

This study was aimed to i) determine the incidence of *L. monocytogenes* in organic chicken piece meats by using classical culture and IMS techniques, ii) confirm the isolates by PCR, iii) make a serotyping of isolates by mPCR and iv) detect the resistance status of the obtained isolates to selected antibiotics.

MATERIAL and METHODS

In the study, 240 pieces of organic chicken meat (thigh, skinless-breast meat, wing) which were sold in Samsun province in packed form were used as material. Each month 80 samples were obtained between October and December of 2018. Samples were purchased at least 500 g and were brought to the laboratory under the cold chain as soon as possible.

Isolation and Identification of Listeria monocytogenes

The IMS-based culture technique recommended by ISO 11290-1 [11] and Dynal [12] was used for the isolation. 25 g of the samples were weighed under aseptic conditions and diluted with 225 mL of Half Fraser Broth (Oxoid-CM0895) and homogenized in the stomacher for 90 sec at medium speed and then incubated at 30°C for 24 h. Following pre-enrichment, 20 µL of the immunomagnetic microparticle solution (Dynabeads anti-Listeria 710.06) homogenized with vortex was placed into the 1.5 mL microcentrifuge tubes according to the manufacturer's instructions and placed into the Dynal magnetic particle port with the magnetic stick removed. Subsequently, 1 mL of homogenized pre-enriched in Half Fraser Broth was added and the ongoing steps were completed in accordance with the recommendations of the manufacturer. From the obtained 100 µL Dynabeads Listeria complex, 50 µL was streaked on MOX (Modified Oxford Agar, Oxoid-CM0856 + Modified Listeria Selective Supplement, Oxoid-SR0206) agar and plates were incubated at 35°C for 24-48 h. After incubation, up to 5 suspected colonies were selected from the plaques and these colonies were cultured into TSA-YE (Tryptic Soy Agar-Yeast Extract, Oxoid-CM131) for biochemical tests and plates were incubated at 30°C for 24 h. The colonies that breed in TSA-YE (Tryptic Soy Agar-Yeast Extract, Oxoid, CM131) were tested by using, respectively; Gram staining, catalase, oxidase activity in SIM medium (Sulphate Indole Motility Medium; Merck 5470), ß-hemolysis and CAMP tests, sugar fermentation and nitrate reduction tests.

Verification of Listeria monocytogenes by PCR

DNA extraction of identified isolates was performed according to the boiling method. In addition, PCR protocol was designed using the primer sequences shown in *Table 1* designed by Bohnert et al.[13] and Doumith et al.[14] for PCR confirmation and serotyping. Electrophoresis of amplicons were performed in 2% agarose at 80 volts.

Antibiotic Resistance

Antibiotic resistance of the isolates were determined by the disc diffusion method on Mueller Hinton Agar (Oxoid, CM0337) based on the methods reported by CLSI [15] and EUCAST [16]. Besides, minimum inhibition concentration (MIC) of isolates resistant to various antibiotics were determined by Etest (Epsilometer test) method.

RESULTS

According to analysis, 60 (25%) of 240 samples were positive for *L. monocytogenes*. Distribution of isolates regarding to sample types was shown in *Table 2* and *Fig. 1*. Consisting of a large amount of portion (71.8%), 1/2a was found to be the dominant serotype. Distribution of all serotypes according to sample type was shown in

Table 1. *Primer sequences used in the study* [13,14]

Target Gene	Primer Sequence	PCR Product (bp)	Serotype
hlyA	F:GAATGTAAACTTCGGCGCAATCAG R:GCCGTCGATGATTTGAACTTCATC	388	*L. monocytogenes*
Imo0737	F: AGGGCTTCAAGGACTTACCC R: ACGATTTCTGCTTGCCATTC	691	1/2a, 1/2c, 3a, 3c
Imo1118	F: AGGGGTCTTAAATCCTGGAA R: CGGCTTGTTCGGCATACTTA	906	1/2c, 3c
ORF2819	F: AGCAAAATGCCAAAACTCGT R: CATCACTAAAGCCTCCCATTG	471	1/2b, 3b, 4b, 4e, 4d
ORF2110	F:AGTGGACAATTGATTGGTGAA R: CATCCATCCCTTACTTTGGAC	597	4b, 4e, 4d

Table 2. *Incidence of L. monocytogenes in organic poultry*

Sample	Number of Samples	Number of *L. monocytogenes* Positive Samples (%)	Number of *L. monocytogenes* Positive Isolates
Thigh	80	24 (30%)	40
Wing	80	20 (25%)	34
Breast	80	16 (20%)	22
Total	240	60 (25%)	96

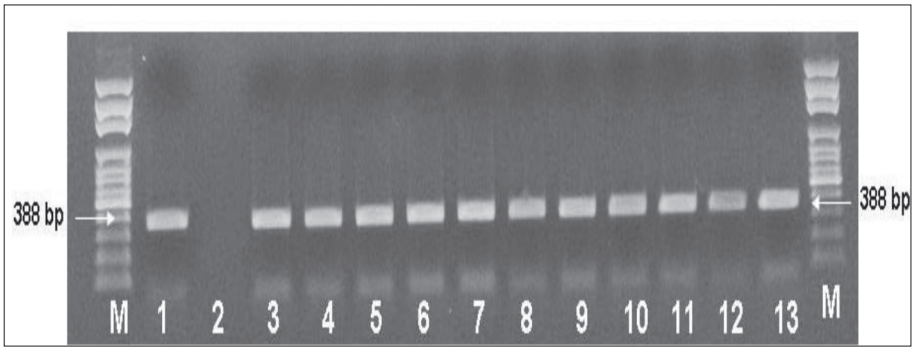

Fig 1. PCR electrophoresis image of *L. monocytogenes* isolates**:** [M: 100 bp DNA ladder, **lane 1:** *L. monocytogenes* positive control *(L. monocytogenes* RSKK 471), **lane 2:** negative control, **lane 3-13:** *L. monocytogenes* positive isolates]

Table 3. *Serotype distribution of L. monocytogenes isolates*

Number of Samples	Number of *L. monocytogenes* Positive İsolates Obtained by IMS-based Conventional Method	Number of *L. monocytogenes* Positive Isolates Verified by PCR (*hlyA* gene)	Distribution of *L. monocytogenes* Serotypes by PCR			
			1/2a (3a)	1/2b (3b)	1/2c (3c)	4b (4d,4e)
Thigh (n: 80)	40	40	31	6	2	1
Wing (n: 80)	34	34	24	9	-	1
Breast (n: 80)	22	22	14	6	-	2
Total (n: 240)	96	96	69	21	2	4

Table 3 and *Fig. 2*. The antibiotic resistance profile of our study revealed that 26 isolates (27%) were resistant to ampicillin. The other portion of the isolates were resistant to meropenem, tetracycline, sulfamethoxazole/trimethoprim, penicillin G, amoxicillin/clavulanic acid, vancomycin, oxytetracycline, erythromycin and chloramphenicol; as 23 (23.9%), 14 (14.5%), 13 (13.5%), 12 (12.5%), 9 (9.3%), 7 (7.2%), 5 (5.2%), 4 (4.1%) and 3 (3.1%) respectively. However, multiple antibiotic resistance profiles were determined in 12 of *L. monocytogenes* isolates *(Table 4, Table 5)*. Datas including the MIC levels were mentioned in *Table 6*.

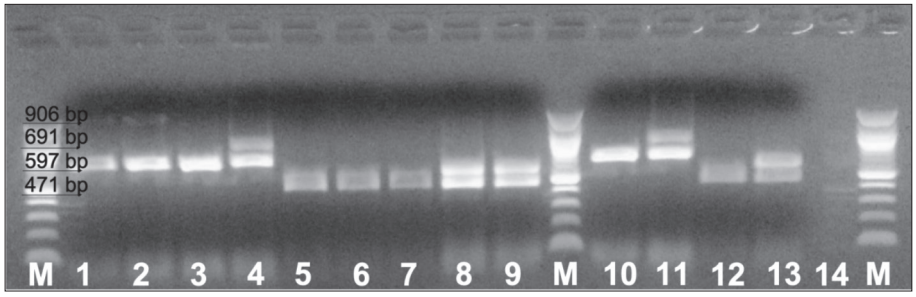

Fig 2. Multiplex PCR electrophoresis image of serotypes: [M: 100 bp DNA ladder, **lane 1-3:** *L. monocytogenes* serotype 1/2a, **lane 4:** *L. monocytogenes* serotype 1/2c, **lane 5-7:** *L. monocytogenes* serotype 1/2b, **lane 8-9:** *L. monocytogenes* serotype 4b, **lane 10:** *L. monocytogenes* serotype 1/2a positive control (*L. monocytogenes* RSKK 471), **lane 11:** *L. monocytogenes* 1/2c positive control *(L. monocytogenes* ATCC 7644), **lane 12:** *L. monocytogenes* serotype 1/2b positive control *(L. monocytogenes* RSKK 472), **lane 13:** *L. monocytogenes* serotype 4b positive control *(L. monocytogenes* RSKK 475), **lane 14:** negative control]

Table 4. *Phenotypic antibiotic resistance profiles of L. monocytogenes isolates*

Antibiotic	Number of Resistant Isolates (%)
Ampicillin (2 µg)	26 (27%)
Meropenem (10 µg)	23 (23.9%)
Tetracycline (30 µg)	14 (14.5%)
Sulfamethoxazol/Trimethoprim (1.25/23.75 µg)	13 (13.5%)
Penicillin G (1U)	12 (12.5%)
Amoxicillin/Clavulanic acid (30 µg)	9 (9.3%)
Vancomycin (30 µg)	7 (7.2%)
Oxytetracycline (30 µg)	5 (5.2%)
Erythromycin (15 µg)	4 (4.1%)
Chloramphenicol (30 µg)	3 (3.1%)

Table 5. *Phenotypic multiple antibiotic resistance profile of L. monocytogenes*

Number of Antibiotics	Antibiotic Profile*	Serotype	Sample Origin
4	AMP, TE, VA, SXT	1/2a	Thigh
4	E, PG, MEM, SXT	4b	Thigh
4	AMC, TE, VA, SXT	1/2a	Thigh
4	C, OT, VA, MEM	1/2a	Wing
3	AMP, OT, MEM	4b	Wing
3	PG, TE, SXT	1/2a	Breast
3	AMC, TE, VA	1/2a	Thigh
3	AMP, TE, MEM	1/2b	Thigh
3	AMC, E, MEM	1/2a	Wing
3	AMP, E, TE	1/2b	Breast
3	AMP, TE, SXT	1/2a	Wing
3	AMP, MEM, SXT	1/2a	Wing

AMC: *Amoxicillin/Clavulanic acid,* ***AMP:*** *Ampicillin,* ***C:*** *Chloramphenicol,* ***E:*** *Erythromycin,* ***OT:*** *Oxytetracycline,* ***PG:*** *Penicillin G,* ***TE:*** *Tetracycline,* ***VA:*** *Vancomycin,* ***MEM:*** *Meropenem,* ***SXT:*** *Sulfamethoxazole/Trimethoprim; * Only one of the same antibiotic group was evaluated*

DISCUSSION

From last few decades to present, studies on the presence of *L. monocytogenes* in poultry meat continue to be important in the worldwide. Unlikely to our findings, in many other studies a high-value prevalence was recorded, like Schafer et al.[17], who detected *L. monocytogenes* in 8.64-44.19% of chicken meat samples; Rahmat et al.[18], in

Table 6. *MIC values of L. monocytogenes serotypes with multiple antibiotic resistance profiles*

Serotype		**1/2a**	**4b**	**1/2a**	**1/2a**	**4b**	**1/2a**	**1/2a**	**1/2b**	**1/2a**	**1/2b**	**1/2a**	**1/2a**
Sample Origin		**Thigh**	**Thigh**	**Thigh**	**Wing**	**Wing**	**Breast**	**Thigh**	**Thigh**	**Wing**	**Breast**	**Wing**	**Wing**
Multiple Antibiotic Resistance Profile		**AMP TE VA SXT**	**E PG MEMSXT**	**AMC TE VA SXT**	**C OT VA MEM**	**AMP OT MEM**	**PG TE SXT**	**AMC TE VA**	**AMP TE MEM**	**AMC E MEM**	**AMP E TE**	**AMP TE SXT**	**AMP MEM SXT**
MIC (µg/mL)	**AMC**	-	-	16	-	-	-	16	-	16	-	-	-
	AMP	2	-	-	-	2	-	-	2	-	2	2	2
	C	-	-	-	32	-	-	-	-	-	-	-	-
	E	-	2	-	-	-	-	-	-	1.5	2	-	-
	OT	-	-	-	32	32	-	-	-	-	-	-	-
	PG	-	3	-	-	-	4	-	-	-	-	-	-
	TE	16	-	24	-	-	16	16	24	-	24	16	-
	VA	4	-	4	4	-	-	4	-	-	-	-	-
	MEM	-	0.5	-	0.5	0.5	-	-	0.75	0.75	-	-	0.5
	SXT	0.064	0.064	0.064	-	-	0.125	-	-	-	-	0.094	0.064

AMC: *Amoxicillin/Clavulanic acid,* **AMP:** *Ampicillin,* **C:** *Chloramphenicol,* **E:** *Erythromycin,* **OT:** *Oxytetracycline,* **PG:** *Penicillin G,* **TE:** *Tetracycline,* **VA:** *Vancomycin,* **MEM:** *Meropenem,* **SXT:** *Sulfamethoxazole /Trimethoprim*

62.5% of 24 carcass samples; Weis [19], in 62.5% of 8 chicken samples; Farber et al.[20], who reported the presence of 50% *L. monocytogenes* in 16 chicken meat; Elmalı et al.[21], who detected *L. monocytogenes* in 45% of chicken wing meat samples. On the other hand, many others reported the presence of *L. monocytogenes* in proportions similar to the findings of our study, like Bailey et al.[22] in 23% of 90 chicken carcasses; Rorvik et al.[23] in 20% to 100% of chicken carcasses from 5 slaughterhouses. In literature review, some of the studies that reported the presence of *L. monocytogenes* at lower values than the results of our study were as follows: Alsheikh et al.[24], 13.6% of the 250 ready-made chicken products; Alsheikh et al.[25], 12.8% of the 500 frozen chicken samples; Genigeorgis et al.[26] 12.5% of 160 chicken meats; Zeinali et al.[27] 18% of 200 fresh chicken carcasses; Basaran Kahraman et al.[28] 0% of 400 chicken carcasses. Although there were no studies on the presence of *L. monocytogenes* in organic poultry in Turkey, a limited number of studies are available in the literature. In a comparasive study of *L. monocytogenes* in organic and conventional poultry, the contaminations levels of products were reported 49.1% to 41% respectively [5].

In the present study, 71.8% of *L. monocytogenes* isolates were detected as serotype 1/2a. In other studies, Carvalho et al.[29] reported mostly 1/2a (94.6%) in chicken meat and chicken-meat processing environment, Oliveira et al.[30] identified 87% of the *L. monocytogenes* isolates as 1/2a in samples of chicken carcasses, and Zeinali et al.[31] determined that 52.77% of *L. monocytogenes* 1/2a serotype were predominant in chicken carcasses, followed by 4a and 4c serotypes (27.77%) in İran. Arslan and Baytur [32] revealed 57.6% of *L. monocytogenes* strains isolated from chicken meat were 1/2a. In contrast, Zeinali et al.[33] and Maung et al.[34] reported 1/2b dominance in samples of chicken meats. In addition, Ayaz and Erol [35] identified 4b as the dominant serotype (51.4%) in samples of turkey meats. Serotype dominance appears to be different due to changes in animal species, geography and seasonal parameters.

In terms of antibiotic resistance, in parallel to our study, in Ireland, Walsh et al.[36] reported that 351 *L. monocytogenes* isolates obtained from various foods were highly resistant to ampicillin, erythromycin, penicillin, and tetracycline. Davis and Jackson [37] investigated the antimicrobial resistance properties of *L. monocytogenes* isolates from human, environmental and food origin in the United States using Sensititre® method and similar to our study, isolates were found to be resistant against ampicillin, penicillin G, erythromycin and tetracycline. Harakeh et al.[38] revealed that 93.33% of *L. monocytogenes* isolates isolated from dairy products in Lebanon were resistant to oxacillin and 90% were resistant to penicillin. Similarly, Rahimi et al.[39] reported that *L. monocytogenes* isolates isolated from milk and dairy products in Iran were resistant to various antibiotics such as nalidixic acid, ciprofloxacin, erythromycin, tetracycline, gentamicin, ampicillin, penicillin, and chloramphenicol. Researchers have linked this high resistance to genetic material transfers that may occur between different species and unconscious drug use. Bilir Ormanci et al.[40], conducted the antibiotic resistance tests of *L. monocytogenes* isolates isolated from turkey meat by disk diffusion method and reported that the isolates they obtained were resistant to penicillin and ampicillin. Ayaz and Erol [35] reported that *L. monocytogenes* isolated from turkey meats were resistant to penicillin and ampicillin and were resistant to erythromycin but they couldn't

detect resistance to tetracycline, chloramphenicol and vancomycin. As an emerging problem of this century, increasing resistance to multiple antibiotics complicates the treatment of infections. In the present study, 12.5% of the isolates were found to be resistant to at least two different antibiotic type and MIC values were determined. In comparasion to other studies conducted, Lemes-Marques et al.[41] determined the MIC values of 13 *L. monocytogenes* isolates obtained from patients with listeriosis in Brazil against ampicillin and vancomycin by microdilution method. They identified that the isolates were not resistant to vancomycin and ampicillin. In our study, also resistance to vancomycin was not detected. Filiousis et al.[42] reported that one of the 30 *L. monocytogenes* isolates obtained from various foods in Greece was resistant to tetracycline and this MIC was determined to be 64 µg/mL. However, they reported that they could not detect any resistance to other antibiotics. Osaili et al.[43] reported that 11% of *L. monocytogenes* isolates obtained from ready-to-eat chicken products in Jordan were resistant to tetracycline and MIC values were determined as 16 µg/mL. Conter et al.[44] searched MIC values against 19 antibiotics including penicillin G, ampicillin, erythromycin, vancomycin and tetracycline by VITEK 2. The researchers reported that they could not detect any resistance to penicillin and erythromycin, but reported that 2% of their isolates were resistant to ampicillin and 0.8% to tetracycline and vancomycin. Yan et al.[45] investigated the antibiotic resistance profiles of 70 *L. monocytogenes* isolates obtained from various foods in China by microdilution method and according to their findings, 14 isolates were resistant to tetracycline, 2 isolates to ampicillin, erythromycin and chloramphenicol, and 1 isolate was resistant to penicillin and vancomycin. Okada et al.[46] mentioned in their study on 201 *L. monocytogenes* isolated from food, environment, animals, and humans in Japan, 31 of 32 isolates found to be resistant to chloramphenicol and had MIC values of 16 µg/mL and 1 isolate had MIC of 32 µg/mL. The researchers found that MIC of 1 isolate found to be resistant to oxytetracycline was 64 µg/mL. Despite the prohibition of antimicrobial use in organic poultry production, several studies have demonstrated that pathogenic and non-pathogenic bacteria have drug resistance properties. In the second half of the twentieth century, glycopeptide (vancomycin) resistance was not reported, but since the 1980s, staphylococci and enterococci suddenly developed resistance to vancomycin [47]. In studies, it was determined that enterococcal and streptococcal plasmids and transposons that carrying antibiotic resistance genes were transferred to *Listeria* species by conjugation. Charpentier and Courvalin [48] reported that plasmid pIP501, which is responsible for the resistance of chloramphenicol, macrolide, lincosamide and streptogramin was found in *Streptococcus agalactie*, and can be transferred to *L. monocytogenes* under *in-vitro* conditions. Similarly, Biavasco et al.[47] reported that the resistance gene from vancomycin-resistant Enterococci strains was transferred to *Listeria* species. In our study, the resistance that we detected for different antibiotics can be attributed to the occurring of mutations in bacteria and to genetic material transfers caused by the interaction between bacteria.

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