DETERMINATION OF PRESENCE AND SOME PATHOGENIC CHARACTERISTICS OF *LISTERIA* SPP. IN GROUND BEEF AND CHICKEN MEAT SOLD IN BOLU BY PHENOTYPIC AND GENOTYPIC METHODS

by

SELİN BAYTUR

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Prof. Dr. Duran KARAKAŞ
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of the Master of Science.

Prof. Dr. Ekrem GÜREL
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Biology.

Prof. Dr. Seza ARSLAN
Supervisor

Examining Committee Members

1. Prof. Dr. Seza ARSLAN .....

2. Prof. Dr. Azra BOZCAARMUTLU .....

3. Assist. Prof. Dr. Mehmet ÖZTÜRK .....

ABSTRACT

DETERMINATION OF PRESENCE AND SOME PATHOGENIC CHARACTERISTICS OF *LISTERIA* SPP. IN GROUND BEEF AND CHICKEN MEAT SOLD IN BOLU BY PHENOTYPIC AND GENOTYPIC METHODS

BAYTUR, Selin
Master of Science, Department of Biology
Supervisor: Prof. Dr. Seza ARSLAN

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Listeria and particularly *Listeria monocytogenes* is an important pathogen that can cause listeriosis with flu-like symptoms in healthy people, and severe complications including bacteremia, meningitis, encephalitis, and endocarditis in immunocompromised people such as children, pregnant women and the elderly. They have been frequently isolated from foods such as meat and meat products. In the present study, a total of 124 food samples composed of ground beef and chicken meat were examined for the presence of *Listeria* spp. Out of the 124 samples, 101 (81.5%) were positive for *Listeria* spp. Overall, out of all samples, 44 (35.5%) isolates were phenotypically identified as *L. innocua*; the remaining isolates were *L. monocytogenes* (26.6%), *L. seeligeri* (15.3%), *L. grayi* (12.9%), *L. welshimeri* (8.9%), *L. ivanovii* subsp. *londoniensis* (3.2%) and *L. ivanovii* subsp. *ivanovii*

(0.8%). The phenotypically identified *L. monocytogenes* strains were also confirmed by *hlyA* virulence gene amplification. The prevalence of *Listeria* spp. was 96.8% in ground beef and 66.1% in chicken meat. *L. innocua* was the most common species in this study that was most frequently isolated in ground beef. All 33 *L. monocytogenes* strains were examined for the virulence genes such as *hlyA, inlB* and *actA*, and serovars. All 33 (100%) *L. monocytogenes* strains were positive for *hlyA* gene. Out of the 33 *L. monocytogenes* strains, 29 (87.9%) and 16 (48.5%) strains harbored *inlB* and *actA* virulence genes, respectively. Nineteen strains (57.6%) belonged to serovar "1/2a, 3a" and 14 (42.4%) belonged to serovar "1/2c, 3c". In our study, the antimicrobial susceptibility of the 128 *Listeria* spp. isolated from ground beef and chicken meat to commonly used antimicrobial agents was examined. Most *Listeria* strains with a rate of 90.6% were resistant to penicillin, 79.7% to ceftriaxone and 71.1% to ampicillin. A multidrug resistance was observed in 112 (87.5%) of the strains to at least three or more antimicrobial agents. Prevalence and antimicrobial resistance of *Listeria* spp. in ground beef and chicken meat samples were determined in the present study. In addition, virulence characteristics and serovars of the 33 *L. monocytogenes* strains obtained from this research were reported. The results of this study indicated that consumption of inadequately cooked ground beef and chicken meat, and products made from raw meat may develop a potential risk of human listeriosis.

Keywords: *Listeria* spp., ground beef, chicken meat, isolation, identification, virulence genes, serovars, antimicrobial resistance.

ÖZET

FENOTİPİK VE GENOTİPİK YÖNTEMLERLE BOLU'DA SATILAN KIYMA VE TAVUK ETİNDE *LISTERIA* TÜRLERİNİN VARLIĞININ VE BAZI PATOJENİK ÖZELLİKLERİNİN BELİRLENMESİ

BAYTUR, Selin
Yüksek Lisans, Biyoloji Bölümü
Tez Danışmanı: Prof. Dr. Seza ARSLAN

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Listeria, özellikle *Listeria monocytogenes* sağlıklı insanlarda grip benzeri semptomlar gösteren; çocuklar, hamileler ve yaşlılar gibi bağışıklık sistemi zayıf olan insanlarda bakteriyemi, menenjit, ensafalit ve endokardit dahil olmak üzere şiddetli komplikasyonlarla listeriosise neden olabilen önemli bir patojendir. *Listeria* spp. et ve et ürünleri gibi besinlerden sıklıkla izole edilir. Bu çalışmada, kıyma ve tavuk etinden oluşan toplam 124 gıda örneği *Listeria* türlerinin varlığı için incelenmiştir. 124 örneğin 101'i (%81,5) *Listeria* spp. için pozitiftir. Toplamda, tüm örneklerden 44 (%35,5) izolat fenotipik testlerle *L. innocua* olarak tanımlanmıştır; geri kalan izolatlar *L. monocytogenes* (%26,6), *L. seeligeri* (%15,3), *L. grayi* (%12,9), *L. welshimeri* (%8,9), *L. ivanovii* subsp. *londoniensis* (%3,2) ve *L. ivanovii*

subsp. *ivanovii* (%0,8) olarak tespit edilmiştir. Fenotipik olarak tanımlanan *L. monocytogenes* suşları aynı zamanda *hlyA* virulans gen amplifikasyonları ile doğrulanmıştır. *Listeria* spp.'nin bulunma sıklığı kıymada %96,8 ve tavuk etinde %66,1 dir. Bu çalışmadaki en yaygın tür olan *L. innocua* en çok kıymadan izole edilmiştir. 33 *L. manocytogenes* suşunun tamamı *hylA, inIB* ve *actA* virülans genleri ve serovarların varlığı için incelenmiştir. Tüm 33 *L. monocytogenes* suşu *hlyA* geni için pozitif bulunmuştur. 33 *L. monocytogenes* suşundan 29'u (%87,9) *inIB* ve 16'sı (%48,5) *actA* virülans genlerini bulundurmuştur. 19 suş (%57,6) "1/2a, 3a" serovarına ve 14 suş (%42,4) "1/2c, 3c" serovarına aittir. Kıyma ve tavuk etinden izole edilen 128 *Listeria* spp.'nin antimikrobiyal ajanlara karşı antimikrobiyal duyarlılığı incelenmiştir. *Listeria* suşlarının çoğu (%90,6) penisiline karşı dirençli bulunmuştur. Bunu seftriakson (%79,7) ve ampisilin (%71,1) direnci takip etmiştir. En az üç ya da daha fazla antimikrobiyal ajana karşı suşların 112'sinde (%87,5) çoklu ilaç direnci gözlemlenmiştir. Kıyma ve tavuk etinde *Listeria* spp.'nin bulunma sıklığı ve antimikrobiyal dirençlilikleri bu çalışmada belirlenmiştir. Bunlara ek olarak, bu araştırmada elde edilen 33 *L. monocytogenes* suşunun virülans özellikleri ve serovarları rapor edilmiştir. Çalışmamızın sonuçları, yeterince pişirilmemiş kıyma ve tavuk etinin ve çiğ etten yapılan ürünlerin tüketiminin insan listeriozisi için potansiyel bir risk oluşturabileceğini göstermiştir.

Anahtar Kelimeler: *Listeria* spp., kıyma, tavuk eti, izolasyon, identifikasyon, virulans genler, serovarlar, antimikrobiyal direnç.

To my dear family…

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1. INTRODUCTION

Bacteria are widely distributed in nature, food and food products; especially meat and poultry and food processing plants (Modzelewska-Kapitula and Maj-Sobotka, 2014). Meat and poultry are good nutrient sources for the growth of microorganisms, especially for pathogens. After consumption of contaminated cooked or raw meat products, these pathogens such as *Listeria* spp. can cause gastrointestinal diseases (Meisel et al., 2014).

All members of the genus *Listeria* are widely distributed in nature. They have been isolated from soil, vegetation, sewage, water, animal feed, fresh and frozen poultry, slaughterhouse wastes, and the feces of healthy humans and animals (Ludwig et al., 2009). Genus *Listeria* has two pathogenic species for humans and animals. These species were *L. monocytogenes* and *L. ivanovii* (Mengaud et al., 1988).

Listeria particularly *L. monocytogenes* is an important foodborne pathogen that can cause listeriosis with flu-like symptoms in healthy people, and severe complications (gastroenteritis, meningitis, neuro-encephalitis, chorioamnionitis, abortions, and neonatal infections) in immunocompromised subjects, children, pregnant women and elderly (Khelef et al., 2006; Pesavento et al., 2010). Listeriosis is associated with a high mortality rate, particularly in immunocompromised individuals (Khelef et al., 2006). *L. ivanovii* has been described to be involved in human pathology only rarely, and *L. seeligeri* has been reported only once to be the

cause of meningitis in a non-immunocompromised adult (Cocolin et al., 2002).

L. monocytogenes can produce a variety of toxins that contribute to its ability to colonize and cause diseases. Internalin, encoded by *inl* gene, is an essential factor for *L. monocytogenes* to adhere to and invade the host cell. *HlyA* gene encodes the hemolysin protein, which allows *L. monocytogenes* to escape from vacuoles by dissolving phagosome membranes (Shi et al., 2013). The intracellular motility of *L. monocytogenes* and spread from cell to cell are due to an actin polymerization protein (actA) (Gelbíčová and Karpíšková, 2012).

Serotyping has been used to characterize *L. monocytogenes* that differs in their virulence and pathogenicity (Soni et al., 2013). The 16 serovars of *Listeria* are based on the expression of somatic (O) and flagellar (H) antigens, with 13 *L. monocytogenes* serovars that can cause disease (Khelef et al., 2006). Ninety-five percent of *L. monocytogenes* strains associated with human listeriosis and food samples belong to serotypes 1/2a, 1/2b, and 4b (Jamali et al., 2013).

The pathogen usually shows susceptibility to a variety of antimicrobials while some multidrug resistant *L. monocytogenes* strains have been isolated from foods and human listeriosis cases (Jamali et al., 2013). The abusive and improper usage of antimicrobials leads to the emergence of antimicrobial resistance, particularly multi-resistance (Wang et al., 2015).

The objectives of this study were to determine the presence of *Listeria* spp. in ground beef and chicken meat, to identify the *L. monocytogenes* using phenotypic and genotypic methods, and to detect virulence genes and characterize serovars of *L. monocytogenes* using the PCR. The present study also aimed to investigate the antimicrobial resistance profiles of *Listeria* spp. from ground beef and chicken meat.

1.1. *Listeria* spp.

1.1.1. Classification and Taxonomy

Numerical taxonomic studies together with biochemical, DNA base composition and 16S rRNA gene sequence studies show that all the species of the genus *Listeria* form a homogeneous group. On the basis of the sequencing of the 16S rRNA gene (as well as other genes), members of the genus *Brochothrix* are the closest relatives to *Listeria*, which is consistent with chemical and numerical taxonomic approaches; these two genera justify family status as *Listeriaceae*. The family *Listeriaceae* shows relatedness to other low-G+C Gram-positive bacteria, especially to species of *Bacillus* (Ludwig et al., 2009).

On the basis of DNA-DNA hybridization, multi-locus enzyme electrophoresis (MEE), rRNA gene restriction patterns, and 16S rRNA sequencing, the genus *Listeria* comprises six species and the two subspecies: *L. monocytogenes*, *L. ivanovii* subsp. *ivanovii*, *L. ivanovii* subsp. *londoniensis*, *L. seeligeri*, *L. innocua L. welshimeri* and *L. grayi. L. monocytogenes* is a human and animal pathogen, and *L. ivanovii* is pathogenic for animals, mainly sheep and cattle. On rare occasions, *L. ivanovii* and *L. seeligeri* were shown to be associated with human infections (Khelef et al., 2006).

1.1.2. The Genus *Listeria*

Members of the genus *Listeria* are Gram-positive, facultative anaerobic, non-spore-forming, nonbranching, regular, short (they are 0.5 to 2 μm long and 0.4 to 0.5 μm wide) rods that occur singly or in short chains (Wellinghausen, 2011).

Colonies of all *Listeria* species show limited variation and usually appear as non-pigmented, round, translucent, low-convex with a smooth surface, entire margin and crystalline central appearance on fresh isolation. After several days, colonies

become less translucent and creamier in color; margins become less entire, and a central depression may form (Ludwig et al., 2009).

Two to six peritrichous flagella are produced by all *Listeria* species grown below 30ºC. Tumbling motility is observed for cultures grown between 20-30ºC, and expression of the structural gene for the flagellin protein *(flaA*) has been shown to be temperature regulated and repressed at 37ºC. Motility can also be demonstrated in semi-solid agar in 'U' tubes. Maximal turbidity in liquid and semi-solid agar is present 2-3 mm below the surface in an umbrella-like pattern (Ludwig et al., 2009).

One of the key properties of *L. monocytogenes* resides in its ability to grow in a wide range of temperatures (1-45ºC), which favors resistance and enrichment in foods. However, optimal growth occurs at 30-37ºC. *Listeriae* are killed at 60ºC, making pasteurization a good technique to eliminate the bacteria from dairy products (Khelef et al., 2006).

1.1.3. Natural Habitats

Listeria species are ubiquitous in nature and are found in soil, water, sewage, silage, plants, and in the intestinal tract of domestic animals (sheep, cattle, goat, etc.). One to five percent of healthy humans are reported to serve as carriers (Bhunia, 2008). Raw meat, dairy foods and non-pasteurized milk are major sources of listeriosis. These foods may act as vehicles of transmission of *Listeria* species (Pesavento et al., 2010). Ready-to-eat or minimally processed foods such as hotdogs, luncheon meats, pâté, smoked fish, some soft cheeses, and cheeses made with unpasteurized milk (ethnic products like Mexican-style soft cheese) were responsible for numerous epidemic or sporadic outbreaks. Post-processing contamination of products is a major concern since those products are eaten without further cooking.

Listeria-associated gastroenteritis has been linked to rice salad, cold corn and tuna salad, corned beef and ham, cold smoked trout, and cheese (Bhunia, 2008).

1.2. *Listeria monocytogenes*

L. monocytogenes is widely distributed in nature, found in soil, mud, sewage, vegetation and in the feces of animals and man. It is pathogenic for animals and man; most cases are food- or feed-borne. In humans, it causes systemic illness (most often septicemia and/or meningitis) in immunocompromised adults or juveniles, as well as infecting the unborn infant (Ludwig et al., 2009).

On the basis of both multilocus enzyme electrophoresis and restriction fragment and sequence analysis of specific genes, three lineages of *L. monocytogenes* have been identified that represent distinct evolutionary branches. These lineages correspond to serovars 1/2a, 1/2c and 3c (lineage I), serovars 4b, 1/2b, and 3b (lineage II) and serovars 4a and 4c (lineage III). Outbreaks of disease are most commonly associated with organisms from Lineages I and II and Lineage III have almost solely been isolated from animal hosts (Ludwig et al., 2009).

1.2.1. Virulence Factors of *L. monocytogenes*

Virulence for *L. monocytogenes* is a multifactorial property and at least nine genes and their products are required for infection, invasion, survival, mobility and cell-to-cell spread, although additional genes (especially those for cell-surface components) are also likely to be involved with the infectious process (Ludwig et al., 2009).

The best characterized virulence factors of *L. monocytogenes* are involved in the four steps of the cell infectious cycle. Entry into epithelial cells is mediated by the internalins InlA and InlB. Lysis of the primary vacuole involves LLO, PLC-A and PLC-B. Intracellular motility is promoted by the ActA protein. Intercellular

spreading involves the ActA protein, LLO, PLC-A, and Mpl, which activates PLC-B. (Khelef et al., 2006).

InlA and InlB are surface proteins that play a critical role in *L. monocytogenes* entry into non-phagocytic cells, in particular in epithelial cells. The secreted pore-forming toxin Listeriolysin O (LLO) and two phospholipases (PlcA and PlcB) promote *L. monocytogenes* escape from the internalized vacuole. Once it has reached the cytosol, *L. monocytogenes* multiplies and ActA triggers actin polymerization around bacteria, forms actin comet tails allowing *L. monocytogenes* motility in the cytosol, the formation of protrusions at the host cell surface and cell-to-cell spread. LLO and actA both play a critical role in *L. monocytogenes* ability to survive in phagocytic cells *in vitro* and *in vivo*, and in *L. monocytogenes* dissemination within tissues (Travier and Lecuit, 2014).

1.2.1.1. Listeriolysin

The virulence factor of *L. monocytogenes* is a specific type of hemolysin, listeriolysin O (LLO). It is produced during the exponential growth of the cells. The pathogens invade different body tissues and multiply inside body cells, releasing the toxin. The toxin causes death of cells (Ray, 2004).

LLO is encoded by *hly* gene, which is under direct control of *prfA*. LLO is a member of the cholesterol-dependent pore-forming toxin, and is related genetically and antigenically to streptolysin O from *Streptococcus pyogenes*, ivanolysin O from *L. ivanovii*, pneumolysin from *Streptococcus pneumoniae*, and seeligerolysin from *L. seeligeri* (Bhunia, 2008).

The gene was undetected in *L. seeligeri*, which is hemolytic but in which the hemolysin is uncharacterized. Thus, *hlyA* is specific for *L. monocytogenes*. It is the first species-specific gene reported in the genus *Listeria*; it is specific for a

pathogenic organism and could be used to detect its presence in food or other environments (Mengaud et al., 1988).

Disruption of vacuole membranes by hemolysin-producing strains of *L. monocytogenes* might be a key mechanism allowing bacteria to escape from phagosomes and to multiply within the cell cytoplasm (Mengaud et al., 1988).

The pathogenic potential of *Listeriae* has been linked to their ability to induce hemolysis, and therefore to the production of LLO. However, despite its fundamental role in virulence, LLO by itself is not sufficient to confer a pathogenic potential to nonpathogenic Gram-positive bacteria. Moreover, *L. seeligeri*, which has weak hemolytic activity, is rarely pathogenic for humans, suggesting that hemolysin is not the only factor required for *L. monocytogenes* virulence (Khelef et al., 2006).

1.2.1.2. Internalins

L. monocytogenes enters both phagocytic and non-phagocytic cells. A listerial surface protein, internalin A has been shown to be involved with the initial stages of invasion of all cell types. A second cell surface protein (internalin B) is required for entry into hepatocyte-like but not into enterocyte-like cells. The receptor for internalin A is E-cadherin which is a mammalian surface protein involved in cell-to-cell adhesion (Ludwig et al., 2009).

The presence of the so-called internalin genes, which are associated with the virulence cycle of *L. monocytogenes*, is a valuable indicator for their virulence potential (Zunabovic et al., 2011).

1.2.1.3. ActA

ActA is one of the most critical and best characterized virulence factors of *L. monocytogenes*. It fulfills many essential functions within host cells, allowing *L. monocytogenes* escape from autophagy and recruiting an actin polymerization

complex that promotes *L. monocytogenes* actin-based motility, cell-to-cell spread and dissemination within host tissues (Travier and Lecuit, 2014).

Actin polymerization protein (ActA) initiates actin polymerization to aid bacterial movement inside the cytoplasm. The moving bacterium forms a pseudopod-like protruding structure, which extends into the neighboring cell. It is suggested that the protruding structure is engulfed by the neighboring cell facilitating bacterial cell-to-cell spread. Bacteria trapped in the double membrane (the outer membrane is from the newly infected cell and the inner membrane from the previously infected cell), is escaped with the aid of phosphatidylcholine-specific PLC (PC-PLC) and LLO (Bhunia, 2008).

1.2.1.4. Phosholipases

Two phospholipases are produced by *L. monocytogenes*, PLC-A and PLC-B. Phospholipases have a membrane damaging activity and are involved in bacterial escape from primary and/or secondary phagosomes. PLC-A has a minor individual role in escape from the primary phagosome but rather acts in synergy with listeriolysin O and PLC-B to fulfill this action. It has no role in cell-to-cell spread, suggesting no role in escape from the double membrane vacuole. PLC-B is encoded by the gene *plcB* and is expressed as an inactive precursor. It can be activated by proteolytic cleavage involving the zinc-dependent metalloprotease Mpl, encoded by the gene *mpl*, and also by cellular proteases. The *plcB* and *mpl* genes are part of an operon, which is controlled by the transcriptional activator PrfA. PLC-B is involved in the lysis of the double membrane vacuole and therefore in cell-to-cell spreading. In certain cell types, such as HenLe 407 and HeLa, but not in macrophages, PLC-B lyses the membrane of primary phagosomes containing *L. monocytogenes* in the

absence of listeriolysin. This suggests that depending on the cell type, PLC-B may act alone or only help LLO to lyse the primary vacuole (Khelef et al., 2006).

1.2.1.5. Other Virulence Factors

Other listerial products such as p60 protein, antioxidant factors, metal ion uptake, and stress response mediators have been identified that also contribute to survival within the host. Although some of them have a major influence on the outcome of the host-parasite interaction, their participation in pathogenesis is more indirect, as they are probably involved in general housekeeping functions also necessary for saprophytic life (Vasquez-Boland et al., 2001).

The p60 protein is encoded by the *iap* (for invasion-associated protein) gene. The p60 protein has a murein hydrolase activity required for normal septum formation and essential for cell viability, which makes it difficult to determine the precise role of p60 in virulence because *iap* mutations are lethal. p60 is a major antigen in the protective response against *L. monocytogenes* (Vasquez-Boland et al., 2001).

Catalase and superoxide dismutase (SOD) enzymes that are produced by bacteria primarily to detoxify the endogenous free oxygen radicals generated during prokaryotic oxidative metabolism but which also play a key role in counteracting the oxygen-dependent microbicidal mechanism of the host during infection, appear to have only a minor involvement, if any, in *Listeria* virulence (Vasquez-Boland et al., 2001).

Iron is a key element for all living cells, including prokaryotic cells, serving as cofactor for a large number of enzymes and essential proteins involved in electron transport processes. In animal host tissues, iron is not freely available because it is sequestered by ferric transferrin in serum and by ferritin and heme compounds within

cells. Thus, bacterial pathogens have evolved specialized mechanisms to capture iron for growth in host tissues, and these mechanisms play an important role in virulence. In addition to its role as an essential nutrient, iron is also used by pathogenic bacteria as a signal molecule for the regulation of virulence gene expression. Expression of *hly* has been shown to be induced in conditions of iron limitation, indicating that this metal ion plays a role in the regulation of virulence gene expression in *L. monocytogenes* (Vasquez-Boland et al., 2001).

A small proportion of *L. monocytogenes* cells survive the stressful, hostile conditions of the phagocytic vacuole and reach the cytoplasm, where they proliferate, leading to the spread of the infection. Survival under stress involves an adaptive response mediated by a set of conserved proteins that are upregulated in vitro upon exposure to heat shock, low pH, oxidative agents, toxic chemical compounds, starvation, and, in general, any situation in which bacterial growth is arrested. These proteins may be chaperones that assist in the proper refolding or assembly of stress-damaged proteins, ensuring that essential physiological pathways function correctly in stressed cells (Vasquez-Boland et al., 2001).

1.2.2. Serotyping

Serovar determination is usually used for the microbiological characterization and a rapid initial screening in epidemiological investigations of *L. monocytogenes*. Furthermore, serotyping is widely used for long-term microbiological surveillance of human listeriosis. Thirteen serovars of *L. monocytogenes* have been identified. These serovars are 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e, and 7, but only serovars 1/2a, 1/2b, 1/2c, and 4b are frequently isolated from foods and patients, with serovars 1/2a, 1/2b, 3b, and 4b causing most cases of human listeriosis (Khelef et al., 2006; Wang et al., 2013).

L. monocytogenes consists of at least four evolutionary lineages (I, II, III, and IV) with different but overlapping ecological niches. Most *L. monocytogenes* isolates belong to lineages I and II, which include the serotypes more commonly associated with human clinical cases, such as serotypes 1/2a (lineage II), and 1/2b and 4b (lineage I). Lineage II strains are common in foods and widespread in the natural and farm environments, and are often isolated from animal listeriosis cases and sporadic human clinical cases. However, most human listeriosis outbreaks are associated with lineage I isolates. Lineage III and IV strains are rare and predominantly isolated from animal sources (Orsi et al., 2011).

Serotyping was originally described based on agglutination reactions with specific antisera, and has more recently been superseded by PCR based procedures (Vitullo et al., 2013).

The traditional agglutination serotyping method allows classifying *L. monocytogenes* into 13 serotypes, while the use of a multiplex PCR assay discriminate the major serovars 1/2a, 1/2b, 1/2c and 4b. However, the uses of classical serological procedure make it possible the identification of the two main phylogenetic lineages: lineage I including serotypes 1/2b, 3b, 3c, 4b and lineage II including serotype 1/2a, 1/2c and 3a (Spanu et al., 2015).

Using PCR, *L. monocytogenes* isolates into four serovars by detecting the genes *lmo0737*, *lmo1118*, ORF2819 and ORF2110. Group I (serovars 1/2a and 3a) is identified by the amplification of the *lmo0737* gene fragment; the *lmo0737* and *lmo1118* genes allow the identification of group II (1/2c and 3c); group III (1/2b, 3b, and 7) is detected by the amplification of ORF2819 DNA fragment; and both ORF2819 and ORF2110 genes are amplified from group IV (4b, 4d, and 4e) (Doumith et al., 2004).

The proposed multiplex PCR profiles do not distinguish, within the species *L. monocytogenes*, serovar 1/2a from 3a, 1/2c from 3c, 1/2b from 3b and 7, or 4b from 4d and 4e. These profiles also do not distinguish *L. monocytogenes* serovars 4a and 4c from the other *Listeria* species. However, serovars 3a, 3b, 3c, 4a, 4c, 4e, 4d, and 7 are very infrequent in food and rarely reported as implicated in human listeriosis (Doumith et al., 2004).

The serovars of *L. monocytogenes* were obtained by PCR from different types of foods in previous studies in some countries. *L. monocytogenes* strains were distributed into serovar "1/2a, 3a", "1/2c, 3c", "1/2b, 3b and 7", "4b, 4d, 4e", "4a, 4c" that isolated from raw meat in USA (Shen et al., 2013). *L. monocytogenes* isolates from turkey meat belonged to "4b, 4d, 4e", "1/2a, 3a", "1/2b, 3b" in Turkey (Erol and Ayaz, 2011). "1/2a, 3a", "1/2b, 3b", "1/2c, 3c" serovars were obtained from *L. monocytogenes* isolates in ready-to-eat meat products in China (Wang et al., 2015).

1.2.3. Clinical Significance

People with normal health, following ingestion of a food contaminated with *L. monocytogenes,* may or may not produce symptoms. Most often, symptoms appear 1 to 7 day following ingestion and include mild flu-like symptoms with slight fever, abdominal cramps, and diarrhea. The symptoms subside in a few days, but the individual sheds *L. monocytogenes* in the feces for some time. The symptoms among sensitive groups are quite different. These groups include pregnant women, unborn fetuses, infants, elderly people with reduced immunity due to diseases, and people taking special medications, such as steroids and chemotherapeutic agents. Initial symptoms are enteric, with nausea, vomiting, abdominal cramps, and diarrhea, along with fever and headache. The pathogens then spread through the bloodstream and

invade tissues in different vital organs, including the central nervous system. In pregnant women, the pathogen can invade organ tissues of the fetus through the placenta. Symptoms include bacteremia (septicemia), meningitis, encephalitis, and endocarditis. The fatality rate in fetuses, infected newborn infants, and immunocompromised individuals is very high. The infective dose is considered to be ca. 100 to 1000 cells, particularly for the highly sensitive individuals (Ray, 2004).

L. monocytogenes infects humans through the ingestion of contaminated food products. The bacteria can cross the intestinal barrier and disseminate from the mesenteric lymph nodes to the spleen and the liver, from which they may reach the brain or the placenta, resulting in meningitis or encephalitis in immunocompromised patients, abortions in pregnant women, or generalized infections in infected neonates (*granulomatosis infantiseptica*). If not controlled properly by the immune system, *L. monocytogenes* infection may also cause septicemia. Highly contaminated food products also infect healthy individuals, resulting mainly in gastroenteritis (Khelef et al., 2006). *L. monocytogenes* causes three forms of disease; gastrointestinal form, systemic listeriosis, and abortion and neonatal listeriosis (Bhunia, 2008).

1.2.3.1. Gastrointestinal Form

Since 1993, seven outbreaks of gastroenteritis caused by *L. monocytogenes* were reported worldwide. The serotype 1/2a, 1/2b, and 4b have been implicated in those outbreaks. The foods involved were rice salad, cold tuna salad, cheese, trout, and chocolate milk. Adults are more susceptible than children and people receiving gastric acid suppressive medications are at greatest risk. The incubation period is less than 24 h; however, it may range from 6 h to 10 days. The exact mechanism of gastroenteritis is not known. However, it appears that the organism causes damage to the absorptive villi affecting absorption of nutrients and promoting fluid secretion.

The gastroenteritis symptoms are characterized by fever, headache, nausea, vomiting, abdominal pain, and diarrhea. Diarrhea is nonbloody but watery (Bhunia, 2008).

1.2.3.2. Systemic Listeriosis

L. monocytogenes causes the disease listeriosis; a rare but fatal disease primarily affected immunocompromised individuals such as young, old, pregnant, and immunologically challenged hosts. AIDS patients, cancer patients receiving chemotherapy, organ transplant patients, diabetic, alcoholics, and patients with cardiovascular diseases are highly susceptible to this bacterium. The organism may colonize for a short duration in the intestine and passes through the intestinal barrier reaching the blood circulation and the lymphatic system. A majority of the organism is distributed in the liver (90%), spleen (10%), gall bladder and the regional lymph nodes within 24 h. After a brief bacteremic phase, *Listeria* crosses the blood-brain barrier and infects the brain causing meningitis and encephalitis. In pregnant women, *Listeria* crosses the placental barrier and infects the fetus. Systemic listeriosis is characterized by fever, headache, malaise, septicemia, meningitis, brain stem encephalitis, ataxia, bacteremia, and liver abscess (Bhunia, 2008).

1.2.3.3. Abortion and Neonatal Listeriosis

In pregnant women, *L. monocytogenes* can cause complications late in the third trimester of pregnancy resulting in stillbirth, or birth of an infected fetus. Pregnancy is associated with suppression of cell-mediated immunity to protect the fetus from rejection, which renders pregnant women extremely susceptible to listeriosis. Complications such as preterm labor, spontaneous abortion, stillbirth, or neonatal infection are seen. Two forms of neonatal listeriosis are reported: early and late onset forms. In the early onset form (1.5 days), organism is acquired in the uterus and the infection is widespread exhibiting very high mortality rate. In the late

forms (14 days), the infection is possibly acquired during birth from the vaginal tract or from the environment. In this form, meningitis is the predominant clinical feature. The symptoms of neonatal listeriosis include respiratory distress, pneumonia, shortness of breath, hyperexcitability, conjunctivitis, rash, vomiting, cramps, and hypo- or hyperthermia (Bhunia, 2008).

1.2.4. Mechanism of Pathogenesis

The pathogenic mechanism of *L. monocytogenes* is a complex process and it can be subdivided into two phases: intestinal and systemic. The intestinal phase of infection involves bacterial colonization in the intestine and consequent translocation through the mucosal barrier to blood circulation or to the lymphatic system for systemic dissemination. During systemic spread, the organism is transported by dendritic cells or macrophages to the liver, spleen, lymph nodes, brain, and to the placenta (in pregnant women). Many surface associated and secreted proteins have been recognized as important virulence factors, which are critical for bacterial persistence in the intestinal tract, to enter the host cells for intracellular movement and cell-to-cell spread, and to evade immune system (Bhunia, 2008).

1.2.5. Transmission

The main route of transmission to both humans and animals is believed to be through consumption of contaminated food or feed. *Listeria* spp. has been isolated from a variety of food products. Meat, poultry, raw milk, dairy products, seafood and vegetable products have all been implicated as vehicles of this pathogen (Gudbjörnsdóttir et al., 2004; Pesavento et al., 2010; Ponniah et al., 2010; Rahimi et al., 2010; Fallah et al., 2012).

Ingestion of, usually heavily contaminated, foods is the principal route of infection. Other routes of transmission for human listeriosis occur including direct

contact with the environment, contact with naturally infected animals, and via cross infection between newborn infants (McLauchlin et al., 2004).

1.2.6. Antimicrobial Susceptibility

While antibiotic sensitivity might vary between strains, most *Listeriae* are sensitive to many antibiotics, such as aminoglycosides, tetracyclines, macrolides, chloramphenicol and penicillins. Some of them are used to treat listeriosis, e.g., usually ampicillin in combination with an aminoglycoside. *Listeria* strains are naturally resistant to antibiotics such as cephalosporins or sulfanamides. Although antibiotic resistance of *Listeriae* is not yet a concern for clinics, new tetracycline- or trimethoprim-resistant strains have recently emerged (Khelef et al., 2006).

Antimicrobial resistance of *L. monocytogenes* may be associated with the presence of a plasmid or determined by genes that are transferred by conjugation (Harakeh et al., 2009).

Listeria isolates from foods were resistant to vancomycin, trimethoprim/sulfamethoxazole, erythromycin, tetracycline, gentamycin, ampicillin and penicillin in some studies (Harakeh et al., 2009; Marian et al., 2012; Soni et al., 2013; Cetinkaya et al., 2014; Wang et al., 2015).

1.3. The Other *Listeria* Species

L. grayi, L. innocua, L. ivanovii, L. seeligeri and *L. welshimeri* are widely distributed in nature, found in soil, mud, sewage, vegetation and in the feces of animals and man. *Listeria grayi* (Two subspecies *Listeria grayi* subsp. *grayi* and *Listeria grayi* subsp. *murrayi*) *L. innocua*, *L. seeligeri* and *L. welshimeri* are not pathogenic to animals or man. *L. ivanovii* which has two subspecies, *Listeria ivanovii* subsp. *ivanovii* and *Listeria ivanovii* subsp. *londoniensis* is pathogenic for animals. (Ludwig et al., 2009).

1.4. Food Association of *Listeria* Species

Food animals are the most important reservoirs for many of the foodborne pathogens. Foodborne listeriosis in humans is mainly sporadic; however, outbreaks have been reported from the consumption of contaminated coleslaw, pasteurized milk, raw milk and dairy products, soft cheeses (Mexican-style, Brie, and Liederkranz), meat pâté, turkey franks, cold cut meats, improperly cooked chicken, smoked mussels. Sporadic listeriosis was also documented from the consumption of these foods either at home or at delicatessen counters. Heat-treated foods were either not properly heated or were contaminated following heating. As many raw foods of both animal and plant origin harbor *L. monocytogenes*, consumption of raw foods or recontaminated heat-processed foods has caused listeriosis. Growth during long refrigerated storage and temperature abuse before eating has been implicated in many cases. Individuals suffering most from listeriosis and fatality from the disease were from the sensitive groups (Ray, 2004; Bhunia, 2008).

A wide variety of meats are contaminated with *L. monocytogenes*, with the incidence of contamination varying greatly. This variation is partly due to differences in methods of detection including such factors as the method used, the sample size, the number of single-colony isolates taken to confirm the presence of hemolytic colonies, and the source from which the samples were purchased (retail or commercial outlets). Most of the observed contamination is on the surface (Farber and Peterkin, 1991).

Following boning, chilled raw meat and ground meat contain microorganisms coming from the carcasses as well as from different equipment used during processing, personnel, air, and water. Some of the equipment used can be important sources of microorganisms, such as conveyors, grinders, slicers, and similar

equipments that can be difficult to clean. However, because the meats are stored at low temperature (-1 to 5ºC), the psychrotrophs constitute major problems. The microbial load of fresh meat varies greatly, with bacteria predominating (Ray, 2004).

Growth of *L. monocytogenes* in meat and poultry products depends on the pH and type of the meat and amount of the background microflora (Farber and Peterkin, 1991). Poultry supports the growth of *L. monocytogenes* better than other meats; roast beef and summer sausage support the least growth. Contamination of animal muscle tissue may occur either from symptomatic or asymptomatic carriage of *L. monocytogenes* by the food animal before slaughter or contamination of the carcass after slaughter. Because *L. monocytogenes* tends to concentrate and multiply in the kidney, mesenteric and mammary lymph nodes, and liver and spleen of infected animals, eating organ meat may be more hazardous than eating muscle tissue. Regardless of the route of contamination of meat, *L. monocytogenes* attaches strongly to the surface of raw meats and is difficult to remove or inactivate (Swaminathan, 2001).

There have been almost no reports of outbreaks of foodborne listeriosis associated with meat consumption. *Listeria* spp. has been isolated from poultry, red meat and meat products in many countries around the world such as Iran (Fallah et al., 2012), Malaysia (Marian et al., 2012), and USA (Shen et al., 2013), although these foods have not been associated with documented outbreaks of human listeriosis. However, presence of *Listeria* spp. in meats is an important risk for consumers because of the unique ability to survive and multiply at refrigeration temperatures. It may increase the hazard of *Listeria* infection from contaminated foods (Fallah et al., 2012). On the other hand, during further transformation processes of raw meat into meat products *L. monocytogenes* can be introduced,

where the amount depends on the extent of cross-contamination, personal and general hygienic measures and the process parameters (Yücel et al., 2005).

Jamali et al. (2013) reported that *Listeria* spp. was isolated in 83 (18.6%) of the 446 raw milk samples. *Listeria* spp. was detected in 33.3% (102 samples) of the 306 raw vegetable samples (Ponniah et al. 2010). In Turkey, 142 homemade white cheeses were studied to find *Listeria* spp. Out of 142 samples, 47 (33.1%) were positive for *Listeria* spp. (Arslan and Özdemir, 2008). In Japan, 12 (13%) of 95 ready-to-eat fish products were positive for *L. monocytogenes* (Nakamura et al., 2004).

1.4.1. Ground Beef

Ground beef is made from meat, trimmings and fat and has a large surface area that favors growth of aerobic bacteria. The knives of meat grinder, if not properly sanitized or washed, may be the source of contamination. Furthermore, one heavily contaminated piece of meat may be sufficient to contaminate an entire lot of ground meat. Pathogens including *L. monocytogenes* are associated with these types of products (Bhunia, 2008).

L. monocytogenes was isolated most frequently from raw meats especially in minced meat. Because surfaces of machines and other surfaces of processing facilities contaminated easily with it (Kramarenko et al., 2013; Modzelewska-Kapitula and Maj-Sobotka, 2014).

Kramarenko et al. (2013) examined 21,574 food samples including minced meat. This pathogen was most frequently isolated from raw meat samples especially from meat cuts (39.0%) and minced meat (37.5%). Marian et al. (2012) examined 140 raw and ready-to-eat samples. Twenty-three of them carried *L. monocytogenes.*

1.4.2. Chicken Meat

Poultry can become contaminated with *Listeria* spp. either environmentally or from healthy carrier birds during production in the processing plant (Osaili et al., 2011). In poultry abattoir and processing plant, improper cleaning and disinfecting of environment and equipments; and also mishandling of the products may lead to *Listeria* contamination of poultry carcasses and the final products (Fallah et al., 2012).

Raw poultry is a well-recognized source of *L. monocytogenes*, and many surveys have confirmed the presence of this pathogen on fresh poultry (Gonzáles-Fandos and Herrera, 2013). Chicken also seems to be heavily contaminated with *L. monocytogenes* (Farber and Peterkin, 1991).

Capita et al. (2001) examined 100 poultry carcasses to find occurrence of *Listeria* spp. Out of 100 samples, 95 (95%) were positive for *Listeria* spp. In another study, 280 raw and ready-to-eat chicken samples were studied. Seventy-six and 65 *Listeria* spp. were isolated from raw broiler chicken and ready-to-eat chicken products, respectively (Osaili et al., 2011).

2. MATERIALS AND METHODS

2.1. Sample Collection

Between July 2013 and March 2014, samples of ground beef and chicken meat were collected from butchers and supermarkets in Bolu. A total of 124 samples were examined; 62 of them were ground beef, 62 of them were chicken meat. The samples were collected and transported to the laboratory under aseptic and refrigerated conditions and analyzed within 1 h of sampling.

2.2. Isolation and Identification of *Listeria* spp.

In this study, ground beef and chicken meat samples were analyzed to identify the *Listeria* species. *Listeria* spp. was isolated using the ISO 11290-1: 1996/Amd 1:2004 method (ISO, 2004). A 25 g portion from each sample was introduced aseptically into a sterile stomacher bag containing 225 mL of Half-Fraser Broth (Merck, Darmstadt, Germany) and homogenized by stomacher (Bagmixer 400, Interscience, Paris, France) followed by incubation for 24 h at 30ºC. Then, 0.1 mL sample from each Half-Fraser Broth culture was added to 10 mL of Fraser Broth (Merck). In addition to this, a loopful of the Half-Fraser Broth culture was streaked on the surface of PALCAM agar (Merck) and incubated for 48 h at 37ºC. After 24 h at 37ºC, a loopful of the Fraser Broth culture was taken and streaked to PALCAM agar and incubated for 48 h at 37ºC. At least five typical suspected colonies were selected and streaked to Tryptone Soya Agar (Oxoid, Basingstoke, UK).

All of the isolates were subjected to Gram staining, oxidase, catalase testing, SIM motility test at 25ºC and 37ºC, esculin hydrolysis, soluble starch test, carbohydrate utilization; such as mannitol, sucrose, rhamnose, xylose, ribose and methyl α-D-glucososide, hemolysis and CAMP test. The isolates were identified according to Bergey's Manual of Systematic Bacteriology, in which data obtained from Seeliger Jones (1986), Kämpfer et al. (1991) and Rocourt and Catimel (1985) (Ludwig et al., 2009). *L. monocytogenes* RSKK 475 was used as a control strain in biochemical tests for identification and *S. aureus* ATCC 25923 were used in CAMP test. Key biochemical tests for identification of *Listeria* spp. isolated in this study are shown in Table 1.

Table 1. Biochemical tests of *Listeria* spp. identified in this study[a].

Biochemical tests	*L. monocytogenes*	*L. innocua*	*L. ivanovii* subsp. *ivanovii*	*L. seeligeri*	*L. welshimeri*	*L. grayi*
Beta-hemolysis	+	-	+	+	-	-
CAMP test with *S. aureus*	+	-	-	+	-	-
D-Mannitol, acid	-	-	-	-	-	+
Methyl α-D-glucososide, acid	+	+	+	+	+	+
L-Rhamnose, acid	+	d	-	-	d	+
Ribose, acid	-	-	+[b]	-	-	+
Sucrose, acid	+	+	+	+	+	-
Soluble starch	-	-	-	d	+	-
D-Xylose, acid	-	-	+	+	+	-

[a]Key tests for the phenotypic differentiation of *Listeria* spp.; d, different strains give different reactions (16-84% positive).
[b]No acid produced by *Listeria ivanovii* subsp. *londoniensis.*

2.3. Molecular Analyses

In this research, the following molecular analyses were performed: DNA extraction of the 33 *L. monocytogenes* strains were obtained for molecular analyses. All *L. monocytogenes* strains were examined to presence of *hlyA, inlB* and *actA* virulence genes. Phenotypically identified *L. monocytogenes* strains were confirmed with genotypic method by determination of presence of *hlyA* gene. Besides, serotyping of all *L. monocytogenes* strains was studied. The selected and used primer pairs, primer sequences, and their product size of virulence genes and serovars are given in Table 2. In molecular analyses, *L. monocytogenes* RSKK 475 was used a positive control for amplification of *hlyA, actA, inlB* virulence genes and serovar primers ORF2819 and ORF2110. *L. monocytogenes* LM6 and LM32 from our culture collection were used as control strains for serovar "1/2c, 3c" (in the amplification of both *lmo0737, lmo1118*) and serovar "1/2a, 3a" (the amplification of *lmo0737*), respectively.

2.3.1. DNA Extraction

In this research, genomic DNA of the 33 *L. monocytogenes* strains was extracted. All *L. monocytogenes* strains were grown on Tryptic Soy Agar (Oxoid) for 24 h at 37ºC. Then, one colony of the culture was inoculated into 5 mL Brain Heart Infusion broth (Merck) and it was incubated at 37ºC for 18 h. DNA extraction was carried out using the method of Ausubel et al. (1991). 1 µL of DNA was used as a template for each 50 µL PCR reaction. The DNA extraction was stored at -20ºC.

2.3.2. Genotypic Virulence Characteristics of *L. monocytogenes*

In this research, presence of *hlyA, inlB* and *actA* virulence genes were tested for the 33 *L. monocytogenes* strains. The primer pair LM1-LM2 of *hlyA* gene was used also to identify *L. monocytogenes* (Border et al., 1990) (Table 2). All

amplifications were performed on a XP Thermal Cycler (Bioer Technology Co., Ltd., Japan). Reactions were carried out in a final volume of 50 µL, 5 µL of 10 X PCR buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, 0.8% (v/v) nonidet P40; Fermentas, Life Sciences, Canada), 2 µL of 25 mM $MgCl_2$ (), 1 µL of 10 mM dNTP mix (Fermentas, Life Sciences, Canada), 0.75 µL 10 µM each of the primers (Biomers, Ulm, Germany), 1 µL of 50 ng/µL template DNA and 0.25 µL of 5 U/µL Taq DNA polymerase (Fermentas, Life Sciences, Canada). The thermal cycling conditions of *hlyA* gene were: initial denaturation (94 ºC for 5 min), 30 cycles of denaturation (94 ºC for 30 s), annealing (50 ºC for 45 s), extention (72 ºC for 1 min 30 s) followed by final extension (72 ºC for 5 min). After PCR, 5 µL of PCR product was analyzed by using electrophoresis in a 1.5% agarose gel. The expected size of a fragment is 702 bp. They were visualized with UV transillumination (DNR Minilumi Bio-imaging Systems Ltd., Mahale HaHamisha, Jerusalem, Israel).

The amplification conditions of *actA* gene were as follows: an initial denaturation of 94 ºC for 2 min 30 s, followed by 40 cycles of 94 ºC for 3 min, 53 ºC for 1 min, 72 ºC for 2 min, with a final extension at 72 ºC for 5 min (Cai et al., 2002). The amplified PCR products were examined by electrophoreses in a 1.5% agarose gel and visualized with UV transilluminator (Table 2).

The presence of *inlB* in *L. monocytogenes* was investigated according to Pangallo et al. (2001) (Table 2). The thermal cycling conditions were as follows: 1 cycle of denaturation at 94 ºC for 2 min; 35 cycles of melting at 94 ºC for 45 s; annealing at 60 ºC for 45 s, and elongation at 72 ºC for 1 min 30 s; and a final extension at 72 ºC for 8 min. A total of 3µL of gel loading dye (Fermentas, Life Science, Canada) was added to 5 µL of each PCR product, and 8 µL of the obtained mixture were analyzed in a 1.5% agarose gel (Merck) using Tris-borate-EDTA

(TBE) (1X) supplemented with ethidium bromide (Applichem), then electrophoresis was carried out for 45-50 min at 80 V. They were visualized with UV transillumination.

2.3.3. Serotyping of *L. monocytogenes* Using PCR

Four major serovars of *L. monocytogenes* including "1/2a, 3a", "1/2b, 3b", "1/2c, 3c" and "4b, 4d, 4e" were examined using primers of *lmo0737*, *lmo1118*, ORF2819, and ORF2110 according to Doumith et al. (2004). Serovar "1/2a, 3a" shows a 691 bp fragment with the primers *lmo0737*. Serovar "1/2c, 3c" displays two fragments, 691 bp and 906 bp when amplified with the primers *lmo0737* and *lmo1118*, respectively. Serovar "1/2b, 3b, 7" shows a 471 bp fragment with the primer ORF2819. Serovar "4b, 4d, 4e" displays two fragments with the primers ORF2819 (471 bp) and ORF2110 (597 bp) (Table 2).

PCR was performed in a final volume of 50 µL containing 5 µL of 10 X PCR buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, 0.8% (v/v) nonidet P40; Fermentas, Life Sciences, Canada), 2 µL of 25 mM $MgCl_2$ (Fermentas, Life Sciences, Canada), 1 µL of 10 mM dNTP mix (Fermentas, Life Sciences, Canada), 0.75 µL 10 µM each of the primers (Biomers, Ulm, Germany), 1 µL of 50 ng/µL template DNA and 0.25 µL of 5 U/µL Taq DNA polymerase (Fermentas, Life Sciences, Canada). Cycling parameters are: an initial denaturation at 94 ºC for 5 min, followed by 35 cycles of 94 ºC for 30 s, 54 ºC for 1 min 15 s, 72 ºC for 1 min 15 s and final extension at 72 ºC for 10 min. The PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized under UV light.

Table 2. Primers used for identification of *L. monocytogenes* and detection of its virulence genes as well as serovars.

Target gene	Specificity	Primer pair	Primer sequence (5'→3')	Product size (bp)	Reference
hlyA	Hemolysin			702	Border et al., 1990
		LM1	CCT AAG ACG CCA ATC GAA		
		LM2	AAG CGC TTG CAA CTG CTC		
actA	Actin-based motility			827	Cai et al., 2002
		01	GCT GAT TTA AGA GAT AGA GGA ACA		
		02	TTT ATG TGG TTA TTT GCT GTC		
inlB	Internalin			343	Pangallo et al., 2001
		inlB-L	CTG GAA AGT TTG TAT TTG GGA AA		
		inlB-R	TTT CAT AAT CGC CAT CAT CAC T		
lmo0737	Serovars 1/2a,1/2c, 3a, and 3c			691	Doumith et al., 2004
		Forward	AGG GCT TCA AGG ACT TAC CC		
		Reverse	ACG ATT TCT GCT TGC CAT TC		
lmo1118	Serovars 1/2c and 3c			906	Doumith et al., 2004
		Forward	AGG GGT CTT AAA TCC TGG AA		
		Reverse	CGG CTT GTT CGG CAT ACT TA		
ORF2819	Serovars 1/2b, 3b, 4b, 4d, and 4e			471	Doumith et al., 2004
		Forward	AGC AAA ATG CCA AAA TCT GT		
		Reverse	CAT CAC TAA AGC CTC CCA TTG		
ORF2110	Serovars 4b, 4d, and 4e			597	Doumith et al., 2004
		Forward	AGT GGA CAA TTG ATT GGT GAA		
		Reverse	CAT CCA TCC CTT ACT TTG GAC		

2.4. Antimicrobial Susceptibility Test

Antimicrobial susceptibility testing of 128 *Listeria* strains were performed by the Kirby-Bauer et al. (1966) disk diffusion method using Mueller Hinton Agar (Oxoid), according to the Clinical and Laboratory Standards Institute (CLSI). Eighteen antimicrobial agents were chosen for the study. They belonged to the following groups: penicillins (penicillin – 10 units; ampicillin – 10 μg), beta-lactamase inhibitors (amoxicillin-clavulanic acid – 30 μg), cephalosporins (ceftriaxone – 30 μg; cephalothin – 30 μg), glycopeptides (vancomycin – 30 μg), aminoglycosides (gentamicin – 10 μg; amikacin – 30 μg; streptomycin – 10 μg), macrolides (erythromycin – 15 μg), tetracyclines (tetracycline – 30 μg), fluoroquinolones (ciprofloxacin – 5 μg), lincosamides (clindamycin – 2 μg), folate pathway inhibitors (trimethoprim/sulfamethoxazole – 25 μg), phenicols (chloramphenicol – 30 μg), streptogramins (quinupristin-dalfopristin – 5 μg), oxazolidinones (linezolid – 30 μg), and steroid (fusidic acid - 10μg).

The turbidity of each bacterial suspension in Mueller-Hinton broth (Oxoid) was adjusted to 0.5 McFarland, and then the bacterial suspensions were spread with a sterile cotton swab confluently over the entire surface of Mueller-Hinton agar. The antimicrobial agent disks were placed on the agar sufficiently separated. The plates were incubated for 24 h at 37ºC. After incubation, the diameter of inhibition zones was measured. The result of all antimicrobial agents was interpreted according to *Staphylococcus* table in CLSI 2006 except the interpretation of streptomycin based on *Enterobacteriaceae* table in CLSI 2006. The result of fusidic acid was recorded according to *Staphylococcus* table in CLSI 2009.

2.5. Culture Media Used in the Study

FRASER Listeria Selective Enrichment Broth (base)…….(Merck 110398)

Proteose peptone, 5 g/L

Peptone from casein, 5 g/L

Yeast extract, 5 g/L

Meat extract, 5 g/L

Sodium chloride, 20 g/L

Di-Sodium hydrogen phosphate, 9.6 g/L

Potassium dihydrogenphosphate, 1.35 g/L

Esculin, 1 g/L

Lithium chloride, 3 g/L

PALCAM Listeria-Selective Agar (base)……(Merck 111755)

Peptone, 23 g/L

Yeast extract, 3 g/L

Starch, 1 g/L

Sodium chloride, 5 g/L

Agar-agar, 13 g/L

D-mannitol, 10 g/L

Ammonium iron (III) citrate, 0.5 g/L

Aesculin, 0.8 g/L

Dextrose, 0.5 g/L

Lithium chloride, 15 g/L

Phenol red, 0.08 g/L

FRASER Listeria Selective Supplement…….....(Merck 100093)

FRASER Listeria Ammonium Iron (III) Supplement……(Merck 100092)

PALCAM Listeria Selective Supplement…….(Merck 112122)

SIM Medium…….....(Merck 105470)

Peptone from casein, 20 g/L

Peptone from meat, 6.6 g/L

Ammonium iron (III) citrate, 0.2 g/L

Sodium thiosulfate, 0.2 g/L

Agar-agar, 3g/L

Bile Aesculin Agar…….(Oxoid CM0888)

Peptone, 8 g/L

Bile salts, 20 g/L

Ferric citrate, 0.5 g/L

Aesculin, 1 g/L

Agar-agar, 15 g/L

Phenol-Red Broth (base)…….....(Merck 110987)

Peptone from casein, 5 g/L

Peptone from meat, 5 g/L

Sodium chloride, 5 g/L

Phenol red, 0.018 g/L

Tryptone Soya Agar…….....(Oxoid CM0131)

Pancreatic digest of casein, 15 g/L

Enzymatic (contains Papain) digest of soya bean, 5 g/L

Sodium chloride, 5 g/L

Agar, 15 g/L

Brain Heart Broth…….(Oxoid CM1135)

Brain infusion solids, 12.5 g/L

Beef heart infusion solids, 5 g/L

Proteose peptone, 10 g/L

Glucose, 2 g/L

Sodium chloride, 5 g/L

Di-sodium phosphate 2.5 g/L

Mueller-Hinton Broth……...(Oxoid CM0405)

Beef, dehydrated infusion, 300 g/L

Casein hydrolysate, 17.5 g/L

Starch, 1.5 g/L

Mueller-Hinton Agar……(Oxoid CM0337)

Beef, dehydrated infusion, 300 g/L

Casein hydrolysate, 17.5 g/L

Starch, 1.5 g/L

Agar-agar, 17 g/L

5% Sheep Blood Agar…….(Salubris MP 325)

2.6. Chemicals, Reagents and Dyes Used in the Study

Crystal violet…….(Merck 115940)

Potassium iodide…….....(BDH 26850)

Iodine.....(Merck 104741)

Fuchsin……..(Merck 115937)

Immersion oil……(Pancreac 251002)

Hydrogen peroxide 30 %.....(Merck 107209)

D (+) Glucose-monohydrate…….(Merck 108342)

D (-) Mannitol……...(Merck 105982)

Sucrose…….....(Merck 107651)

L (+) Rhamnose-monohydrate……..(Merck 104736)

D (+) Xylose…….....(Fluka 95730)

D (-) Ribose……(Applichem A2219)

Methyl α-D-Glucopyranoside……(Sigma M9376)

Starch ……...(Merck 101252)

Lysozyme from chicken egg white ……(Sigma L6876)

Sodium dodecyl sulfate (SDS)……(Fluka 71729)

Proteinase K……(Thermo scientific E00491)

NaCl……..(Merck 106404)

Cetyl Trimethyl Ammonium Biomide (CTAB)…….(Sigma H5882)

Chloroform……(Merck 102431)

Isoamyl alcohol……(Fluka 59090)

Phenol solution……...(Sigma P4557)

Isopropanol……...(Birpa 603001006)

Ethanol……..(Merck 100983)

Tris (hydroxyl-methyl) aminomethane…….(Merck 108387)

Boric acid……..(Merck 100165)

EDTA……(BDH 280214S)

Sodium hydroxide…….(Merck 106495)

Ethidium bromide……...(Applichem A1151)

Glycerol……(Merck 104092)

Bromophenol blue……(BDH 200152E)

Agarose……..(Merck 116801)

2.7. Antimicrobial Agents Used in the Study

Ampicillin (10 µg)…….(Oxoid CT0003B)

Amoxicillin/Clavulanic Acid (30 µg)……....(Oxoid CT0223B)

Sulphamethoxazole/Trimethoprim (25 µg)……...(Oxoid CT0052B)

Chloramphenicol (30 µg)……..(Oxoid CT0013B)

Tetracycline (30 µg)……..(Oxoid CT0054B)

Gentamicin (10 µg)……...(Oxoid CT0024B)

Amikacin (30 µg)……..(Oxoid CT0107B)

Ciprofloxacin (5 µg)……..(Oxoid CT0425B)

Cephalotin (30 µg)…….(Oxoid CT0010B)

Ceftriaxone (30 µg)……...(Oxoid CT0417B)

Vancomycin (30 µg)……..(Oxoid CT0058B)

Erythromycin (15 µg)……(Oxoid CT0020B)

Clindamycin (2 µg)……...(Oxoid CT0064B)

Linezolid (30 µg)…….....(Oxoid CT1650B)

Fusidic Acid (10 µg)……..(Oxoid CT0023B)

Streptomycin (10 µg)…….(Oxoid CT0047B)

Quinupristin/Dalfopristin (15 µg)…….(Oxoid CT1644B)

Penicillin G (10 U)……(Oxoid CT0043B)

3. RESULTS

3.1. Prevalence and Identification of *Listeria* spp. in Ground Beef and Chicken Meat

In this research, a total of 124 meat samples; 62 ground beef and 62 chicken meats were tested for the presence of *Listeria* spp. Out of the 124 food samples, 101 (81.5%) were contaminated with *Listeria* spp. The positive samples of *Listeria* spp. was found higher in ground beef than in chicken meat. The prevalence rates of *Listeria* spp. were 96.8% (60) in the 62 ground beef and 66.1% (41) in the 62 chicken meat samples. Overall, out of 124 samples, 44 (35.5%) isolates were phenotypically identified as *L. innocua*, 33 (26.6%) as *L. monocytogenes*, 19 (15.3%) as *L. seeligeri*, 16 (12.9%) as *L. grayi*, 11 (8.9%) as *L. welshimeri*, 4 (3.2%) as *L. ivanovii* subsp. *londoniensis*, and 1 (0.8%) as *L. ivanovii* subsp. *ivanovii*. In our study, out of 101 (81.5%) samples contaminated with *Listeria* spp., 128 isolates were obtained since at least one or more species were identified from each sample. The prevalence of *Listeria* spp. among ground beef and chicken meat is given in Table 3.

Table 3. The prevalence of *Listeria* spp. among ground beef and chicken meat.

Food type	Analyzed samples	*L.innocua*	*L. monocytogenes*	*L. seeligeri*	*L. grayi*	*L. welshimeri*	*L. ivanovii* subsp. *londoniensis*	*L. ivanovii* subsp. *ivanovii*	Number of strains[c]
Ground beef	62	27[a] (43.5)[b]	26 (41.9)	14 (22.6)	6 (9.7)	2 (3.2)	4 (6.5)	1 (1.6)	80
Chicken meat	62	17 (27.4)	7 (11.3)	5 (8.1)	10 (16.1)	9 (14.5)	0 (0)	0 (0)	48
Total	124	44 (35.5)	33 (26.1)	19 (15.3)	16 (12.9)	11 (17.4)	4 (3.2)	1 (0.8)	128

[a]number of positive strains

[b]percentage of positive strains

[c] At least one or more strains were isolated from one positive sample.

In the 60 *Listeria* positive ground beef samples, the most frequent isolated species was *L. innocua* 27 (45%). The following strains were: *L. monocytogenes* 26 (43.3%), *L. seeligeri* 14 (23.3%), *L. grayi* 6 (10%), *L. ivanovii* subsp. *londoniensis* 4 (6.7%), *L. welshimeri* 2 (3.3%), *L. ivanovii* subsp. *ivanovii* 1 (1.7%). *L. ivanovii* was found only in ground beef. Distribution of *Listeria* species in the 60 positive ground beef samples is shown in Figure 1.

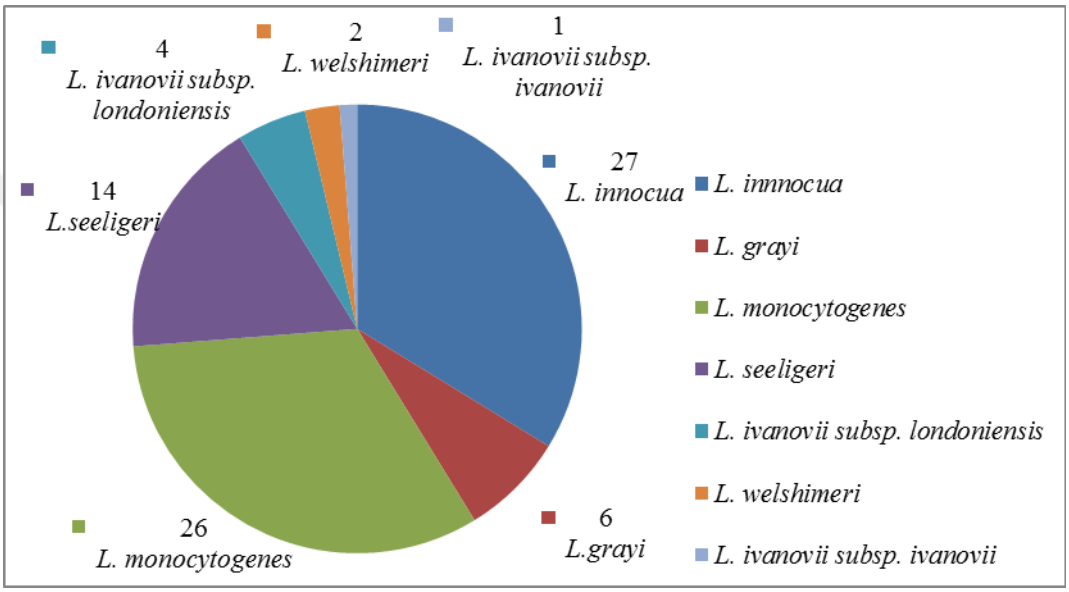

Figure 1. Distribution of *Listeria* species in the 60 ground beef samples.

In the 41 *Listeria* positive chicken meat samples, the most prevalent species was also *L. innocua* 17 (41.5%). The following species were: *L. grayi* 10 (24.4%), *L. welshimeri* 9 (22%), *L. monocytogenes* 7 (17.1%), and *L. seeligeri* 5 (12.2%). The number of *L. welshimeri* was significantly higher in chicken meat than in ground beef. Distribution of *Listeria* species in the 41 positive chicken meat samples has been summarized in Figure 2.

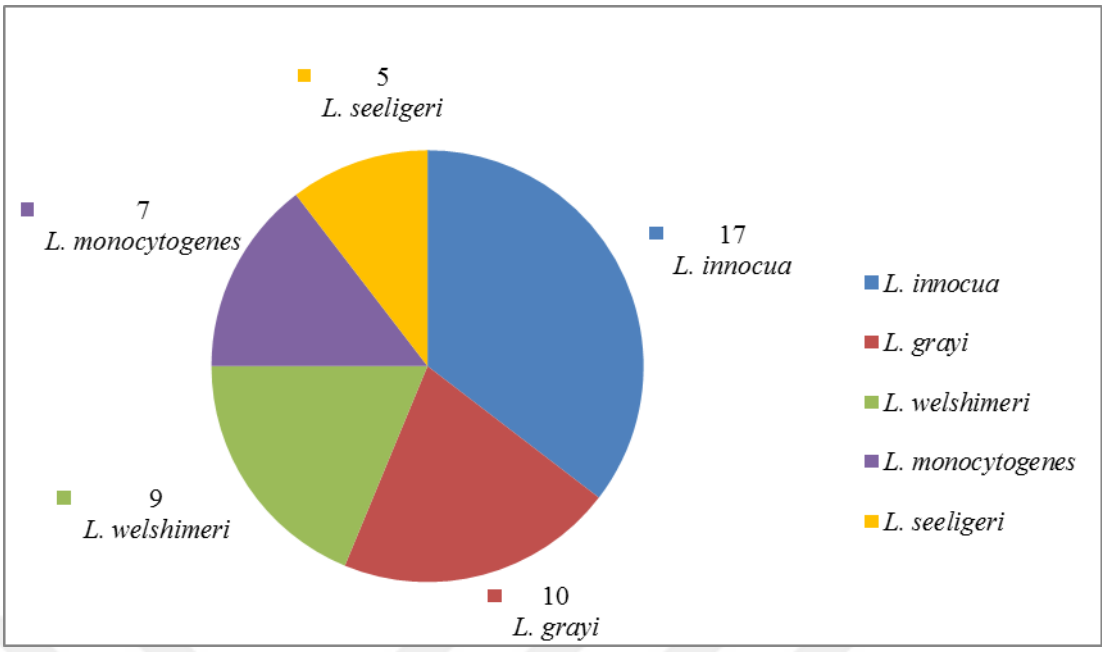

Figure 2. Distribution of *Listeria* species in the 41 chicken meat samples.

It is important that co-contamination with different species of *Listeria* was detected in analyzed samples. *L. monocytogenes* plus *L. innocua* was the most frequent combination. Eleven ground beef and 5 chicken meat samples were contaminated with *L. monocytogenes* plus *L. innocua*. Distribution of all *Listeria* spp. with combinations in 101 positive ground beef and chicken meat samples is shown in Table 4.

Table 4. Distribution of *Listeria* spp. with combinations in 101 positive meat samples.

Listeria species	Ground beef	Chicken meat	No. of positive samples (%)
L. innocua	12	12	24 (23.8)
L. monocytogenes and *L. innocua*	11	5	16 (15.8)
L. monocytogenes	12	2	14 (13.9)
L. seeligeri	10	3	13 (12.9)
L. grayi	3	9	12 (11.9)
L. welshimeri	0	8	8 (7.9)
L. ivanovii subsp. *londoniensis*	3	0	3 (3)
L. monocytogenes and *L. grayi*	2	0	2 (2)
L. innocua and *L. seeligeri*	2	0	2 (2)
L. seeligeri and *L. grayi*	1	1	2 (2)
L. monocytogenes and *L. welshimeri*	1	0	1 (1)
L. innocua and *L. welshimeri*	1	0	1 (1)
L. innocua and *L. ivanovii* subsp. *ivanovii*	1	0	1 (1)
L. seeligeri and *L. ivanovii* subsp. *londoniensis*	1	0	1 (1)
L. seeligeri and *L. welshimeri*	0	1	1 (1)
Total	60	41	101 (100)

Thirty-three (32.7%) of the 101 *Listeria* positive meat samples harbored *L. monocytogenes*. Fourteen (13.9%) of these contained *L. monocytogenes*, 19 (18.8%) *L. monocytogenes* and other *Listeria* species in combination. Sixty-eight (67.3%) of 101 positive samples were also contaminated with other *Listeria* species. Phenotypically identified the 33 *L. monocytogenes* strains were also confirmed with genotypically by using *hlyA* gene. Distribution of *L. monocytogenes* and other *Listeria* spp. either alone or with combination in the 101 positive *Listeria* meat samples is shown in Table 5.

Table 5. Distribution of *L. monocytogenes* and other *Listeria* either alone or with combination in the 101 positive *Listeria* samples among ground beef and chicken meat.

Analyzed Sample	*L. monocytogenes*	*L. monocytogenes* + Other *Listeria*	Other *Listeria*
Ground Beef (n = 60)	12[a] (20)[b]	14 (23.3)	34 (56.7)
Chicken Meat (n = 41)	2 (4.9)	5 (12.2)	34 (83)
Total (n = 101)	14 (13.9)	19 (18.8)	68 (67.3)

[a]number of positive strains

[b]percentage of positive strains

n, number of strains tested

3.2. Virulence Genes of *L. monocytogenes*

L. monocytogenes carry a basic set of virulence genes and have the potential for invading the host epithelial cells. Hemolysin, internalins and actin-based motility have significant roles in the pathogenicity of *L. monocytogenes*. In this study, genotypic virulence characteristics of *L. monocytogenes* were examined by PCR for the presence of virulence genes such as *hlyA, inlB* and *actA.* All the 33 *L. monocytogenes* strains harbored *hlyA* virulence gene encoded listeriolysin O. The presence of internalin gene, which is associated with internalization of *L. monocytogenes* into the host cells, is an important indicator for its virulence potential. In our research, out of the 33 *L. monocytogenes* strains, 29 (87.9%) were found to be positive for *inlB* gene. Strains that carried *inlB* gene were higher rates in ground beef (92.3%) than in chicken meat (71.4%). *actA* gene recruits an actin polymerization complex that promotes *L. monocytogenes* actin-based motility, cell-to-cell spread and dissemination within host tissues. We found that 16 (48.5%) of all *L. monocytogene*s strains were positive for *actA* gene. Consequently, presence of *actA* gene was 50% in ground beef and 42.9% in chicken meat. Moreover, 15 (45.5%) of *L. monocytogene*s strains from all food samples carried *hlyA*+ *actA*+ *inlB* gene combinations. Percentages of positive virulence markers of the 33 *L. monocytogenes* isolated from ground beef and chicken meat samples are presented in Table 6. Figure 3 shows the photographs of PCR products of some *L. monocytogenes* strains obtained from our study that harbored *hlyA*, *actA*, and *inlB* virulence genes.

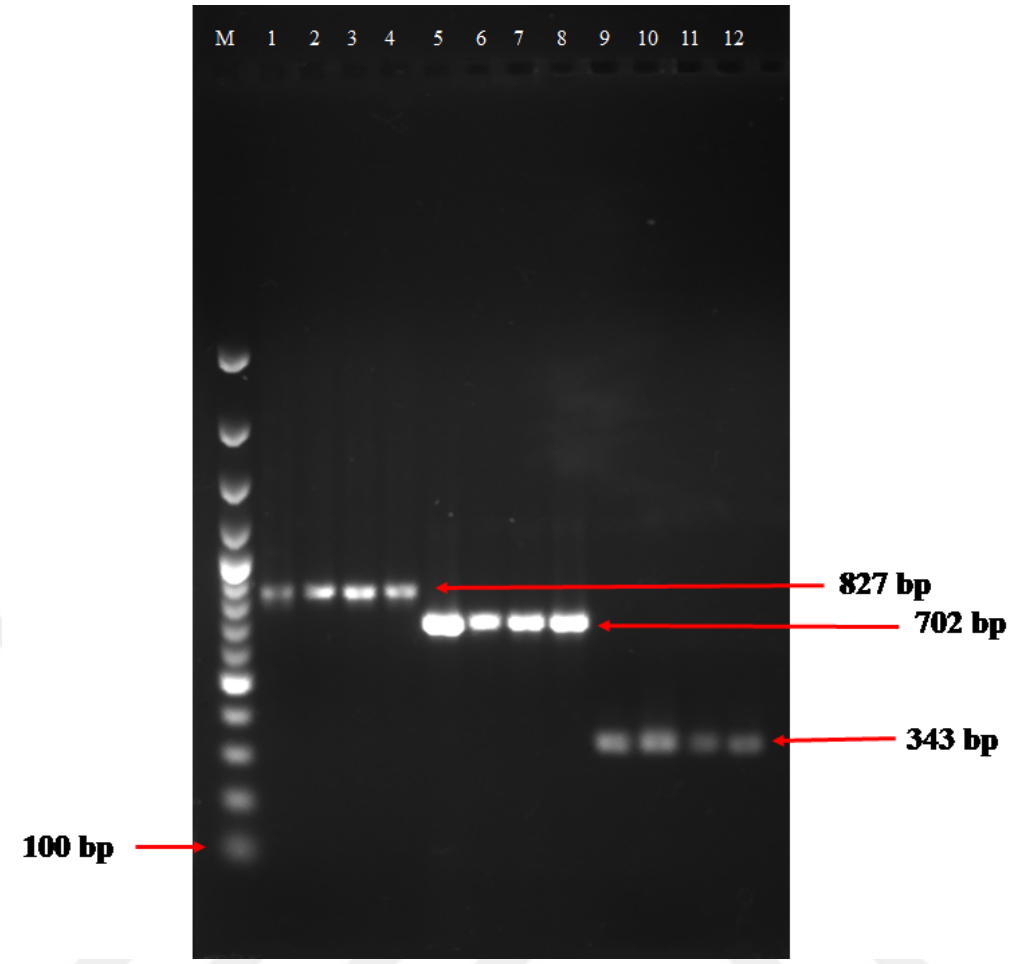

Figure 3. Amplification of *hlyA, actA,* and *inlB* virulence genes from *L. monocytogenes* strains. M: 100 bp DNA Ladder. Lane 1: *L. monocytogenes* RSKK 475, positive control for *actA* (827 bp), Lanes 2 to 4: our strains harbored *actA.* Lane 5: *L. monocytogenes* RSKK 475, positive control for *hlyA* (702 bp). Lanes 6 to 8: our *hlyA* positive strains. Lane 9: *L. monocytogenes* RSKK 475, positive control for *inlB* gene (343 bp). Lanes 10 to 12: our strains carried *inlB.*

3.3. Serovar Identification of *L. monocytogenes* Using PCR

Serotyping is important in the determination of *L. monocytogenes*' virulence and pathogenicity. Although 13 serovars of *L. monocytogenes* can cause disease, only three serovars (1/2a, 1/2b and 4b) are frequently isolated from the clinical cases of which "1/2a" is mostly isolated from food and "4b" from human epidemics.

Serotyping of 33 *L. monocytogenes* strains was performed by PCR. Out of 26 strains isolated from ground beef, 12 (46.2%) belonged to serovar "1/2a, 3a" and 14 (53.8%) belonged to serovar "1/2c, 3c". Seven *L. monocytogenes* strains (100%) were distributed into "1/2a, 3a" that were isolated from chicken meat. Frequency of serovar "1/2a, 3a" (57.6%) was higher than of serovar "1/2c, 3c" (42.4%). In addition, serovars "1/2b, 3b" and "4b, 4d, 4e", which are frequently found in the clinical cases, were not determined in this study. Serovar percentages of the 33 *L. monocytogenes* strains isolated from ground beef and chicken meat are presented in Table 6. Figure 4 shows a representative gel of DNA amplicons generated by PCR for identification of *L. monocytogenes* serovars. In addition, profiles of *L. monocytogenes* strains for virulence genes and serovars are shown in Table 7.

Table 6. Percentages of positive virulence markers and serovars of the 33 *L. monocytogenes* among ground beef and chicken meat.

	Virulence genes				Serovars	
Analyzed samples	***hlyA***	***actA***	***inlB***	***hlyA* + *actA* + *inlB***	**1/2a, 3a**	**1/2c, 3c**
Ground beef (n=26)	26[a] (100)[b]	13 (50)	24 (92.3)	13 (50)	12 (46.1)	14 (53.8)
Chicken meat (n=7)	7 (100)	3 (42.9)	5 (71.4)	2 (28.6)	7 (100)	0 (0)
Total (n=33)	33 (100)	16 (48.5)	29 (87.9)	15 (45.5)	19 (57.6)	14 (42.4)

[a]number of positive strains

[b]percentage of positive strains

n, number of strains tested

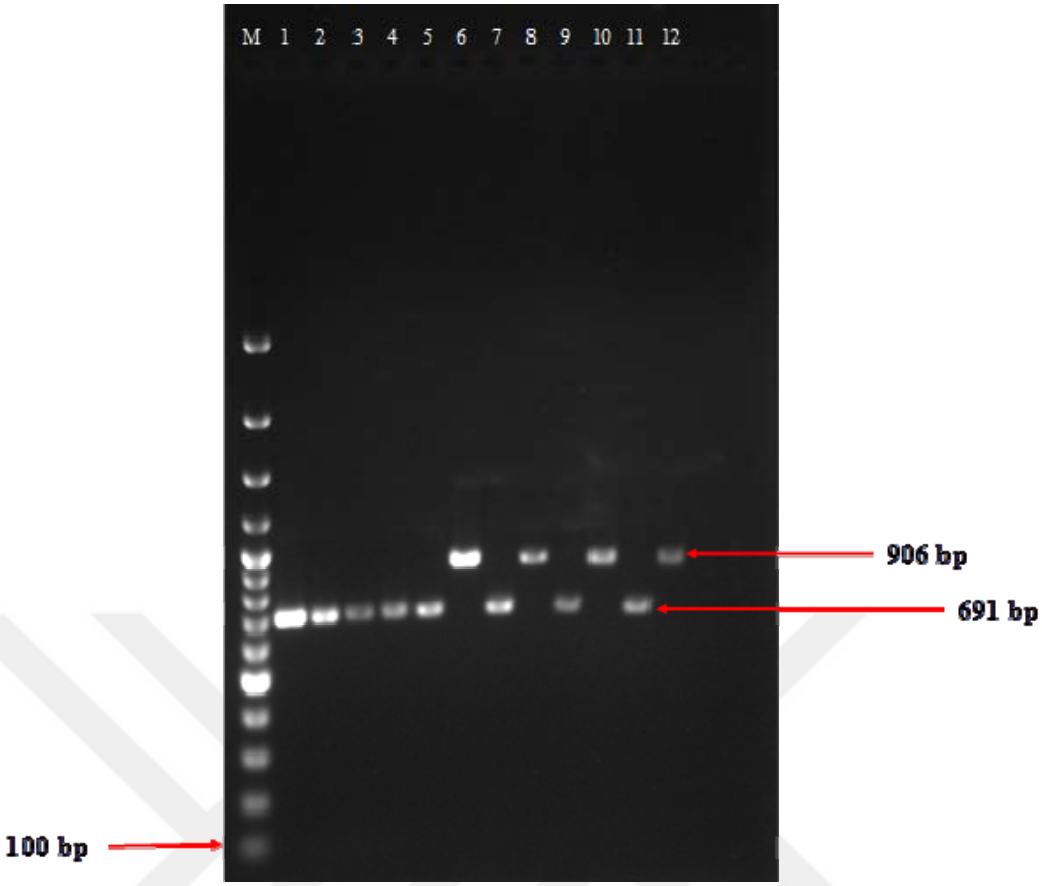

Figure 4. A representative gel of DNA amplicons generated by PCR for identification of *L. monocytogenes* serovars. Serovar "1/2a, 3a" is indicated by the amplicon of 691 bp (*lmo0737*), serovar "1/2c, 3c" by two amplicons 691 bp (*lmo0737*) and 906 bp (*lmo1118*). Lane M, 100 bp molecular size marker; Lane 1: *L. monocytogenes* LM32, positive control for serovar "1/2a, 3a"; Lanes 2 to 4: serovar "1/2a, 3a" positive strains in our study; Lanes 5 and 6: *L. monocytogenes* LM6, positive control for serovar "1/2c, 3c"; Lanes 7 to 12: serovar "1/2c, 3c" positive strains in our study.

Table 7. Profiles of *L. monocytogenes* strains for virulence genes and serovars.

No	Isolates	Sample	*hlyA*	*actA*	*inlB*	*lmo0737*	*lmo1118*	ORF2819	ORF2110	Serovars
1	K3/3	Ground beef	+	+	+	+	-	-	-	1/2a, 3a
2	K5/1H	Ground beef	+	-	-	+	-	-	-	1/2a, 3a
3	K6/1H	Ground beef	+	-	+	+	+	-	-	1/2c, 3c
4	K9/3H	Ground beef	+	+	+	+	-	-	-	1/2a, 3a
5	K11/3H	Ground beef	+	+	+	+	-	-	-	1/2a, 3a
6	K13/1	Ground beef	+	+	+	+	-	-	-	1/2a, 3a
7	K16/1H	Ground beef	+	+	+	+	+	-	-	1/2c, 3c
8	K17/2H	Ground beef	+	+	+	+	+	-	-	1/2c, 3c
9	K21/2H	Ground beef	+	+	+	+	+	-	-	1/2c, 3c
10	K24/1H	Ground beef	+	+	+	+	+	-	-	1/2c, 3c
11	K25/5	Ground beef	+	-	+	+	-	-	-	1/2a, 3a
12	K27/2H	Ground beef	+	-	+	+	-	-	-	1/2a, 3a
13	K28/1	Ground beef	+	-	+	+	+	-	-	1/2c, 3c
14	K30/2H	Ground beef	+	-	+	+	+	-	-	1/2c, 3c
15	K33/2H	Ground beef	+	-	+	+	-	-	-	1/2a, 3a
16	K37/3H	Ground beef	+	-	+	+	-	-	-	1/2a, 3a
17	K40/2H	Ground beef	+	+	+	+	+	-	-	1/2c, 3c

Table 7. Profiles of *L. monocytogenes* strains for virulence genes and serovars (continued).

18	K41/2H	Ground beef	+	+	+	+	+	-	-	1/2c, 3c
19	K43/2H	Ground beef	+	-	+	+	+	-	-	1/2c, 3c
20	K49/1H	Ground beef	+	-	+	+	+	-	-	1/2c, 3c
21	K52/2H	Ground beef	+	+	+	+	+	-	-	1/2c, 3c
22	K54/2H	Ground beef	+	+	+	+	+	-	-	1/2c, 3c
23	K56/3	Ground beef	+	-	+	+	-	-	-	1/2a, 3a
24	K57/3H	Ground beef	+	+	+	+	+	-	-	1/2c, 3c
25	K59/3H	Ground beef	+	-	-	+	-	-	-	1/2a, 3a
26	K60/3H	Ground beef	+	-	+	+	-	-	-	1/2a, 3a
27	T2/3	Chicken meat	+	-	+	+	-	-	-	1/2a, 3a
28	T11/1H	Chicken meat	+	-	+	+	-	-	-	1/2a, 3a
29	T25/3	Chicken meat	+	+	+	+	-	-	-	1/2a, 3a
30	T46/2H	Chicken meat	+	-	+	+	-	-	-	1/2a, 3a
31	T55/2H	Chicken meat	+	+	-	+	-	-	-	1/2a, 3a
32	T59/3H	Chicken meat	+	+	+	+	-	-	-	1/2a, 3a
33	T61/1H	Chicken meat	+	-	-	+	-	-	-	1/2a, 3a

3.4. Antimicrobial Susceptibility of *Listeria* Species

In this research, the antimicrobial susceptibility of the 128 *Listeria* spp. isolated from ground beef and chicken meat to 18 various antimicrobial agents was examined by the standard disk diffusion method.

Most *Listeria* strains with a rate of 90.6% were resistant to penicillin, 79.7% to ceftriaxone and 71.1% to ampicillin. All *Listeria* strains were susceptible to vancomycin. Among all *Listeria* strains, the highest susceptibility was found to amoxicillin/clavulanic acid (99.2%) followed by linezolid (97.7%), cephalothin (96.9%), chloramphenicol (96.9%), gentamicin (96.9%) quinupristin/dalfopristin (96.9%), amikacin (92.2%), tetracycline (90.6%), erythromycin (89.1%). Only one strain was susceptible to all antimicrobial agents. Antimicrobial susceptibility rates of 18 antimicrobial agents tested against 128 *Listeria* strains in ground beef and chicken meat are presented in Figure 5.

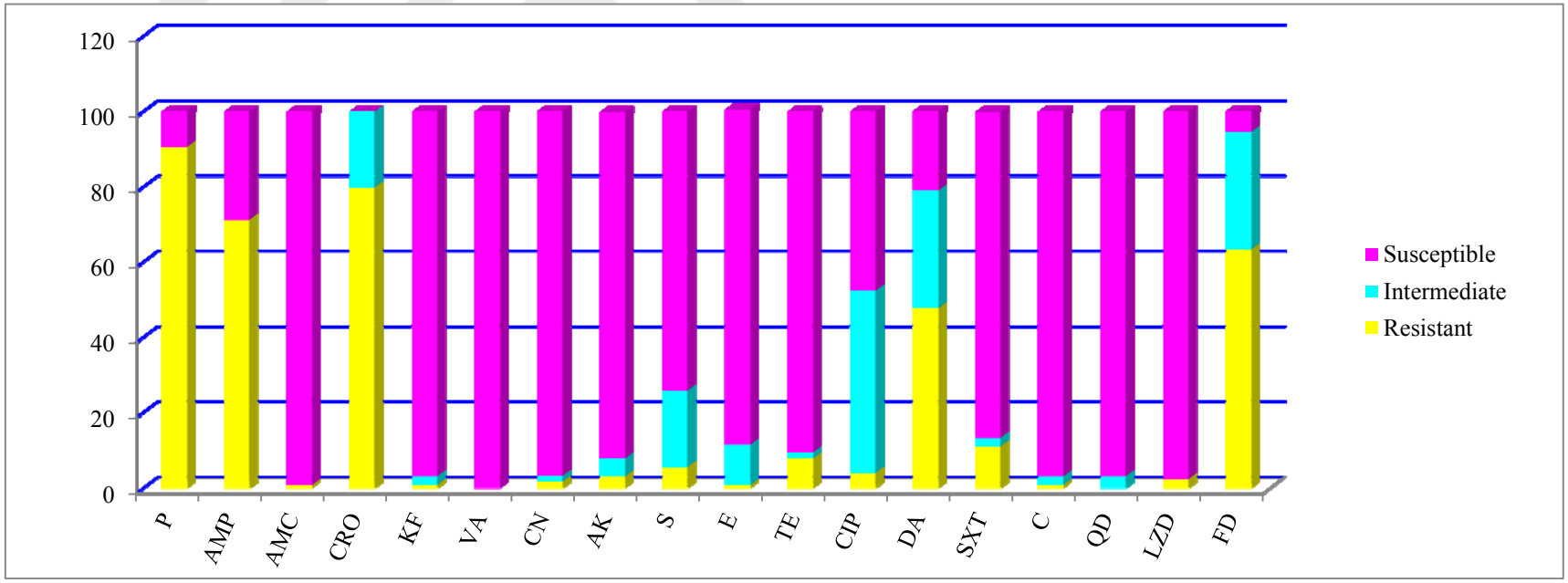

Figure 5. Antimicrobial susceptibility of 128 *Listeria* strains isolated from ground beef and chicken meat.

P, penicillin; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CRO, ceftriaxone; KF, cefalothin; VA, vancomycin; CN, gentamycin; AK, amikacin; S, streptomycin; E, erythromycin; TE, tetracycline; CIP, ciprofloxacin; DA, clindamycin; SXT, trimethoprim/sulfamethoxazole; C, cholaramphenicol; QD, quinupristin/dalfopristin; LZD, linezolid; FD, fusidic acid.

In the present study, a total of 127 (99.2%) of the 128 *Listeria* spp. strains were resistant at least one or more antimicrobial agents. Besides, 112 (87.5%) of the 128 *Listeria* strains were resistant to three or more antimicrobial agents, but 63.3% of them to four or more antimicrobial agents. Among the 128 *Listeria* strains, 1 was resistant to one antimicrobial agent, 14 to two, 31 to three, 44 to four, 27 to five, 7 to six, 2 to seven and 1 to eleven. Multiple antimicrobial resistance profiles of *Listeria* spp. and *L. monocytogenes* in ground beef and chicken meat are shown in Table 8.

Table 8. Multiple antimicrobial resistance profiles of the 128 *Listeria* spp. and the 33 *L. monocytogenes*.

Antimicrobial resistance profiles	No. of antimicrobial agents	*Listeria* spp. n = 128 (%)	*L. monocytogenes* n = 33 (%)
AMP, CRO, FD	3	1 (0.8)	0 (0)
AMP, CRO, P	3	9 (7)	5 (15.2)
AMP, FD, P	3	9 (7)	4 (12.1)
CRO, DA, FD	3	2 (1.6)	0 (0)
CRO, DA, P	3	5 (3.9)	0 (0)
CRO, FD, P	3	4 (3.1)	0 (0)
DA, FD, P	3	1 (0.8)	0 (0)
AMP, CIP, CRO, P	4	1 (0.8)	1 (3)
AMP, CRO, DA, P	4	5 (3.9)	2 (6.1)
AMP, CRO, FD, P	4	22 (17.2)	4 (12.1)
AMP, CRO, P, SXT	4	2 (1.6)	2 (6.1)
AMP, DA, FD, P	4	1 (0.8)	0 (0)
AMP, DA, P, SXT	4	1 (0.8)	1 (3)
CIP, CRO, P, SXT	4	1 (0.8)	0 (0)
CRO, DA, FD, P	4	8 (6.3)	0 (0)
CRO, DA, P, SXT	4	1 (0.8)	0 (0)
CRO, FD, P, SXT	4	2 (1.6)	0 (0)
AMP, CRO, DA, FD, P	5	20 (15.6)	0 (0)
AMP, CRO, FD, LZD, P	5	1 (0.8)	1 (3)

Table 8. Multiple antimicrobial resistance profiles of *Listeria* spp. and *L. monocytogenes* (continued).

AMP, CRO, FD, P, SXT	5	1 (0.8)	1 (3)
AMP, CRO, FD, P, TE	5	1 (0.8)	0 (0)
C, CRO, DA, P, TE	5	1 (0.8)	0 (0)
CIP, CRO, DA, P, SXT	5	1 (0.8)	0 (0)
CRO, DA, FD, P, SXT	5	1 (0.8)	0 (0)
CRO, DA, LZD, P, SXT	5	1 (0.8)	0 (0)
AK, AMP, CN, DA, P, S	6	2 (1.6)	2 (6.1)
AK, AMP, CRO, FD, S, TE	6	1 (0.8)	0 (0)
AMP, CRO, DA, FD, P, SXT	6	1 (0.8)	0 (0)
AMP, CRO, DA, FD, P, TE	6	2 (1.6)	0 (0)
AMP, CRO, DA, P, S, TE	6	1 (0.8)	0 (0)
AMC, AMP, CRO, DA, FD, P, TE	7	1 (0.8)	0 (0)
DA, KF, LZD, P, S, SXT, TE	7	1 (0.8)	0 (0)
AK, AMP, CIP, CRO, DA, E, FD, P, S, SXT, TE	11	1 (0.8)	0 (0)
Total		**112 (87.5)**	**23 (69.7)**

n, number of strains tested

The results of the research indicated a high resistance of *L. monocytogenes* to some antimicrobial agents including penicillin and ampicillin, commonly used the drugs for the treatment of listeriosis. Antimicrobial resistance profile of the 33 *L. monocytogenes* is shown in Figure 6.

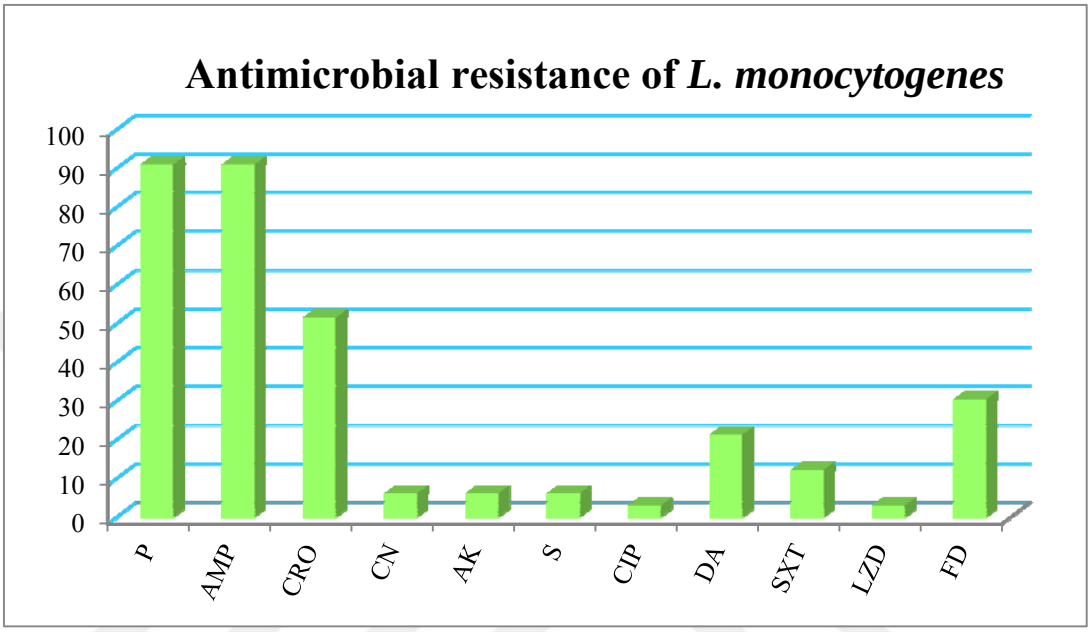

Figure 6. The percentages of antimicrobial resistance of the 33 *L. monocytogenes.* P, penicillin; AMP, ampicillin; CRO, ceftriaxone; CN, gentamicin; AK, amikacin; S, streptomycin; CIP, ciprofloxacin; DA, clindamycin; SXT, trimethoprim/sulfamethoxazole; LZD, linezolid; FD, fusidic acid.

4. DISCUSSION

Food is the primary vehicle of infection, and meat, fish, dairy products and vegetables are potential source of transmission. Among the food sources, meat and poultry is convenient for the growth of microorganisms. Genus *Listeria* is widely distributed in food and has been isolated frequently from meat and poultry (Bhunia, 2008; Meisel et al., 2014). Many studies have implicated the presence of *Listeria* species in meat and poultry products (Capita et al., 2001; Vitas and Garcia-Jalon, 2004; Yücel et al., 2005; Pesavento et al., 2010; Osaili et al., 2011; Fallah et al., 2012; Adzitey et al., 2013).

The rate of contamination with *Listeria* spp. in ground beef and chicken meat samples was found to be 81.5% in our study. Similar rate (80.9%) was reported by Yücel et al. (2005) for *Listeria* spp. in 68 ground beef and chicken meat samples in Turkey. In Iran, a total of 402 poultry product samples composed of raw, ready-to-cook and ready-to-eat products were analyzed for the presence of *Listeria* spp. Out of a total of 402 poultry product samples, 134 (33.3%) were found to be contaminated with *Listeria* spp. (Fallah et al., 2012). On the other hand, Capita et al. (2001) investigated the occurrence of *Listeria* spp. on the skin of 100 fresh chicken carcasses in Spain. *Listeria* species were found in 95% of the samples.

The contamination level of different types of food with *Listeria* species has been changed. In this study, contamination level of ground beef (96.8%) was higher than of chicken meat (61.1%). In Turkey, Yücel et al. (2005) examined raw meat samples and the prevalence of *Listeria* spp. was found to be 86.4% in minced meat, 69.0% in chicken meat and 73.6% in beef. In another study, Pesavento et al. (2010) analyzed two groups of products, classified in raw meat (beef, poultry and pork) and retail products (fresh soft cheeses, ready salads, smoked salmons, ham and sandwiches) in Italy. They reported that prevalence of *Listeria* spp. was significantly higher in raw meat (21.4%) than in retail products (3.61%).

Results of this research suggest that meats, ground beef and chicken meat, can heavily be contaminated with *Listeria* spp. In the present study, isolates were identified as: *L. innocua* (35.5%), *L. monocytogenes* (26.6%), *L. seeligeri* (15.3%), *L. grayi* (12.9), *L. welshimeri* (8.9%), *L. ivanovii* subsp. *londoniensis* (3.2%) and *L. ivanovii* subsp. *ivanovii* (0.8%) (Table 3). The reported distribution of identified species was variable in other studies. In Spain, the presence of *Listeria* spp. was investigated in a total of 3685 food samples including fresh raw products (meat, milk and poultry) and treated products (cooked and cured meats, frozen vegetables and smoked salmon). Out of 3685 samples, 307 (8.3%) were contaminated with *L. monocytogenes,* followed by *L. innocua* 479 (13.0%), *L. welshimeri* 59 (1.6%), *L. seeligeri* 11 (0.3%), *L. ivanovii* 5 (0.08) and *L. grayi* 1 (0.03%) (Vitas and Garcia-Jalon, 2004). In another research, the occurrence of *Listeria* species in poultry carcasses marketed in Spain was studied. From a total of 100 samples studied, 95 samples (95%) were contaminated with *Listeria* spp. *L. monocytogenes* was found in 32% of all samples tested, *L. innocua* in 66%, *L. welshimeri* in 7%, *L. grayi* in 4%

and *L. ivanovii* in 2%. Ten (10%) poultry carcasses yielded *Listeria* species, which could not be identified at species level (Capita et al., 2001).

Prevalence of *Listeria* species among different types of food samples varied in this research (Table 3). In the 62 ground beef, the isolated species were *L. innocua* (43.5%), *L. monocytogenes* (41.9%), *L. seeligeri* (22.6%), *L. grayi* (9.7%), *L. ivanovii* subsp. *londoniensis* (6.5%), *L. welshimeri* (3.2%), and *L. ivanovii* subsp. *ivanovii* (1.6%). In the 62 chicken meat, distribution of species was *L. innocua* (27.4%), *L. grayi* (16.1%), *L. welshimeri* (14.5%), *L. monocytogenes* (11.3%), and *L. seeligeri* (8.1%). *L. ivanovii* was isolated only from ground beef (Table 3). Osaili et al. (2011) tested 160 raw fresh broiler chickens and 120 ready-to-eat chicken product samples to obtain *Listeria* species in Jordan. They isolated 76 *Listeria* spp. including *L. ivanovii* (48), *L. monocytogenes* (15), *L. grayi* (8), *L. seeligeri* (4) and *L. welshimeri* (1) from 160 raw fresh broiler chicken. However, 65 *Listeria* spp. was identified including *L. monocytogenes* (36), *L. ivanovii* (25), *L. grayi* (2), *L. seeligeri* (1) and *L. welshimeri* (1) from 120 ready-to-eat chicken products. In another study, Pesavento et al. (2010) observed 1268 raw meat (beef, poultry and pork) and retail products (fresh soft cheeses, ready salads, smoked salmons, ham and sandwiches) to find *Listeri*a spp. In raw meat samples, *L. innocua* (43.6%), *L. monocytogenes* (23.6%), *L. welshimeri* (22.1%), *L. ivanovii* (7.14%), *L. seeligeri* (2.14%), and *L. grayi* (1.43%) were isolated. In retail product samples, *L. innocua* (53.6%), *L. monocytogenes* (25.0%), *L. welshimeri* (14.3%) and *L. grayi* (7.14%) were obtained. The differences between the results of various studies for the prevalence of *Listeria* spp. in different types of foods may be due to several factors such as isolation methods, original source of the food, environment, water, the quality of technique used to slaughter animals, the sanitary conditions under which the product was

handled or processed and the conditions for subsequent packaging, handling, storage, and distribution, temperature, season, geographical location. These factors affect the prevalence of *Listeria* spp. in food samples (Ray, 2004; Bhunia, 2008).

In our study, out of 101 (81.5%) samples contaminated with *Listeria* spp., 128 isolates were obtained since at least one or more species were identified from each sample. Our results showed that *L. monocytogenes* and other *Listeria* spp. were frequently isolated either alone or in association from ground beef and chicken meat samples. *L. monocytogenes* plus *L. innocua* was found the most frequent combination in this study (Table 4). In Spain, co-contamination with different species of *Listeria* was detected in raw products (meat, milk and poultry) and treated products (cooked and cured meats, frozen vegetables and smoked salmon). *L. monocytogenes* plus *L. innocua* was the most frequent combination (Vitas and Garcia-Jalon, 2004).

Prevalence of *L. innocua* was obviously much higher than the other species in this study. In fact *L. innocua* was detected in 43.5% of ground beef and in 27.4% of chicken meat. As other studies indicated (Capita et al., 2001; Vitas and Garcia-Jalon, 2004; Yücel et al.,2005; Yadav and Roy, 2009; Pesavento et al., 2010; Rahimi et al., 2010; Fallah et al., 2012; Jamali et al., 2013; Spanu et al., 2015), *L. innocua* was the most common species in different types of samples.

L. monocytogenes is a serious foodborne pathogen that can cause a severe human disease, listeriosis. Consumption of food contaminated with this pathogen has been reported as the main route of transmission to humans (Farber and Peterkin, 1991; Bhunia 2008). From a total of 124 ground beef and chicken meat samples examined, 33 samples (26.6%) were contaminated with *L. monocytogenes*. The prevalence rates of this study were similar to the results of other studies. Jamali et al.

(2013) reported that the frequency of *L. monocytogenes* was 21.7% in 446 raw milk samples. A total of 140 food samples included raw and ready-to-eat food samples were analyzed for the presence of *L. monocytogenes*. The percentage of *L. monocytogenes* was 16.4% (Marian et al., 2012). A total of 306 raw vegetable samples collected from the markets in Malaysia were screened for the presence of *L. monocytogenes*. Contamination with *L. monocytogenes* was detected in 22.5% of the total samples (Ponniah et al., 2010). The prevalence of this pathogen in the food samples tested was within the range (2.6-17.4%) found in previous studies (Nakamura et al., 2004; Vitas and Garcia-Jalon, 2004; Arslan and Özdemir, 2008; Filiousis et al., 2009; Yan et al., 2010; Atıl et al., 2011; Gelbíčová and Karpíšková, 2012; Sant'Ana et al., 2012; Adzitey et al., 2013; Ieren et al., 2013; Kramarenko et al., 2013; Cetinkaya et al., 2014; Shi et al., 2015). Others (Rahimi et al., 2010; Spanu et al., 2015) have reported that *L. monocytogenes* from cheese making plants (44.5%) and milk and dairy products (32.7%) was recovered in high percentages, respectively.

A number of virulence factors have an important role in the pathogenicity of *L. monocytogenes*. Listeriolysin O (LLO) encoded by *hlyA* gene is a pore-forming toxin secreted by *L. monocytogenes*. In addition, being a secreted protein, its detection can serve as an indicator of the presence of *L. monocytogenes* in a food sample (Churchill et al., 2006). The primer pair LM1-LM2 (Border et al., 1990) of *hlyA* gene was the one that only amplified the specific fragment to identify *L. monocytogenes* strains (Aznar and Elizaquível, 2008). In previous studies, these primer pairs were used to confirm *L. monocytogenes* genotypically and for its virulence identity (Conter et al., 2009; Ponniah et al., 2010; Atıl et al., 2011; Marian et al., 2012; Jamali et al., 2013). In this research, phenotypically identified *L.*

monocytogenes strains were confirmed genotypically by specific primers LM1-LM2. All *L. monocytogenes* strains (n=33) harbored this virulence gene (Table 6).

Virulence gene *inlB* promotes invasion into several cell types. In the present study, a high percentage of strains was positive for *inlB* virulence gene (87.9%) (Table 6). In Malaysia, 32 *L. monocytogenes* isolates were obtained from ready-to-eat foods. All isolates harbored *inlB* virulence gene (Jamali and Thong, 2014). In Czech Republic, 12 *L. monocytogenes* was isolated from the wild, farm environment, and vegetation. These all strains were carriers of the virulence gene *inlB* (Gelbíčová and Karpíšková, 2012).

actA gene plays a key role in intracellular movements of *L. monocytogenes*, cell-to-cell spread, and consequently bacterial dissemination into host tissues (Khelef et al., 2006). Out of 33 *L. monocytogenes* strains, 16 (48.5%) were found to be positive for *actA* gene in our research (Table 6). In previous studies, *actA* gene was also performed for virulence determinant of *L. monocytogenes.* In India, of 7 *L. monocytogenes*, 4 (57.1%) were carried *actA* virulence gene (Yadav and Roy 2009). In another studies, *actA* gene was harboured in all examined *L. monocytogenes* strains (Gelbíčová and Karpíšková, 2012; Soni et al., 2013).

L. monocytogenes was comprised of 13 serovars including 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e, and 7 that can cause disease. Serovar 4b is found in most of the invasive foodborne outbreaks worldwide and in up to 50% of the sporadic cases of listeriosis, while serovars 1/2a, 1/2b and 1/2c are mostly associated with sporadic cases (Khelef et al., 2006). A total of two different serovars were identified among ground beef and chicken meat samples (Table 6). Nineteen (57.6%) strains belonged to serovar "1/2a, 3a" which is encoded by *lmo0737* gene. In another words, all of them possessed only *lmo0737* gene as shown by the presence of the

691bp PCR amplicon. Fourteen (42.4%) strains were amplified both *lmo0737* and *lmo1118* genes so they belong to serovar "1/2c, 3c". Serovar "1/2b, 3b, 7" and "4b, 4d, 4e" were not detected in any strains in this study.

Although 13 serovars of *L. monocytogenes* have been described, only 1/2a, 1/2b and 4b account for the majority of clinical cases (Khelef et al., 2006). The result of previous studies demonstrated that serovar 1/2a was the most frequently isolated serovar from the food (Zhang et al., 2007; Wang et al., 2013). In our study, serovar "1/2a, 3a" (57.6%) was the predominant in *L. monocytogenes* strains. The high percentage rate of serovar "1/2a, 3a" in the current study is in agreement with earlier findings (Jamali et al., 2013; Shen et al., 2013; Wang et al., 2013; Cetinkaya et al., 2014; Jamali and Thong, 2014; Shi et al., 2015). Furthermore, in this study, serovar "1/2a, 3a" (100%) was found much higher in chicken meat than in ground beef (46.2%). Wang et al. (2015) detected "1/2b, 3b" serogroup in 48.5% of 628 ready-to-eat meat products in China. In another study, serotype "4b, 4d and 4e" (51.4%) was found much higher than other serotypes in Turkey (Erol and Ayaz, 2011). The rank of each serogroup may indicate differences in several studies due to the fact that they were conducted in different locations (Wang et al., 2015).

Antimicrobial resistance has been very important public health problem in the world (Fallah et al., 2012). Excessive use of antimicrobials as therapeutic agent or growth promoters in animal husbandry is the risk of presence of antibiotic-resistant bacteria into the environment and their transfer to humans through the food chain (Cetinkaya et al., 2014). In this study, *Listeria* species showed varied degree of resistance to antimicrobial agents (Figure 5). *Listeria* spp. strains showed a high resistance to penicillin (90.6%), ceftriaxone (79.7%) and ampicillin (71.1%). In a previous study, the resistance of *Listeria* species to penicillin was 34.5% (Rahimi et

al., 2010), whereas in another study the resistance was 43.4% (Jamali et al., 2013). On the other hand, the result of antimicrobial susceptibility testing in a previous study indicated that there was a low resistance to ampicillin (12.7%) (Rahimi et al., 2010).

In our study, all 128 *Listeria* strains were susceptible to vancomycin. This result is in agreement with those previously reported by Rahimi et al. (2010) and by Jamali et al. (2013). Among the *Listeria* strains, the highest susceptibility was found to amoxicillin/clavulanic acid (99.2%) followed by linezolid (97.7%) cephalothin (96.9%), chloramphenicol (96.9%), gentamicin (96.9%) and quinupristin/dalfopristin (96.9%), amikacin (92.2%), tetracycline (90.6%), erythromycin (89.1%), trimethoprim/sulfamethoxazole (86.7%).

The emergence of *L. monocytogenes* resistant to multiple drugs is a major concern worldwide. Ampicillin and penicillin is the first-choice antibiotics used alone or in combination with aminoglycosides for treatment of human listeriosis (Fallah et al., 2013). The results of our study indicated the highest resistance of *L. monocytogenes* to ampicillin (90.9%) and penicillin (90.9%). Similar finding has been recorded from Ieren et al. (2013) who have observed 92.9% resistance to ampicillin. Besides, Soni et al. (2013) reported that all strains of *L. monocytogenes* were resistant to ampicillin. In a different study, researchers found 100% resistance to both penicillin and ampicillin (Marian et al., 2012). On the contrary, susceptibility to ampicillin and penicillin was found 100% in a different study (Wang et al., 2015). On the other hand, Shi et al. (2015) reported that their all strains showed susceptibility to penicillin.

A combination of trimethoprim/sulfamethoxazole is generally used for treatment of listeriosis in patients having allergy to penicillin. Erythromycin is

another choice to treat the pregnant women infected with *L. monocytogenes* (Fallah et al., 2013). In this study, *L. monocytogenes* strains showed a high susceptibility to erythromycin (89.1%) and trimethoprim/sulfamethoxazole (86.7%), as similar results obtained by Harakeh et al. (2009). In previous studies, all isolates were susceptible to trimethoprim/sulfamethoxazole (Fallah et al., 2012; Jamali and Thong, 2014). On the other hand, Wang et al. (2015) reported that their all *L. monocytogenes* isolates were resistant to trimethoprim/sulfamethoxazole.

Vancomycin is one of the last therapeutic options for the treatment of human listeriosis (Fallah et al., 2013). In our research, all *L. monocytogenes* isolates were susceptible to vancomycin. This is supported by Rahimi et al. (2010), Fallah et al. (2012) and Jamali et al. (2013) in Iran and Cetinkaya et al. (2014) in Turkey. On the other hand, 26.7% vancomycin resistant strains were obtained from dairy-based products by Harakeh et al. (2009). Jamali and Thong (2014) reported that 9.4% vancomycin resistant strains were found in ready-to-eat foods.

5. CONCLUSION

Illness caused due to the consumption of contaminated foods has a wide economic and public health impact worldwide. Meat and meat products, seafood and fish products, milk and dairy products, and vegetables have all been associated with microorganisms' contamination such as *Listeria* spp. It is important to note that meat and meat products are the major source of foodborne infections and the most important link between food-producing animals and humans. In order to minimize the risk of infection for consumers, microbiological control of the food chain is being increasingly applied.

Listeria species are widely distributed in natural environment and isolated frequently from foods. *L. monocytogenes* and *L. ivanovii* were two pathogenic species of the genus *Listeria*. *L. monocytogenes* is a well-known cause of abortion, encephalitis and septicemia in man and animals. *L. ivanovii* is of major veterinary importance with a vast majority of reported isolations from abortions, stillbirths and neonatal septicemias in sheep and cattle, and a rare human infection.

In the present study, a total of 124 food samples including ground beef and chicken meat were examined for the presence of *Listeria* spp. Besides, *L. monocytogenes* strains were also investigated for the determination of virulence genes and serovars. In addition, all *Listeria* strains were tested for antimicrobial resistance. Out of the 124 samples, *Listeria* spp. was isolated from 101 (81.5%)

samples. Thirty-three (26.6%) samples were contaminated with *L. monocytogenes*. The other identified species were as follows: *L. innocua* 44 (35.5%), *L. seeligeri* 19 (15.3%), *L. grayi* 16 (12.9%), *L. welshimeri* 11 (8.9%), *L. ivanovii* subsp. *londoniensis* 4 (3.2%), *L. ivanovii* subsp. *ivanovii* 1 (0.8%). Phenotypically identified *L. monocytogenes* strains were confirmed with genotypic method by determination of presence of *hlyA* gene. *L. monocytogenes* strains tested were carriers of *hlyA* (100%), *inlB* (87.9%) and *actA* (48.5%) virulence genes. Moreover, 19 isolates (57.6%) belonged to serovar 1/2a, 3a and 14 (42.4%) belonged to 1/2c, 3c. The results of the antimicrobial susceptibility test revealed that *Listeria* species were resistant to most of the antimicrobials used in the study. Resistance to at least three or more antimicrobial agents was observed in 87.5% of the *Listeria* isolates from ground beef and chicken meat.

Detection of *Listeria* in food samples using biochemical and molecular methods generally identifies the contaminating *Listeria* to the species level. The presence of *Listeria* spp., particularly *L. monocytogenes*, in foods could be a potential risk for consumers. The results of this study provide information about the contamination status of ground beef and chicken meat samples with *Listeria* spp. Moreover, the presence of virulence-associated genes and serovar 1/2a, 3a in strains suggested that those virulent strains may have the potential to invade host cells and cause listeriosis. Besides, monitoring of the resistance of *Listeria* spp. to commonly used antimicrobials is important because antimicrobial resistant pathogens, especially *L. monocytogenes*, can constitute a serious hazard for public health.

Animal origin foods may be contaminated with pathogenic bacteria. High density of contamination occurs during slaughter due to the animal hide, equipment,

water, and utensils. However, meat can be further contaminated or cross-contaminated by various pathogenic bacteria including *Listeria* spp. after the slaughter process during chilling, cutting, deboning, and slicing. It may be necessary to improve hygienical and provide for adequate storage conditions from producers through retail establishments to avoid growth of the pathogen to high levels, because cross-contamination represents the major factor in the introduction of *Listeria* spp. to foods.

The excessive use of antimicrobials has led to the emergence of antimicrobial resistant bacteria in the environment. Antimicrobials used as growth promoters in animal feed have reduced the impact of infectious diseases but led to the dissemination of antimicrobial resistant *Listeria* into the environment which is a great hazard to public health due to transmission of resistant strains to humans via the food chain. It needs to remind consumers, sellers, and producers increasing of the awareness to importance of controlling use of antibiotics. Regular monitoring of multidrug resistance in microorganisms isolated from environment and food products is necessary in order to understand the patterns of resistance to antibiotics; and plan the strategies for prevention of their environmental spread.

The existence of *Listeria* spp., especially *L. monocytogenes*, in raw meats would pose a threat only if the meat were eaten without any cooking or insufficiently cooked. Consumers should be very careful for certain food, and had better take sufficient temperature/time combination to kill the pathogen before consumption. Consequently, the wide range of efforts to achieve food safety include better monitoring and reporting of foodborne diseases by government agencies, routine food sampling and testing, inspection at food processing facilities, training of food workers and general awareness among consumers about food safety.

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