Isolation and identification of *Listeria* spp. in chicken carcasses marketed in northeast of Iran

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**Abstract**

The aim of the present study was to evaluate the contamination of chicken carcasses with *Listeria* spp. Also, the antibiotic susceptibility of the *Listeria monocytogenes* isolates were investigated. In this study, 200 fresh chicken carcasses were examined for the presence of *Listeria* spp. Presumptive isolated *Listeria* colonies were confirmed by m-PCR. From 200 fresh chicken carcasses samples which were collected randomly from different supermarkets and butcheries, 80 samples (40%) were detected as contaminate with *Listeria* spp. and 18% of the isolates identified as *Listeria monocytogenes* (*L. monocytogenes*) using multiplex PCR assay. Conventional methods were used to differentiate other species of the *Listeria* genus. The results showed the most prevalent isolates is *L. monocytogenes* (18%). Other isolates were detected as *Listeria innocua* (11.5%), *Listeria grayi* subspp. *murrayi* (8%), *Listeria grayi* subspp. *grayi* (1.5%) and *Listeria welshimeri* (1%). The Majority of the isolates had multidrug resistance to commonly used antibiotics. Most of them were resistant to erythromycin (52.77%), followed by Tetracycline (44.44%), Clindamycin (41.66%), and Trimethoprim (25%). Some of them showed resistance to chloramphenicol (16.66%). The results indicate the noticeable contamination of fresh chicken carcasses with *Listeria* spp. Resistance of the *L. monocytogenes* isolates to antimicrobials commonly used to treat human listeriosis, which could be a potential health hazard for consumers.

**Keywords**

Listeria monocytogenes
Antibiotic resistance
Chicken

**Introduction**

The genus *Listeria* consists of a group of Gram-positive bacteria with low G + C content. *Listeria* species are gram positive, non-spore forming bacilli found in a variety of food and environment. The genus *Listeria* contains eight species: *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, *L. grayi*, *L. marthii* (Graves et al., 2010) and *L. rocourtiae* (Leclercq et al., 2009). Among these different species, only *L. monocytogenes* is a human pathogen and *L. ivanovii* is an animal pathogen, which is rarely pathogenic in human (Warriner and Namvar, 2009). *Listeria* species are recognized as foodborne organisms, because of their ability to grow across a broad range of temperatures, withstanding osmotic stress and survival under mild preservation techniques (Warriner and Namvar, 2009). *L. monocytogenes* causes a severe foodborne disease with a high mortality rate (20%) in humans which is associated with consumption of contaminated dairy products, raw vegetables, under-cooked meat, seafood and poultry products (Painter and Slutsker, 2007; Todd and Notermans, 2011). Due to the ability of multiplication of *Listeria* at refrigerator temperature, it could not be act as a safe preservation technique for the contaminated products (Warriner and Namvar, 2009). *Listeria* spp. can contaminate Poultry either environmentally or from healthy carrier birds during breeding in the farm (Skovgaard and Morgen, 1988). Improper cleaning and disinfecting of environment and equipments in poultry abattoir and processing plant; and also mishandling of the products may lead to *Listeria* contamination of poultry carcasses (Loura et al., 2005).

Nowadays, the excessive use of antibiotics has led to the emergence of antibiotic-resistant bacteria (Charpentier and Courvalin, 1999). Although, *L. monocytogenes* is usually susceptible to wide range of antimicrobial agents, but several studies have reported
multidrug resistance of this species (Charpentier and Courvalin, 1999; Rahimi et al., 2012). In recent decades, poultry farmers use antimicrobial agents during poultry breeding to reduce the risk of infectious disease, it may lead to dissemination of antimicrobial-resistant bacteria including resistant strains of Listeria in the environment (Filiousis et al. 2009). Some commonly used antibiotics in local poultry farms are Felorfenicol, Doxycycline, Linco-Spectin, Tyllosin and Thiamolin. The transmission of the resistant strains to human via contaminated poultry products may has a public health consequence (Filiousis et al. 2009). The aim of the present study was to determine the occurrence of Listeria spp. in fresh chicken carcasses in northeast of Iran and the resistance profile of L. monocytogenes to selected antibiotics.

Materials and Methods

Sampling
A total of 200 fresh chicken carcasses were collected randomly from different supermarkets and butcheries in Mashhad, from August to December, 2013. Each Chicken carcass was placed in a sterile, large plastic bag with 250 ml sterile distilled water and massaged inside the bag for 1 min. The samples were immediately transported to the laboratory inside a portable ice-chest and bacterial analyses started within 1-4 hours.

Isolation and identification of bacteria
After filtration with sterilized cheese cloth and centrifugation at 3000 rpm for 10 minutes of each rinsed fluid, the supernatant fluid was removed. The pellets obtained by centrifugation were unifed and resuspended in 9 ml of Listeria enrichment broth (LEB, Merck, Germany) containing 15 mg/L acriflavin, 40 mg/L nalidixic acid and 50 mg/L cycloheximide. All samples were incubated at 30°C for 48 h. After incubation, 0.1 ml of the enriched culture was spread on Oxford agar plate supplemented with Natamycin 25 mg/L, Colistinsulphate 20 mg/L, Acriflavine 5 mg/L, Cefotetan 2 mg/L, and Fosomycin 10 mg/L (Listeria Selectival-SV33 Series-Mast Diagnostic, Germany) which was incubated at 30°C for 48 h (Hitchins and Jinneman, 2013). Colonies that hydrolyzed aesculin were streaked onto another Oxford agar plate and incubated at 30°C for 48 h. The amplified products were visualized by standard gel electrophoresis using 3 µl of the final reaction mixture with 1 µl of loading dye (Cinnagene, Sinaclon, Iran) on 1.5% agarose gel in TAE buffer containing 1 µg ml⁻¹ ethidium bromide (Gibco, UK) for 45 min at 100 V. A 100 base-pare (bp) DNA ladder molecular weight marker (Fermentas, UK.) was included in each electrophoretic run to allow identification of the amplified products. PCR products were visualized under UV illumination and catalogued with a gel documentation system.

Gel electrophoresis
The amplified products were visualized by standard gel electrophoresis using 3 µl of the final reaction mixture with 1 µl of loading dye (Cinnagene, Sinaclon, Iran) on 1.5% agarose gel in TAE buffer containing 1 µg ml⁻¹ ethidium bromide (Gibco, UK) for 45 min at 100 V. A 100 base-pare (bp) DNA ladder molecular weight marker (Fermentas, UK.) was included in each electrophoretic run to allow identification of the amplified products. PCR products were visualized under UV illumination and catalogued with a gel documentation system.

Biochemical identification of other Listeria spp.
Suspected colonies which were confirmed as Listeria spp. but not as Listeria monocytogenes, in d-PCR assay, were differentiated by the following biochemical tests: β-haemolytic activity, nitrate reduction, and acid production from L-rhamnose,
D-xylose, and D-manitol (Roberts and Greenwood, 2003). All the isolates identified as *L. monocytogenes* were confirmed by PCR assay.

**Antimicrobial susceptibility test**

The antimicrobial susceptibility of *Listeria monocytogenes* isolates were examined by use of disc-diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2013) on Mueller-Hinton agar (Merck, Darmstadt, Germany). The following antimicrobial discs were used: ampicillin (10 µg), tetracycline (30 µg), erythromycin (15 µg), ciprofloxacin (5 µg), clindamycin (2 µg), penicillin (10 U/IE), chloramphenicol (30 µg), gentamycin (10 µg), vancomycin (30 µg), trimethoprim (5 µg) and rifampin (5 µg). The diameter of growth inhibition zone around each antimicrobial disc was measured after an incubation period of 24 h at 37°C. The results were interpreted according to the Clinical and Laboratory Standards Institute (2013) recommendations, isolates categorized in 3 groups: sensitive, intermediate and resistant. 

Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 29213 were used as reference strains.

**Results**

**Occurrence of Listeria spp. in chicken carcasses**

In the present study, a total of 200 samples of fresh chicken carcasses were examined for *Listeria* spp. which were isolated from 80 out of 200 (40%) samples. The most *Listeria* isolates determined as *L. monocytogenes* (36 out of 80). Figure 1 shows the m-PCR detection of *Listeria* spp. and *L. monocytogenes*. The second and the third major isolates were *Listeria innocua* and (23 out of 80) *Listeria grayi* subspp. *murrayi* (16 out of 80), respectively. Only 3 and 2 isolates were identified as *Listeria grayi* subspp. *grayi* and *Listeria welshimeri*, respectively.

**Antimicrobial susceptibility of L. monocytogenes**

Twelve isolates were sensitive to all antimicrobial agents. All of the *L. monocytogenes* isolates were sensitive to Ampicillin and Vancomycin. Overall, 21 of 36 *L. monocytogenes* isolates (58.33%) were resistant to more than one antimicrobial agent. Four isolates were resistant to two antibiotics, but some of the isolates (25%) showed resistance to more than four antimicrobial agents and this defined as multidrug resistance. Resistance to erythromycin was the most common finding (52.77%) and one isolate had intermediate resistance, followed by resistance to Tetracycline (44.44%), Clindamycin (41.66%), and Trimethoprim (25%). Resistance to Gentamycin and Chloramphenicol were observed in six (16.66%) isolates. Five isolates were resistant to Ciprofloxacin and Rifampin. Seven isolates showed intermediate resistance to Tetracycline. 4 isolates had intermediate resistance to Trimethoprim, Clindamycin, and Rifampin. Intermediate resistance to Ciprofloxacin observed in 3 isolates. Only two isolates were resistant to Penicillin.

**Discussion**

In the present study, *Listeria* spp. was detected in 40% (80) of fresh chicken carcasses in northeast of Iran. Fallah *et al.* (2012) reported the prevalence of *Listeria* from chicken carcasses about 40.7% in Iran. In another study by Jalali *et al.* (2004) *Listeria* spp. was isolated from 3 of 66 fresh poultry meat (Jalali *et al.*, 2004). In other studies, the occurrence of *Listeria* spp. in various poultry products ranged from 8% to 99% (Lawrence and Gilmour 1994; Yücel *et al.*, 2005; Chen *et al.*, 2009; Pesavento *et al.*, 2010; Osaili *et al.*, 2011; Sakaridis *et al.*, 2011).

In our study, *Listeria monocytogenes* was predominant among isolated *Listeria* spp. It was detected in 18% (36) of fresh chicken carcasses. Fallah *et al.* (2012) reported that, 52 out of 402 poultry product samples (12.9%) were positive for *L. monocytogenes*. Otherwise, it was detected in 14.1% of raw poultry products. In another study by Kosek-Paszkszewska *et al.* (2005), 43 out of 70 samples of raw poultry meat were contaminated with *Listeria* spp. and 6 of them were defined as *L. monocytogenes*. In other studies, the rate of contamination with *L. monocytogenes* in raw poultry products was found to be 41% in Portugal (Antunes *et al.*, 2002), 38.2% in Belgian markets (Uyttendaele *et al.*, 1999), 38% in
northern Greece (Sakaridis et al., 2011), 34% in Sri Lanka (Ganasena et al., 1995), and 22% in the Nordic countries (Gudbjörnsdóttir et al., 2004); which are higher than the results of the present study. However, the reported rate of L. monocytogenes contamination in Italy (Pesavento et al., 2010), Turkey (Yücel et al., 2005), Gauteng, South Africa (Niero et al., 2005) and Jordan (Osaily et al., 2011) are consistent with our results.

In our study, Listeria innocua was the second major species among isolated Listeria spp. It was detected in 28.75% (23 out of 200) of samples. In another study in Iran, 16.1% of raw poultry meat was contaminated with L. innocua (Fallah et al., 2012). The rate of contamination with this bacterium in Jalali et al. (2004) investigation was as 4.5%, and L. innocua was the predominant species, which is different from our results.

In the present study, Listeria murrayai (16 out of 200) was detected in 8% of samples. This species was not detected in other studies in Iran (Jalali et al., 2004; Fallah et al., 2012). Although, we didn’t find any Listeria ivanovii in our samples but, Fallah et al. (2012) found it in 2 of 22 samples. Also, Jalali et al. (2004) didn’t find this species in their study, which is in agreement with our results. The difference in the rate of contamination which has been reported in other studies may be due to the methods of sampling and bacterial isolation, and also different geographical arias of sampling.

Over the 36 Listeria monocytogenes isolates from fresh chicken carcasses, twelve isolates were sensitive to all antimicrobial agents. The acquisition of mobile genetic elements such as self-transferable and mobilizable plasmids; and conjugative transposons is the cause of emergence of antimicrobial resistance of Listeria spp. (Charpentier and Courvalin, 1999). In the present study, all of the isolates were sensitive to Ampicillin and Vancomycin. Fallah et al. (2012) and Ayaz and Erol (2010) observed resistance to Vancomycin, but all of their isolates were sensitive to Vancomycin.

Resistance to erythromycin was the most common finding (52.77%), and one isolate had intermediate resistance to this antibiotic but, in a study by Fallah et al. (2012), this resistance was reported in 15.2% of the isolates. Resistance to erythromycin is conferred by a broad-host-range plasmid pAMB1 of Enterococcus faecalis, which could be transferred successfully by conjugation (Flamm et al., 1984). This plasmid is able to replicate in the new host and conjugative transferred between strains of L. monocytogenes. Plasmid pAMB1 was also compatible with two cryptic plasmids of L. monocytogenes (Flamm et al., 1984).

In our study, only 2 isolates were resistant to penicillin while in the report of other researchers, a high resistance to this agent was reported (Ayaz and Erol, 2010; Fallah et al., 2012). In contrast, the other researchers (Davis and Jackson 2009; Sakaridis et al., 2011; Alonso-Hernando et al., 2012) reported a high susceptibility of L. monocytogenes to ampicillin and penicillin, which is in agreement with the results of present study. Dhanashree et al. (2003) in India reported that all the isolates of L. monocytogenes were susceptible to ampicillin and penicillin.

In this study, resistance to Tetracycline was 44.44% which was consistent with the other reports (Fallah et al., 2012; Dharmendra et al., 2013). Diverse species of Enterococcus and Streptococcus harboring conjugative plasmids and transposons are also present at very high numbers in the digestive tract in humans and animals; where L. monocytogenes is frequently found (Khachatourarians, 1998). The results of high antimicrobial resistance rate to tetracycline in this study could be explained by the frequent use of this antibiotic in order to treatment of infection in Iranian poultry farms. Moreover, use of antibiotics including tetracycline in human may lead to discharge of contaminated waste water in the environment. Also, agricultural residual of antibiotics (e.g., as growth promoting and prophylactic agents in animals) could lead to the emergence of antibiotic resistance bacteria (Khachatourarians, 1998). This observation reinforces the notion that the intestinal tract represents an ecosystem most favorable to direct exchange of genetic information between these two bacterial genera (Enterococcus-Streptococcus and Listeria).

In our study resistance to Gentamycin and Chloramphenicol were observed in six (16.66%) isolates. Conter et al. (2009) indicated that L. monocytogenes isolated from food and environmental samples were highly sensitive to ampicillin and gentamicin. Moreover, resistance of some isolates to chloramphenicol could be due to the illegal use of this antimicrobial in some poultry farms. Plasmid pIP501 has a broad host range and confers resistance to chloramphenicol, macrolides, lincosamides, and streptogramins (Evans and Macrina, 1983).

Administration of ampicillin or penicillin G combined with an aminoglycoside as like as gentamicin is the standard therapy for human listeriosis. At the second line of therapy is combination of trimethoprim (TMP) and sulfamethoxazole (SMX) (Charpentier and Courvalin, 1999). The results of the present study indicate a high susceptibility of L. monocytogenes to ampicillin and penicillin, the drugs
of choice for treatment of listeriosis. Resistance to Trimethoprim was observed in 25% of the isolates. Moreover, 4 isolates had intermediate resistance to this agent. A 3.7-kb plasmid (pIP823) containing a gene (dfrD) coding for a high-level trimethoprim-resistant dihydrofolate reductase was correspondence to resistance to trimethoprim in L. monocytogenes (Charpentier and Courvalin, 1997). PIP823 is a broad host range plasmid, and conjugative mobilization of pIP823 was obtained by self-transferable plasmids between different species (Charpentier et al., 1999).

The presence of antimicrobial-resistant Listeria in chicken meat has an important public health implication, since listeriosis is transmitted primarily via contaminated foods, especially in developing countries, where there is frequent and uncontrolled use of antibiotics. Further study on genotypic characterization of all Listeria isolates by molecular techniques will be needed to determine whether antimicrobial resistance observed in this study is associated with particular genotypes. Therefore, the result of the present study indicates the prevalence of multi-drug resistant isolates of L. monocytogenes in poultry samples, and underlines the need for broad surveillance of their antibiotic resistance for selection of appropriate treatment, especially for those cases of food-borne listeriosis with severe or prolonged symptoms or in immunocompromised patients.

**Conclusion**

In conclusion, presences of multidrug resistance of L. monocytogenes in the chicken meat indicate the potential risk of infection with these bacteria, especially people consuming chicken meat barbecue, which is a popular meal in Northeast of Iran.

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**References**


