Prevalence, genotyping and antibiotic resistance of *Listeria monocytogenes* and *Escherichia coli* in fresh beef and chicken meats marketed in Zanjan, Iran

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ABSTRACT

Background and Objectives: The aim of present study was to evaluate the prevalence of *Listeria monocytogenes* and *Escherichia coli*, characterization and antimicrobial resistance of their serotypes and genotyping profiles in fresh beef and poultry meats marketed in Zanjan, Iran.

Materials and Methods: A total of 90 (45 chicken and 45 beef) samples were collected from January to June 2018 focusing on retail meat stores of Zanjan city, Iran. Foodborne pathogen detection and antimicrobial resistance of isolates performed by PCR and disc diffusion methods, respectively. Simplex PCR method was used for screening *hly* and *uidA* genes in *L. monocytogenes* and *E. coli* isolates, respectively.

Results: Findings revealed high contamination in beef and chicken meats with *E. coli* (68.89% and 88.89%, respectively) and *L. monocytogenes* (53.33% and 46.67%, respectively). The most likelihood of *E. coli* isolates belonged to *E. coli* 13479 serotype. All *L. monocytogenes* isolates from beef and chicken meat samples had high similarity with serotypes *L. monocytogenes* strain NCTC 10357 and strain MF 4545, respectively. Multi drug resistance (MDR) was seen in both *L. monocytogenes* and *E. coli* isolates.

Conclusion: This study shows an insight of the current status of beef and chicken meat contamination maketed in Zanjan, Iran with *E. coli* and *L. monocytogenes* isolates (high contamination rate), their genotypic profile, epidemiological relationship and antimicrobial resistance (AMR) that should be considered as a significant public health concern in Zanjan, Iran.

Keywords: Antibiotic resistance; *Escherichia coli*; Genotyping; *Listeria monocytogenes*; Meat hygiene

INTRODUCTION

Escherichia coli as a commensal bacterium of

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intestinal tract in human and animals and *Listeria monocytogenes* as an ubiquitous bacterium are of major concern to human health and the most commonly studied foodborne pathogens (1). Both *L. monocytogenes* and *E. coli* are common in food and clinical samples due to their high prevalence and ability to grow in different conditions such as low temperature (2). Direct contact with the reservoir animals such as ruminants, contaminated unprocessed and processed foods such as meat products, dairy products, ready to eat (RTE) foods, fruits and vegetables are sourc-

es to transmission of foodborne pathogen bacteria to human including *L. monocytogenes* and *E. coli* (1, 2). High rate of meat consumption (beef, chicken) and high contamination probability of them increase the risk of foodborne pathogens to public health associated with morbidity and mortality (3). Antibiotic therapy is necessary in some cases but it can be complicated by antimicrobial resistance (AMR) of pathogens. Antibiotics have been used successfully in livestock breeding for growth promotion and therapeutics use. In this respect, chicken and cattle can harbor and transmit antimicrobial-resistant strains to human (4). It is demonstrated that antimicrobial resistance has closely related with presence of virulence factors in pathogens which connected to public health and risk of pathogens in food chain (1).

Standard conventional methods for detection of pathogens in food products is time consuming and require skilled staff. Therefore, using rapid and precise methods to detect foodborne pathogens are preferred in food industry (5). PCR has been established as one of the noteworthy and alternative method to the traditional procedures due to its rapidity, simplicity, sensitivity and cost-effectiveness and provides possibility to detect specific genes related to pathogen virulence (5-7). It has been shown that some molecular markers are more specific to detect pathogens. The *uidA* and *hly* genes have been shown to be very specific to *E. coli* and *L. monocytogenes*, respectively (8, 9). The *uidA* gene encodes the beta-glucuronidase enzyme and routinely used to assay β-D-glucuronidase activity and unequivocally identify *E. coli* (10). *hly* gene encoded Listeriolysin O, a pore-forming toxin protein considered as a virulence factor that is crucial for the virulence of *L. monocytogenes* (9). In this study, 16S-rRNA sequencing was performed to detect and classification of bacteria.

Several studies have been implemented on different food such as chicken meat and beef to determine molecular characterization and antibiotic resistance of pathogens throughout the world including Iran (11-13). Existent information about the distribution, variety of serotypes, AMR and genetically diversity of foodborne pathogens in contaminated foods including meat are rare in Iran. In this regard, this study aimed to evaluate occurrence of *E. coli* and *L. monocytogenes* contaminations and determination of their antimicrobial resistance (AMR) and genotyping profiles in fresh beef and chicken meats marketed in Zanjan city, Iran.

MATERIALS AND METHODS

Sample collection. In this cross-sectional study, a total of 90 (45 chicken and 45 beef) fresh non-branded samples were collected according to randomized cluster sampling plan from January to June 2018 on retail meat stores of Zanjan city, Iran, and based on the number of the retail meat stores including butcher shops and supermarkets in each cluster in compare with previous studies. 500 g fresh meat sample was collected from each retail store and transported in a sterile plastic bag to the laboratory maintaining cold chain. All samples were processed as soon as possible otherwise preserved at 4°C. Normal physical attributes are mentioned during sampling including odor, color and density.

Isolation and identification of bacteria. For *E. coli*, 10 grams of each sample was aseptically added to 90 ml of 0.1% buffered peptone water (BPW) (Merck, Darmstadt, Germany) and homogenized at room temperature for 2 min, at 200 rpm speed in a Stomacher blender (Bagmixer, Interscience, France) and serial dilution was prepared. 1 ml of each dilution was inoculated into MacConkey agar. After incubation at 37°C for 24 hr, the Red/pink colonies were considered as suspicious ones and transported to Eosin methylene blue agar (EMB) and incubated at 35°C for 24 hr (14). EMB is a selective and differential medium used to isolate coliforms like *E. coli* (15). The resulting colonies that produced the metallic green sheen isolated and stored in Brain Heart Infusion (BHI) broth which were incubated at 37°C for 24 hr. PCR was used for confirmation.

Listeria monocytogenes. Preparation of originally homogenized suspension and serial dilutions performed such as *E. coli*. 1 ml of each dilution was inoculated into Listeria CHROM agar (DRG International, Inc., Springfield, USA) at 37°C for 24 hr. Then, Blue colonies with halos were selected and characterized by the Gram staining. After the confirmation, the confirmed colonies were transported to Brain Heart Infusion (BHI) broth tubes, which were incubated at 37°C for 24 hr (7). PCR assay used for confirmation.

Polymerase chain reaction assays. Identification and verification of isolated strains of *L. monocytogenes* and *E. coli* by classic methods were performed using PCR and genotyping methods. Simplex PCR

method was used for screening *hly* and *uidA* genes in *L. monocytogenes* and *E. coli* isolates, respectively according to described method of Karimiazar et al. 2019 with some modification (16). YTA DNA extraction kit (Yekta Tajhiz Azma, Tehran, Iran) was used for extraction of DNA from overnight culture of isolates based on kit protocol. Previously published studies were used for selection of primers listed in Table 1 (17, 18). Purified DNA from reference strains of *L. monocytogenes* (ATCC13932) and *E. coli* (ATCC 25922) was used as a template in PCR to ensure its specificity and testing primers. PCR mixture for identification of *L. monocytogenes* and *E. coli* isolates, based on *hly* and *uidA*, is showed in Table 1. PCR cycling conditions for both *L. monocytogenes* and *E. coli* isolates is showed in Table 2. PCR conditions for amplification of selected 16S-rRNA genes are summarized in Table 2.

PCR products were detectable by electrophoresis on 1.5% agarose gel. To determine the size of PCR products, a 100 bp Ladder was used. Sequencing of *16S-rRNA* gene is known as a basic step to identify and classification of bacteria (20). DNA fragments was verified at 1450 bp were considered for sequencing based on *16S-rRNA* gene. The PCR mixture was same for amplification of all genes (Table 1).

Table 1. Primers, PCR mixture for detection *L. monocytogenes* and *E. coli* isolates based on *hly, uidA* and *16S-rRNA* genes

Molecular marker	Sequence of nucleotides	Primers Size (bp)	Gene size (bp)
hly gene *(L. monocytogenes)*	F: GCAGTTGCAAGCGCTTGGAGTGAA	24	495
	R: GCAACGTATCCTCCAGAGTGATCG	24	
uidA gene *(E. coli)*	F: GCGAAAACTGTGGAATTGAT	20	250
	R: GCGAAAACTGTGGAATTGGG	20	
16S-rRNA	F: AGAGTTTGATCCTGGCTCAG	20	1450
	R: ACGGTACCTTGTTACGACTT	20	
Composition	**Stock**	**Content in final volume (20 μl)**	**Final concentration in a reaction**
Master Mix	× 20	10 μL	1×
Forward primer	10 μM	μl 1	0.5 μM
Reverse primer	10 μM	μl 1	0.5 μM
DDW*	-	μl 7	-
DNA	-	μl 1	-

**Deionized Distilled Water

Table 2. The PCR cycling conditions for detection of *E. coli* and *L. monocytogenes* isolates based on *uidA* and *hly* genes, respectively and to detect both pathogens based on *16S-rRNA* genes in this study

The PCR cycling conditions for detection of *E. coli* and *L. monocytogenes* isolates based on *uidA* and *hly* genes						
Steps	**Temperature (ºC)**		**Time (Sec)**		**Cycles**	
	E. coli	***L. monocytogenes***	***E. coli***	***L. monocytogenes***	***E. coli***	***L. monocytogenes***
Denaturation	95	94	30	60	34	35
Annealing	65	60	30	120		
Extension	72	72	30	60		
Final extension	72	72	300	420	1	1
The PCR cycling conditions based on *16S-rRNA* genes in this study to detect both pathogens						
Stage	**Temperature (ºC)**		**Time (s) Cycle**		**Cycle**	
Initial denaturation	95		900		1	
Denaturation	95		30		35	
Annealing	66		30			
Extension	72		60			
Final extension	72		420		1	

To sequence the amplified genome of identified isolates, 20 μl of purified PCR products of *16S-rRNA* gene and primers of both *hly* and *uidA* genes were sent to Macrogen inc. (South Korea). Received profiles of sequencing were analyzed by Mega 6.0 software and blasted in NCBI database to determine and comparing similarity with reported and existing strains in NCBI gene bank.

Antimicrobial susceptibility testing. Disc diffusion method was used to determine AMR of the isolates using commercial antimicrobial susceptibility discs (Hi-Media, Mumbai, India) on Mueller Hinton Agar (Merck, Darmstadt, Germany) based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2018-M100-S28). The used antibiotic discs were ciprofloxacin, crimethoprim/sulfamethoxazole, tetracycline, streptomycin, vancomycin, carbenicillin, erythromycin, penicillin, cephalexin, cephalothin, gentamycin, chloramphenicol. The growth inhibition zone (GIZ) for each disk was measured after incubation at 37°C for 24 hr and evaluated according to the CLSI guidelines. The reference strains, *L. monocytogenes* (ATCC13932) and *E. coli* (ATCC 25922) were included as a quality controls.

Statistical analysis. Microsoft Excel software was used to capture and sorting of obtained data and a simple descriptive statistic was performed to determine frequency and percentage using this software.

RESULTS

Prevalence in chicken and beef. The prevalence of *L. monocytogenes* and *E. coli* isolated from examined samples was showed in Table 3. A single band at the expected size generated for each of the pathogen-specific primer sets (Fig. 1). According to Iran national standard, maximum allowed limit of *E. coli* in beef and chicken meat is 0 and 50 CFU/gr respectively. This limit for *L. monocytogenes* is 10^2 CFU/gr of beef and chicken meat samples (15, 19).

Table 3. Prevalence of bacterial pathogens in meat samples

Bacteria	Beef (%)	Chicken meat (%)	Total (%)
E. coli	31 (68.89)	40 (88.89)	71 (78.94)
L. monocytogenes	21 (46.67)	24 (53.33)	45 (50.20)

Genotyping. The PCR technique was able to verify and identification *L. monocytogenes* and *E. coli* isolates by amplify 250 bp and 495 bp fragment of the targeted gene (*hly* and *uidA* for *L. monocytogenes* and *E. coli*, respectively) from their genomic DNA, successfully. Screening of isolated bacteria was conducted by genus specific PCR (*16S rRNA* gene of *L. monocytogenes* and *E. coli*) and amplification of 1450 bp fragments of *hly* and *uidA* genes (Fig. 1). In general, sequencing of *16S-rRNA* PCR products showed that all sequencing profiles in beef and chicken meat samples samples for both *E. coli* and *L. monocytogenes* isolates were similar. Neighbor-joining method and *16S rRNA* sequence analyzing of *E. coli* in beef samples showed two main clusters in which clusters 1 and 2 were composed of one and 21 isolates, respectively (Fig. 2B). This phylogenetic relationship was in two clusters for thirteen isolates in chicken meat samples (Fig. 2A). The most likelihood of *E. coli* isolates in both beef and chiken meat belonged to *E. coli* 13479 serotype (Fig. 2).

16S rRNA sequence analyzing using neighbour-joining method for *L. monocytogenes* in beef and chicken meat samples showed two and one clusters with phylogenetic relationship of 3 and 21 isolates in beef and chicken meat samples, respectively (Fig. 3). All isolates of *L. monocytogenes from* beef showed high similarity with serotype *L. monocytogenes* strain NCTC10357 (Fig. 3B), whereas all isolated *L. monocytogenes* from chicken meat had high similarity with serotype *L. monocytogenes* strain MF4545 (Fig. 3A).

Antimicrobial resistance. Antimicrobial resistance profile of *E. coli* and *L. monocytogenes* isolates are shown in Table 4. All isolates of *E. coli* were resistant to vancomycin and penicillin. In addition, most of them (96.6% and 87.5% of isolated strains from meat and chicken samples, respectively) were semi-sensitive to streptomycin and susceptible to other antibiotics. All of the isolated *E. coli* strains from meat and chicken had same antibiotic resistance profile (Table 4). All isolates of *L. monocytogenes* were resistant to trimethoprim/sulfamethoxazole, tetracycline, penicillin and gentamycin. In addition, some of these isolates were semi-sensitive to carbenicillin (71.4% and 91.7% of isolated strains from meat and chicken samples, respectively), erythromycin (33.3% and 37.5% of isolated strains from meat and chicken samples, respectively) and susceptible to other antibiotics (Ta-
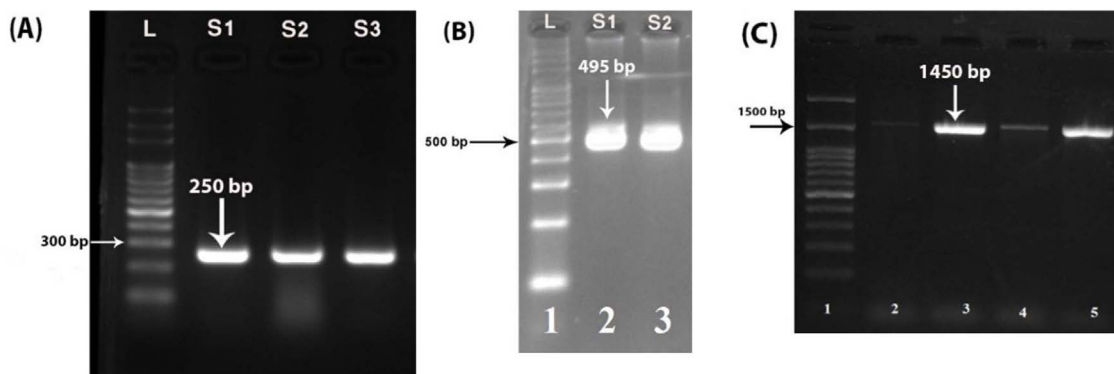
Fig. 1. Analysis of the PCR products in a 1.5% agarose gel. PCR products of target gene in: (A) *E. coli*, S1-S3 are positive samples. (B) *Listeria monocytogenes*; S1-S2 are positive samples. (C) PCR product of *16S-rRNA* amplification. L: molecular mass marker (100 bp ladder)

ble 4). All *L. monocytogenes* isolates from meat and chicken showed similar antibiotic resistance profile.

DISCUSSION

Escherichia coli. According to this study, prevalence of *E. coli* was very high. Out of 90 samples about 79% were found to be positive for *E. coli*. The occurrence of *E. coli* in chicken (88.89%) was higher than in beef (68.89%). This prevalence in beef and chicken meat is very higher than reported occurrence in previous studies in Iran conducted by Momtaz and Jamshidi (2013) (34.59% in chicken meat) (20), Jafareyan-Sedigh and Doosti, 2011 (29.1% in beef) (8), Momtaz et al. (2013) (29.02% in ruminant's meat) (21). Occurrence rate of *E. coli* strains in this study was inconsistent with that reported in other countries. In the United States *E. coli* was isolated from 38.7% of chicken meat, 19.0% of beef meat, and 16.3% of pork meat samples (7). Another study in Egypt showed 6.67 and 16.67% contamination rate of beef and chicken meat samples, respectively (22).

The antimicrobial susceptibility of *E. coli* isolates was demonstrated in Table 4. Antibiotic resistance in Gram-negative bacteria is on the rise in Iran, particularly in *E. coli*. Different patterns of antibiotic resistance were seen in various regions of Iran. More than 90% of *E. coli* isolates were resistant to penicillin (ampicillin or amoxicillin) in Tehran (capital) (23).

Generally, the *Enterobacteriaceae* are resistant to erythromycin, penicillin, ampicillin, streptomycin, tetracycline and kanamycin. AMR were lowest to chloramphenicol and gentamycin (31). *E. coli* is

the most common producers of Extended-Spectrum Beta-Lactamases (ESBLs) (24). Presence of ESBLs enzymes compromises the efficacy of all β-lactams, except cephamycins and carbapenems, by hydrolysis of the β-lactam ring and play a major role in the inhibition of penicillin-binding protein targets (25). In this study, high contamination rate in beef and chicken meat samples indicates the low attention to health regulations during the processing, packaging, storage and distribution of meat products. On the other hand, the staff hands of the distribution and sales centers, as well as the water used to wash the slaughtered carcasses, may have been contaminated by *E. coli*.

Listeria monocytogenes. Reports from Iran show until 2018 prevalence of *L. monocytogenes* in Iranian food samples was estimated to range from 0-50% (26). In this study, the overall prevalence of *L. monocytogenes* in chicken meat (53.33%) was higher than in beef (46.67%). Reported prevalence of *Listeria* in poultry products was lower in Iran (33.4%) (27). Prevalence of *L. monocytogenes* in raw meat samples were reported 41.9%, 8.5%, 11% and 8.5-19.8% from turkey, China, Bangladesh and Iran, respectively that is lower than obtained results in this study (28-31). The most prevalent serotypes of *L. monocytogenes* isolated from beef and chicken samples were 1/2a, 1/2b and 4b which is in accordance with reported document of Ranjbar and Halaji (2018) (26). Contamination rate depends on respecting to hygienic measures in processing by food handlers and the extent of cross-contamination (32). Several parameters allow this pathogen to reach to hazardous levels prior to consumption including growth ability at cold tem-
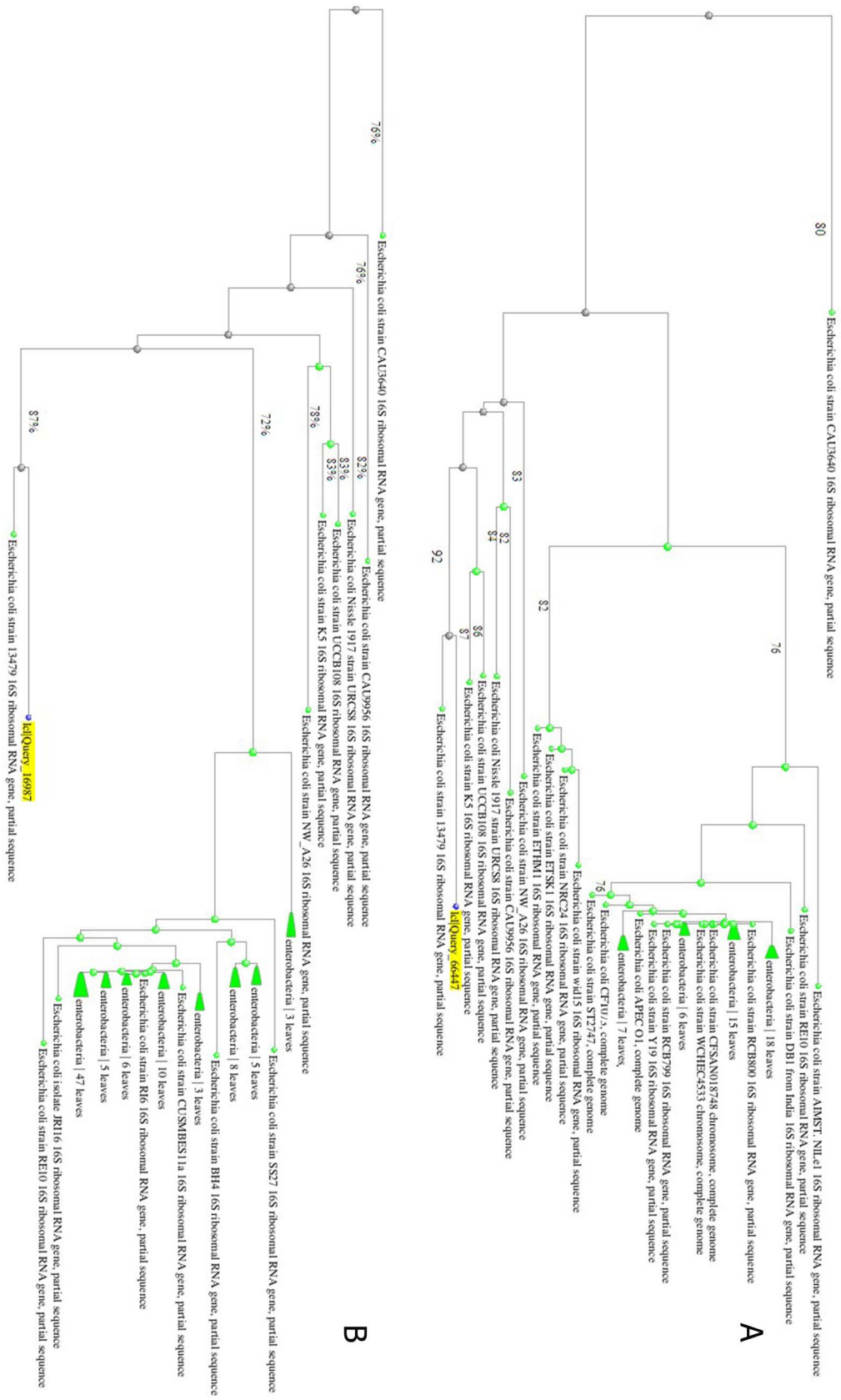
Fig. 2. Maximum likelihood phylogenetic tree of *E. coli* isolated from (A) Chicken meat and (B) Beef
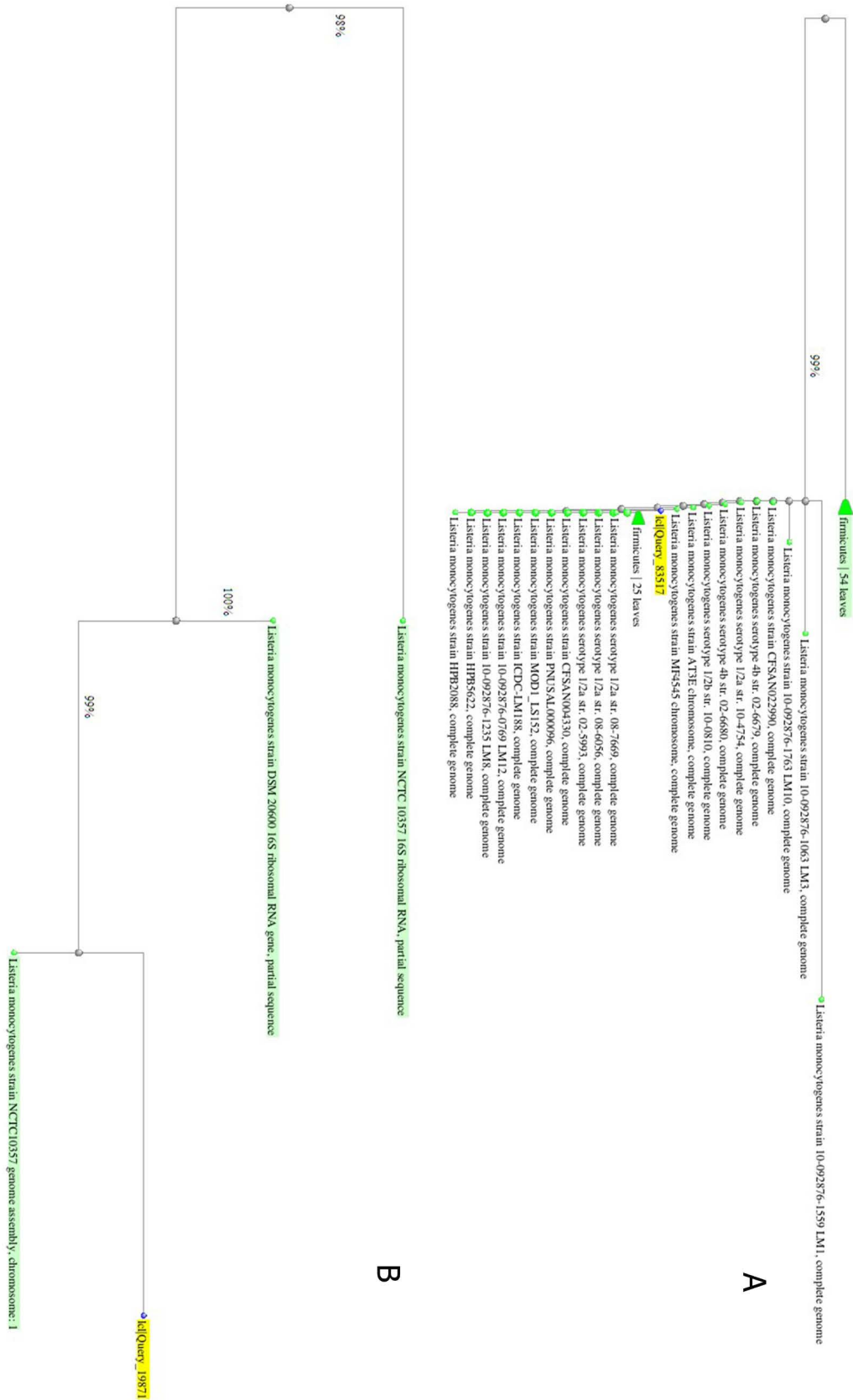
Fig. 3. Maximum likelihood phylogenetic tree of *L. monocytogenes* isolated from (A) chicken meat and (B) beef

Table 4. Antimicrobial resistance pattern of isolated *E. coli* and *L. monocytogenes* from beef /chicken meat samples

No	Antibiotics	Concentration and diameter of inhibition zone	Isolates	Sensitive No. (%) of isolates		Intermediate No. (%) of isolates		Resistance No. (%) of isolates	
				beef	chicken	beef	chicken	beef	chicken
1	Ciprofloxacin	5 µg, ≥21 mm	*E. coli*	28 (90.3)	40 (100)	3 (9.7)	0	0	0
			L. monocytogenes	19 (90.4)	18 (75)	2 (9.6)	6 (25)	0	0
2	Trimethoprim/ sulfamethoxazole	1.25/23.75 µg, ≥16 mm	*E. coli*	26 (83.6)	35 (87.5)	5 (16.4)	5 (22.5)	0	0
			L. monocytogenes	0	0	0	2 (8.3)	21 (100)	22 (91.7)
3	Tetracycline	30 µg≥15 mm	*E. coli*	24 (77.4)	30 (75)	7 (23.6)	10 (25)	0	0
			L. monocytogenes	0	0	0	2 (8.3)	21 (100)	22 (91.7)
4	Streptomycin	10 µg, ≥15 mm	*E. coli*	2 (3.4)	5 (2.5)	29 (96.6)	35 (87.5)	0	0
			L. monocytogenes	18 (85.7)	24 (100)	3 (14.3)	0	0	0
5	Vancomycin	30 µg, ≥14 mm	*E. coli*	0	0	0	0	31 (100)	40 (100)
			L. monocytogenes	21 (100)	24 (100)	0	0	0	0
6	Carbenicillin	100 µg, ≥17 mm	*E. coli*	31 (100)	40 (100)	0	0	0	0
			L. monocytogenes	0	0	15 (71.4)	22 (91.7)	6 (28.6)	2 (8.3)
7	Erythromycin	30 µg, ≥23 mm	*E. coli*	21 (67.7)	35 (87.5)	10 (32.3)	5 (22.5)	0	0
			L. monocytogenes	14 (66.7)	15 (62.5)	7 (33.3)	9 (37.5)	0	0
8	Penicillin	10 U, ≥21 mm	*E. coli*	0	0	0	0	31 (100)	40 (100)
			L. monocytogenes	0	0	0	0	21 (100)	24 (100)
9	Cephalexin	30 µg, ≥15 mm	*E. coli*	30 (96.8)	40 (100)	1 (3.2)	0	0	0
			L. monocytogenes	21 (100)	24 (100)	0	0	0	0
10	Cephalothin	30 µg, ≥23 mm	*E. coli*	28 (90.3)	40 (100)	3 (9.7)	0	0	0
			L. monocytogenes	21 (100)	24 (100)	0	0	0	0
11	Gentamycin	10 µg, ≥15 mm	*E. coli*	26 (83.9)	40 (100)	5 (16.1)	0	0	0
			L. monocytogenes	0	0	0	0	21 (100)	24 (100)
12	Chloramphenicol	30 µg, ≥18 mm	*E. coli*	31 (100)	40 (100)	-	0	0	0
			L. monocytogenes	21 (100)	24 (100)	0	0	0	0

perature, and desirable conditions at the retail level, during transport, and home storage (2).

Antimicrobial resistance pattern of *L. monocytogenes* isolates is shown in Table 4. According to our results, some of isolate was multidrug-resistant (≥3 antibiotics). These results are in agreement with previous studies (32, 33). Horizontal gene transfer between *L. monocytogenes* and commensal microorganisms found in food and obtaining various antibiotic resistance genes can be the main reason of increasing number and type of new antibiotic resistant strains of this bacterium reported worldwide (16). On the other hand, genetic mutation in the new strains is possible, too. AMR is a global concern of public health. Therefore, continuous surveillance and taking some preventive control measures about resistant bacteria are necessary.

Inappropriate, excessive dose, and extensive use of antibiotics in human and veterinary medicine are known as the main causes of antibiotic resistance. Existence of genetic elements such as integrons and transposons are often the main reason of multi-resistant phenotypes among bacteria (22). Conjugative transposons and mobile plasmids can be the reasons to multi drug resistance with the ability to identify and capturing carriers of related genes (mobile gene cassettes) (16).

Genotyping. Rapid and cost effective methods play a prominent role to detect foodborne pathogens, particularly in the foods, immediately and are able to reduce human errors in their detection. However, rapid methods have some advantages and limitations. To accurate molecular detection of pathogens, choosing the specific gene amplicons is critical (10). In this study, *hly* and *uidA* genes were used to characterize *L. monocytogenes* and *E. coli* isolates but identification of species was based on sequencing of *16S*

rRNA gene. This method depends on matching level of the primers in different species which can lead to bias in the detection of species (34). The main limitation for application of *16S rRNA* genes is alteration in gene sequence within a genome or closely related taxa and undetermined copy numbers in bacterial genomes. In present study, due to high diversity of closely related taxa and use of universal PCR primers, determination of precise identity is impossible and unexpected results can be occurred. Direct sequencing of *16S rRNA* is the best way to overcome on this diversity (16, 34).

To better assessment of the prevalence and antibiotic resistance and decline potential hazards to public health of *E. coli* and *L. monocytogenes*, continuous and constant monitoring programs and comprehensive studies are needed. There are many issues have made it difficult to risk assessments of foodborne diseases in most countries including Iran such as the rare available epidemiological and quantitative data on occurrence of foodborne diseases and pathogens in the food chain. High rate contamination of meat and meat products, particularly chicken meat with foodborne pathogens remains a significant public health concern. Several issues such as geographic and regional features of animal feeding systems, various meat processing environments, sampling method, frequency and quantity of sampling, seasonal variation and etc. can impress on contamination rate (27).

In conclusion, the results of this study revealed a high contamination rate with *L. monocytogenes* and *E. coli* in chicken and beef meat in Zanjan, Iran. Most of the isolates were antibiotic-resistant which can threaten public health in this area by consuming these products. Neighbor-joining method and *16S rRNA* sequence analyzing of *E. coli* in beef and chicken meat samples showed that the most likelihood of *E. coli* isolates belonged to *E. coli* 13479 serotype. These methods for *L. monocytogenes* that all beef isolates had high similarity with serotype *L.monocytogenes* strain NCTC10357, whereas all isolated *L. monocytogenes* from chicken meat had high similarity with serotype *L. monocytogenes* strain MF4545.

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