

## Article

# Prevalence, phylogroups, antimicrobial susceptibility and genetic diversity of *Escherichia coli* isolates from food products

Babak Pakbin <sup>1,2</sup>, Samaneh Allahyari <sup>1</sup>, Zahra Amani <sup>1</sup>, Wolfram Manuel Brück <sup>2,\*</sup>, Razzagh Mahmoudi <sup>1,\*</sup> and Amir Peymani <sup>1</sup>

<sup>1</sup> Medical Microbiology Research Center, Qazvin University of Medical Sciences, Qazvin, Iran.; [b.pakbin@ut.ac.ir](mailto:b.pakbin@ut.ac.ir); [z.amani@ut.ac.ir](mailto:z.amani@ut.ac.ir); [samaneh.alahyari66@gmail.com](mailto:samaneh.alahyari66@gmail.com); [r.mahmodi@yahoo.com](mailto:r.mahmodi@yahoo.com); [a.peymani@gmail.com](mailto:a.peymani@gmail.com)

<sup>2</sup> Institute for Life Technologies, University of Applied Sciences Western Switzerland Valais-Wallis, 1950 Sion 2, Switzerland; [wolfram.bruck@hevs.ch](mailto:wolfram.bruck@hevs.ch)

\* Correspondence: [wolfram.bruck@hevs.ch](mailto:wolfram.bruck@hevs.ch); [r.mahmodi@yahoo.com](mailto:r.mahmodi@yahoo.com)

**Abstract:** Background: The emergence of multi-drug resistant *E. coli* is an important matter of increasing considerable concern to global public health. The aim of this study was to investigate the incidence, antibiotic resistance pattern, phylogroups and genetic variation of *E. coli* isolates from raw milk, vegetable salad and ground meat samples. Methods: Culture-based techniques, Kirby-Bauer disk diffusion susceptibility testing, PCR and RAPD assays were used to determine the incidence rate, antimicrobial resistance pattern, phylogenetic groups and genetic diversity of the *E. coli* isolates. Results: *E. coli* isolates were highly resistant to amoxicillin (79.16%), trimethoprim-sulfamethoxazole (70.83%), amoxicillin-clavulanic acid (62.50%), tetracycline (54.16%), chloramphenicol (54.16%), nitrofurantoin (54.16%), ampicillin (45.83%), streptomycin (45.83%), and kanamycin (33.33%); and completely susceptible to norfloxacin and azithromycin. 70.83% of the isolates were multi-drug resistant. Most *E. coli* isolates (46%) belonged to phylogroup A. RAPD with UBC245 primer categorized the isolates into 11 clusters. A high level of genetic diversity was found among the isolates; however, 33.3% of the isolates were grouped in a major cluster (R5). Conclusions: Antibiotic resistance patterns are randomly distributed among the genetic clusters. Novel, practical, efficient food safety control and surveillance systems of multi-drug resistant foodborne pathogens are required to control the foodborne pathogen contamination.

**Keywords:** *Escherichia coli*; Antimicrobial resistance; Food samples; Phylogenetic group; Genetic diversity

## 1. Introduction

Foodborne diseases are defined as disorders caused by agents (bacteria, fungi, viruses, parasites and chemicals) that are usually either toxic or infectious in nature and enter the human body through the ingestion of food or drinks [1]. Foodborne bacteria are the main microbial factors causing foodborne diseases with significant adverse effects on human health and economic well-being. Foodborne pathogens can induce mild to severe both intestinal and extra-intestinal symptoms in humans [2]. WHO estimated that considered foodborne hazards caused more than 600 million foodborne diseases leading to more than 33 million disability-adjusted life years (DALYs) and 420 000 deaths annually worldwide [3]. The global burden of foodborne illnesses is comparable to those of the main infectious diseases such as tuberculosis, HIV and malaria; and certain risk factors including unimproved sanitation, air pollution and dietary risk factors [4]. At least 90% of all foodborne diseases are caused by diarrheal disease agents, indicating that many diarrheal illnesses are pathologically benign. The most prevalent foodborne pathogens contributing to the global burden of diarrheal illnesses have been reported to be Noroviruses, *Campylobacter* spp., *Escherichia coli*, *Salmonella* spp., *Shigella*

spp., *Giardia* spp., and *Entamoeba histolytica*. *E. coli* is one of the main challenges and concerns in food safety and public health [3].

*Escherichia coli* is gram-negative rod-shaped facultative anaerobic bacteria belonging to the Enterobacteriaceae family. *E. coli* is a commensal bacterium and typical inhabitant of gastrointestinal tract of warm-blooded animals such as mammals, including human, cattle, and pigs, amongst others [5]. *E. coli* mostly remains confined to the human or animal gastrointestinal lumen. However, these bacteria can also cause severe intestinal and extraintestinal infections in immunocompromised or debilitated hosts [6]. Diarrheal diseases and extraintestinal infections such as hemorrhagic uremic syndrome (HUS), sepsis and meningitis in both humans and animal are caused by specific groups of *E. coli* [7]. Nevertheless, these bacteria have been generally regarded as an indicator of human fecal contamination due to its consistent presence in human feces. Contamination with either pathogenic or non-pathogenic *E. coli* may occur during any farm-to-table processing steps from animal, environmental or human sources and cause foodborne diseases and outbreaks in human and animals [8]. Investigation of serogroups, pathotypes, sequence types and phylogroups in *E. coli* strains allow characterization of potential pathogenicity and virulence of the isolates from human, animal, food, water and environments [9].

A classification system based on phylogenetic cluster characterization of *E. coli* has previously been developed by Clermont et al. (2000) for tracking microbial source, determination of clonal relationship and determination potential pathogenicity among *E. coli* isolates [10]. Phylogenetic analysis of *E. coli* isolates has indicated that *E. coli* clones are divided into four main distinct groups, including A, B2, B2 and D; and seven main subgroups consisting of A0, A1, A2, B22, B23, D1 and D2. A refined phylogroup classification has recently been proposed by Clermont et al. (2013), and four more phylogroups including C, E, F and *Escherichia* cryptic clade I were added [11]. *E. coli* strains belonging to the phylogroup B2, and a lesser extent to the phylogroup D, are main causes of extra-intestinal infections in human. Also, the strains belonging to the phylogroup A are mostly commensal [12]. *E. coli* strains causing diarrheal diseases are most probably of the phylogroups B2 and E. There is a strong link between the virulence and phylogeny in *E. coli* infections. Phenotypic and genotypic properties of *E. coli* strains belonging to all phylogroups are entirely different. These properties include sugar fermentation, growth temperature, presence or absence of virulence encoding genes and antibiotic resistance pattern [13].

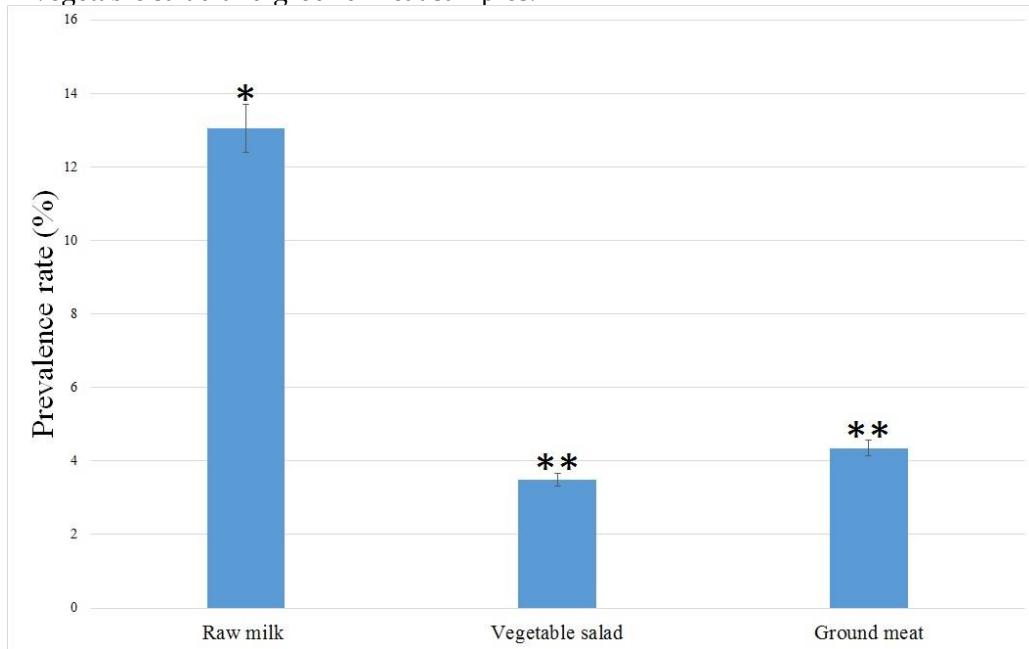
Antibiotics have a major role in treatment of bacterial infections in humans and animals by decreasing mortality and morbidity associated with the infectious diseases. Consequently, antimicrobial resistance in foodborne pathogens, particularly those identified in animal origin foods have been increased around the world [14]. Several causative factors have been attributed to this increase including use of antibiotics for growth promotion of farm animals, addition of clinical antibiotics to the farm animal feeds, and overuse of antimicrobial agents in humans and animals [15]. Emergence of multidrug resistant (MDR) foodborne pathogens has been considered one of the main concerns in public health [16]. *E. coli* strains are more likely to be resistant to several classes of antibiotics as a MDR foodborne pathogen. MDR *E. coli* has been recognized as one of the most significant challenges in food safety [17]. Little is known about phylogroups, antimicrobial susceptibility and genetic relatedness of *E. coli* strains isolated from food products [18]. The purpose of this study was to determine the prevalence rate, phylogroups, antimicrobial susceptibility patterns and genetic diversity in *E. coli* strains isolated from food products including vegetable salad, raw cow milk and ground meat samples.

## 2. Results

### 2.1. Isolation and identification of *E. coli* in food products

A total of 24 (6.95%) *E. coli* strains were isolated and confirmed from 345 food samples. Prevalence rates of *E. coli* isolated from raw milk, vegetable salad, and ground meat samples are illustrated in Figure 1. Among all *E. coli* isolates, 15 (54.16%) of 24, 5

(20.83%) of 24 and 4 (16.66%) of 24 isolates were detected in raw cow milk, ground meat and vegetable salad samples, respectively. All *E. coli* isolates were primarily isolated and detected using culture-based methods and then confirmed by biochemical tests. Prevalence rate of the *E. coli* isolates was significantly ( $P < 0.01$ ) higher in raw com milk than that in vegetable salad and ground meat samples.



**Figure 1.** Prevalence rates of *E. coli* in different food samples. \* and \*\* indicates significant differences ( $P > 0.05$ ).

## 2.2. Antimicrobial susceptibility evaluation the of *E. coli* isolates

All twenty-four *E. coli* isolates were tested for their antimicrobial resistance against nine different common classes of antibiotics and nineteen different commercial antibiotics. The results of phenotypic resistance tests to antibiotics of the isolates are shown in Table 1. Amoxicillin (19 isolates; 79.16%), trimethoprim-sulfamethoxazole (17 isolates; 70.83%), amoxicillin-clavulanic acid (15 isolates; 62.50%), tetracycline (13 isolates; 54.16%), chloramphenicol (13 isolates; 54.16%); nitrofurantoin (13 isolates; 54.16%), ampicillin (11 isolates; 45.83%); streptomycin (11 isolates; 45.83%) and kanamycin (8 isolates; 33.33%) resistance were the dominant resistance phenotypes among the *E. coli* isolates. However, the lowest antibiotic resistance phenotypes were observed against cefoxitin (4 isolates; 16.66%), colistin (4 isolates; 16.66%), cefepime (3 isolates; 12.50%), imipenem (1 isolate; 4.16%), amikacin (1 isolate; 4.16%), gentamicin (1 isolate; 4.16%), nalidixic acid (1 isolate; 4.16%) and levofloxacin (1 isolate; 4.16%). All *E. coli* isolates were completely sensitive to norfloxacin and azithromycin. No significant differences were seen among the antibiotic resistant patterns of the *E. coli* strains isolated from raw milk, vegetable salad, and ground meat samples. 17 of 24 *E. coli* strains (70.83%) expressed resistance to at least three different classes of antimicrobial agents and were considered as MDR *E. coli* isolates (Table 2). In this study, resistance to five (20.8%) or six (20.8%) antibiotic classes simultaneously were the most frequent patterns of multidrug resistance for the *E. coli* isolates. The multidrug resistance pattern of  $\beta$ -lactams, nitroheterocyclics, aminoglycosides, folate pathway antagonists, lipopeptides, tetracyclines and phenicols was the most resistance profile (n=2; 8.3%). In summary, most of the *E. coli* isolates (n=14; 58.3%) were resistant to four, five or six classes of antibiotics simultaneously, whereas, only 4.1% (n=1) and 8.3% (n=2) of the isolates were simultaneously resistant to three and seven classes of antimicrobial agents, respectively.

**Table 1.** Antibiotic resistance phenotype of *E. coli* isolated from food samples.

Antibiotic class	Antibiotic Agent	n (%)			
		Raw milk (n=15)	Ground meat (n=5)	Vegetable salad (n=4)	Total (n=24)
$\beta$ -Lactams	cefoxitin	3 (20.0)	0 (0)	1 (25.0)	4 (16.66)
	imipenem	1 (6.66)	0 (0)	0 (0)	1 (4.16)
	amoxicillin	12 (80.0)	5 (100)	2 (50.0)	19 (79.16)
	ampicillin	8 (53.33)	2 (40.0)	1 (25.0)	11 (45.83)
	cefepime	2 (13.33)	0 (0)	1 (25.0)	3 (12.50)
	amoxicillin-clavulanic acid	11 (73.33)	3 (60.0)	1 (25.0)	15 (62.50)
Aminoglycosides	streptomycin	7 (46.66)	4 (80.0)	0 (0)	11 (45.83)
	kanamycin	8 (53.33)	0 (0)	0 (0)	8 (33.33)
	amikacin	1 (6.66)	0 (0)	0 (0)	1 (4.16)
	gentamicin	1 (6.66)	0 (0)	0 (0)	1 (4.16)
Quinolones and fluoroquinolones	nalidixic acid	0 (0)	1 (20.0)	0 (0)	1 (4.16)
	norfloxacin	0 (0)	0 (0)	0 (0)	0 (0)
	levofloxacin	1 (6.66)	0 (0)	0 (0)	1 (4.16)
Macrolides	azithromycin	0 (0)	0 (0)	0 (0)	0 (0)
Tetracyclines	tetracycline	10 (66.66)	3 (60.0)	0 (0)	13 (54.16)
Lipopeptides	colistin	4 (26.66)	0 (0)	0 (0)	4 (16.66)
Phenicols	chloramphenicol	9 (60.0)	3 (60.0)	1 (25.0)	13 (54.16)
Nitroheterocyclics	nitrofurantoin	10 (66.66)	0 (0)	3 (75.0)	13 (54.16)
Folate pathway antagonists	trimethoprim-sulfamethoxazole	12 (80.0)	4 (80.0)	1 (25.0)	17 (70.83)

**Table 2.** Multidrug resistance class patterns of *E. coli* isolates from food samples.

No. classes of antibiotics	Multidrug resistance patterns <sup>a</sup> (No. isolates in each pattern)	No. total isolates (%) (n=24)
One	$\beta$ Ls (n=3)	3 (12.5)
Two	$\beta$ Ls-NHCs (n=4)	4 (16.6)
Three	$\beta$ Ls-AGs-FPAs (n=1)	1 (4.1)
Four	$\beta$ Ls-TCs-PNs-FPAs (n=1)	4 (16.6)
	$\beta$ Ls-LPs-NHCs-FPAs (n=1)	
	$\beta$ Ls-AGs-NHCs-FPAs (n=1)	
	$\beta$ Ls-PNs-NHCs-FPAs (n=1)	
Five	$\beta$ Ls-AGs-TCs-PNs-FPAs (n=3)	5 (20.8)
	AGs-TCs-LPs-PNs-FPAs (n=1)	
	$\beta$ Ls-AGs-TCs-NHCs-FPAs (n=1)	
Six	$\beta$ Ls-AGs-TCs-NHCs-PNs-FPAs (n=3)	5 (20.8)
	$\beta$ Ls-AGs-TCs-QNs-PNs-FPAs (n=2)	
Seven	$\beta$ Ls-AGs-TCs-LPs-NHCs-PNs-FPAs (n=2)	2 (8.3)

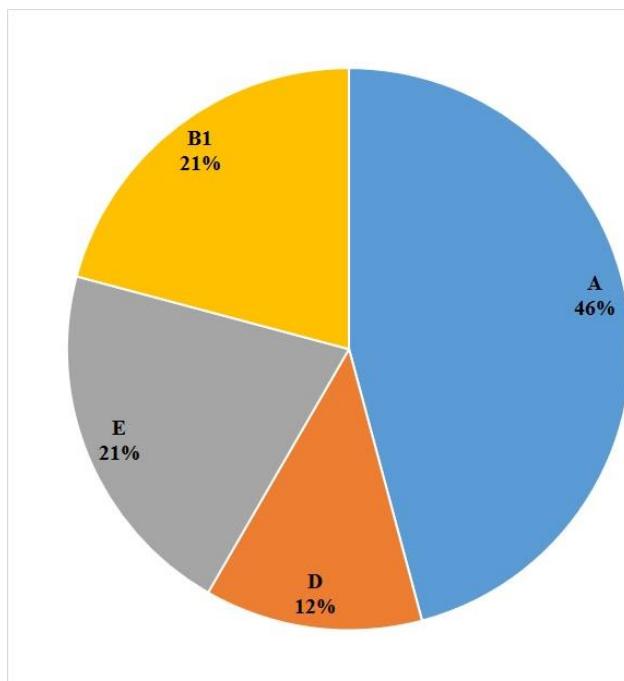
<sup>a</sup>  $\beta$ Ls,  $\beta$ -Lactams; NHCs, Nitroheterocyclics; AGs, Aminoglycosides; FPAs, Folate pathway antagonists, LPs, Lipopeptides; TCs, Tetracyclines; PNs, Phenicols; QNs, Quinolones and fluoroquinolones.

### 2.3. Phylogroups of the *E. coli* isolates

According to phylogenetic grouping of the 24 *E. coli* isolates in this study, 11 (46%), 5 (21%), 5 (21%) and 3 (12%) of the isolates were assigned to the phylogenetic groups of A, E, B1 and D, respectively (Figure 2). As shown in Table 3, phylogenetic groups of A (7 isolates; 29.16%) and E (4 isolates; 16.66%) were the most prevalent phylogroups among the *E. coli* strains isolated from raw milk samples. No D and E phylogenetic group *E. coli* strains were isolated from vegetable salad samples. Also, there are not any significant differences in distribution of phylogenetic grouping among the *E. coli* strains isolated from each food products.

**Table 3.** Distribution of phylogroups among the *E. coli* isolates obtained from different food products.

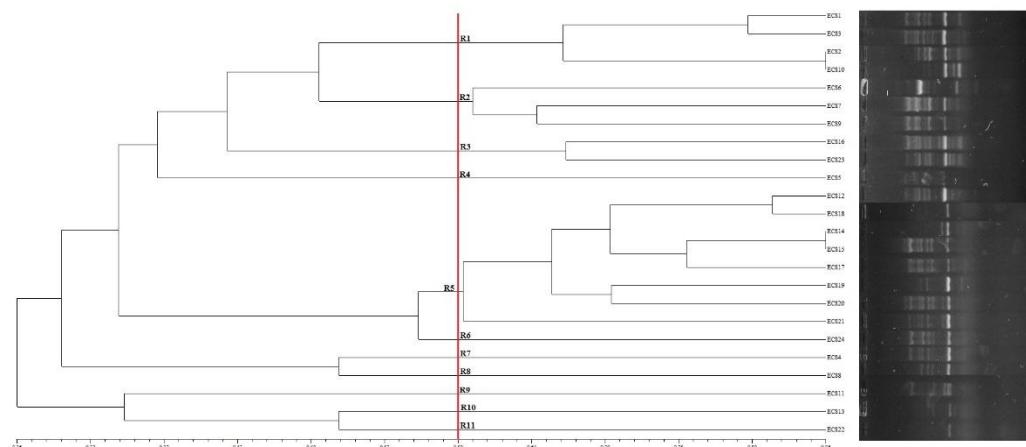
Food product	n (%) of the <i>E. coli</i> isolates (n=24)			
	A	B1	D	E
Raw milk	7 (29.16)	2 (8.33)	2 (8.33)	4 (16.66)
Vegetable salad	2 (8.33)	2 (8.33)	0 (0)	0 (0)
Ground meat	2 (8.33)	1 (4.16)	1 (4.16)	1 (4.16)

**Figure 2.** Phylogenetic group distribution of *E. coli* strains isolated from food samples.

#### 2.4. Genetic diversity among the *E. coli* isolates

At the present study, genetic diversity, and genotypic polymorphism of twenty-four *E. coli* isolates from raw milk, ground meat and vegetable salad samples were evaluated by RAPD-PCR assay using the UBC245 primer. Amplification of the *E. coli* isolates with the UBC245 arbitrary oligonucleotide resulted in RAPD-PCR patterns composed of two to seven bands ranged in size from 400 to > 2000 bp (Figure 3). As shown in Figure 4, UBC245 primer discriminated the twenty-four *E. coli* isolates into eleven distinct clusters (R1-R11) of genetically identical *E. coli* isolates with at least 59% RAPD-PCR profile similarity (a cut-off value at 59% similarity). All RAPD-PCR groups were compared using t test and the significant differences ( $P > 0.05$ ) were not found. Genetic diversity within the *E. coli* isolates was evaluated using the Simpson's index of diversity. The diversity Simpson's index for the isolates was calculated to be 0.86, showing a high level of genetic diversity among the *E. coli* isolates in this study. As shown in Table 4, clusters R4 and R6 - R11 included one *E. coli* isolate each one; however, only cluster R5 contained 8 isolates. Also, clusters R1 - R3 contained 4, 3 and 2 isolates, respectively. Ground meat isolates were just grouped in clusters R3 and R5. However, other isolates were also included in these clusters. B1 phylogroup strains were only grouped in the clusters R1 and R5. *E. coli* isolated from raw milk samples mostly were observed more resistant to antimicrobial agents than isolates from vegetable salad and ground meat samples. In this study, *E. coli* strains isolated from each food product were not grouped in a same RAPD-PCR cluster and phylogroup, indicating a high level of genetic and phylogenetic diversity among the isolates from each food product. Also, as it can be seen in Table 4, mostly

diverse patterns of antibiotic resistance can be found among the isolates from each food product.



**Figure 3.** UPGMA Dendrogram with the Dice coefficient of *E. coli* isolates from food samples in the basis of RAPD-PCR analysis.

**Table 4.** Distribution of phylogroups among the *E. coli* isolates obtained from different food products.

No.	Isolate	Food sample	Resistance phenotype <sup>a</sup>	Phylogroup	RAPD-PCR type
1	ECS1	Vegetable salad	FOX, NIT	B1	R1
2	ECS2	Raw milk	AMC, IPM, AMX, TET, CHL, SXT	B1	R1
3	ECS3	Raw milk	AMC, KAN, AMX, TET, LVX, FEP, CHL, SXT	E	R1
4	ECS4	Raw milk	SPT, AMC, AMX, AMP, TET, FEP, CHL, NIT, SXT	E	R7
5	ECS5	Raw milk	FOX, AMC, KAN, AMX, TET, CHL, NIT, SXT	E	R4
6	ECS6	Raw milk	AMC, CST, NIT, SXT	A	R2
7	ECS7	Raw milk	KAN, TET, CST, CHL, SXT	D	R2
8	ECS8	Vegetable salad	AMC, AMX, AMP, NIT	A	R8
9	ECS9	Raw milk	SPT, AMC, KAN, AMX, AMP, TET, CHL, SXT	A	R2
10	ECS10	Raw milk	FOX, SPT, AMX, AMP, GEN, NIT, SXT	A	R1
11	ECS11	Raw milk	SPT, AMC, KAN, AMX, AMP, TET, CST, CHL, NIT SXT	A	R9
12	ECS12	Raw milk	SPT, AMC, KAN, AMX, AMP, TET, CHL, NIT SXT	D	R5
13	ECS13	Raw milk	FOX, AMX, NIT	A	R10
14	ECS14	Ground meat	SPT, AMC, AMX, SXT	A	R5
15	ECS15	Ground meat	SPT, AMC, AMX, CHL, SXT	E	R5
16	ECS16	Ground meat	NAL, SPT, AMC, AMX, AMP, TET, CHL, SXT	D	R3
17	ECS17	Vegetable salad	AMX, CHL, NIT, SXT	B1	R5
18	ECS18	Raw milk	AMX	A	R5
19	ECS19	Vegetable salad	FEP	A	R5
20	ECS20	Ground meat	AMX	B1	R5
21	ECS21	Raw milk	SPT, AMC, KAN, AMX, AMK, AMP, TET, CST, CHL, NIT, SXT	B1	R5
22	ECS22	Raw milk	SPT, AMC, KAN, AMX, AMP, TET, NIT, SXT	E	R11
23	ECS23	Ground meat	SPT, AMX, TET, CHL, SXT	A	R3
24	ECS24	Raw milk	AMC, AMX, AMP, NIT	A	R6

<sup>a</sup> NAL, nalidixic acid; FOX, cefoxitin; SPT, streptomycin; AMC, amoxicillin-clavulanic acid; IPM, imipenem; KAN, kanamycin; AMX, amoxicillin; AMK, amikacin; AMP, ampicillin; TET, tetracycline; LVX, levofloxacin; FEP, cefepime; GEN, gentamicin; CST, colistin; CHL, chloramphenicol; SXT, trimethoprim-sulfamethoxazole; NIT, nitrofurantoin.

### 3. Discussion

Most of *E. coli* strains attach and harmlessly colonize the human and animal colonic region of gastrointestinal tract and only seldomly cause mild to severe intestinal and extraintestinal diseases in immunocompromised individuals [7]. Conversely, diarrheal diseases caused by pathogenic *E. coli* are a severe public health problem and concern, a major cause of mortality and morbidity especially in children and infants [19]. Because of poor living conditions such as poor sanitation, environmental hygiene, and insuffi-

cient education, diarrheal diseases with lethal outcomes mostly caused by different strains of *E. coli* are mainly prevailing in developing and low-income countries particularly in Asia, Africa, and Latin America [20]. Several studies reported that food animal origin might be an important source of human-acquired MDR pathogenic *E. coli* [17]. Poultry and meat products can be widely contaminated with pathogenic or non-pathogenic groups of *E. coli* of animal origins including MDR strains [21]. Because the main reservoir of this bacteria are intestinal tract and feces of warm-blooded animals and humans, the presence of *E. coli* strains (pathogenic or non-pathogenic) in foods, drinks, water and other environments has been used as an indicator of fecal contamination, poor hygiene and sanitation standards during production, processing, and distribution [22].

Few current studies are available regarding the prevalence rate *E. coli* strains in food products [18]. We aimed to provide determination of *E. coli* prevalence rate in food products including vegetable salad, raw milk, and ground meat samples collected from restaurants and local markets located in different areas Qazvin province, Iran. Total prevalence rate of *E. coli* was 6.95% (24 out of 345 samples) in this study which was significantly higher than that reported from Mexico (1.08%; 56 out of 5162 food samples)[23], Colombia (2.1%; 8 out of 380 food samples)[24], Korea (2.2%; 96 out of 4330 food samples)[25], Iran (4.0%; 4 out of 100 samples)[26], and Japan (6.0%; 20 out of 333 samples)[27]. Frequencies reported here were lower than those reported from China (11.0%; 83 out of 750 total raw milk samples)[28] and Finland (43.0%; 53 out of 120 food samples)[29]. The results of *E. coli* prevalence rate in this study indicated that poor hygiene and low standards of sanitation practices and behaviors have been employed during food production, processing, and distribution [27-29]. The results reported here also suggested that higher incidence rates of *E. coli* may not necessarily imply considerable *E. coli* contamination in food in Iran. Instead, using the efficient detection and identification methods might have led to significantly higher recovery of *E. coli* isolates from food samples [22,26,30].

In this study, 54.16, 20.83, and 16.66% of all *E. coli* isolates (15, 5 and 4 out of 24 isolates) were isolated from raw cow milk, ground meat and vegetable salad samples, respectively. We found that the incidence rate of *E. coli* strains in raw milk samples was higher than that in vegetable salad and ground meat samples. Previously in Mexico, Canizalez-Roman et al. (2013) reported higher prevalence rate of diarrheagenic *E. coli* in dairy products (28.4%), as the most contaminated food products, than that in meat (24.0%), seafood and fish (17.0%), prepared foods (10.9%), and beverage and ice products (3.3%) [23]. Recently, Ombarak et al. (2016) in Egypt demonstrated significantly higher incidence rate of *E. coli* contamination in raw milk samples (76.4%) than that in Karish (74.5%) and Ras (21.7%) cheese samples among dairy products [30]. Raw milk could be considered as the most potential food vehicle of transmission for *E. coli* strains in Iran and around the world [31]. Higher prevalence rate and presence of *E. coli* strains in raw milk samples is an important indicator of poor hygiene practices, sanitation and fecal contamination in raw milk production and distribution. It is fairly reasonable to assume that the main source of *E. coli* contamination could be the consequence of human fecal contamination of those raw milk products during production or distribution activities [32].

One of the main concerns in food safety and public health is the emergence of antibiotic resistant foodborne bacterial pathogens. Resistance genes could be transferred from environments and animal food sources to the opportunistic pathogens, commensal microbiota and normal bacterial gut flora in human through food chains [33]. A wide range of antimicrobial agents are currently being employed worldwide for growth promotion, diseases prevention, and treatment of sick animals allowing the development of MDR foodborne pathogens [34]. A previous study in Korea reported high level of antibiotic resistance among *E. coli* strains isolated from food and food animals, finding that 15.6, 12.5, 10.4, 9.4, and 9.4% of the *E. coli* isolates were resistant against tetracycline, streptomycin, ampicillin, ticarcillin and nalidixic acid antibiotics, respectively [25]. A

study which was conducted in Mexico reported *E. coli* strains isolated from food samples resistant to tetracycline (34%; 19 out of 56), cefepime (30%; 17 out of 56) and ampicillin (29%; 16 out of 56) [23]. A study in Iran by Mazaheri et al. (2014) reported high resistance to tetracycline and ampicillin in shiga-toxin producing *E. coli* (STEC) strains isolated from lettuce samples [26]. Another study which has been performed recently by Wang et al. (2017) showed that 49, 28, 24, 20 and 18% of *E. coli* strains isolated from retail food samples were resistant to tetracycline, nalidixic acid, ampicillin, sulfamethoxazole-trimethoprim and cephalothin, respectively [27]. Moreover, Yu et al. (2020) in China isolated *E. coli* from raw milk samples with high resistance to penicillin, acetylspiramycin, oxacillin, lincomycin, sulphamethoxazole, cephalosporin and ampicillin [28]. Recent study of Elmonir et al. (2021) in Egypt also showed a high resistance to nalidixic acid, ampicillin and streptomycin in STEC strains isolated from raw milk and beef samples [35].

*E. coli* isolates in this study were resistant to amoxicillin (79.16 %), trimethoprim-sulfamethoxazole (70.83%), amoxicillin-clavulanic acid (62.5%), tetracycline (54.16%), chloramphenicol (54.16%), nitrofurantoin (54.16%), ampicillin (45.83%), streptomycin (45.83%) and kanamycin (33.33%). All isolates were completely susceptible to norfloxacin and azithromycin. We also found that *E. coli* isolates from raw milk samples were more resistant to antibiotics than isolates from vegetable salad and ground meat samples. Obviously, resistance to different classes of antimicrobial agents among the *E. coli* isolates from food products have recently increased worldwide [18,28,35]. From the regional point of view, antibiotic resistance patterns of *E. coli* isolates from different food products at the present study are partly in agreement with previous studies in Iran [26]. In this study, 70.83% (17 out of 24) of *E. coli* isolates expressed resistance to at least three different classes of antibiotics and were regarded as MDR *E. coli* strains. The MDR rates of *E. coli* isolated from food samples reported in this study was significantly higher than that reported in Korea (12.5%) [25], Turkey (20%) [36], and Egypt (51.42%) [35], and lower than that reported in China (100%) [28] and Mexico (92.4%) [23]. The continuous global resistance among *E. coli* strains of food origins have been considered a serious threat to the public health and a major concern in food safety [16]. Indiscriminate and irrational use of antibiotics in the water and feeds of livestock (for infection treatment and/or growth promotion) may contribute to multidrug resistance in *E. coli* strains [18,27,28]. This may be regarded as the main reason for high prevalence of MDR *E. coli* isolates from food samples in this study.

Approximately, only 2000 of the 4721 genes in *E. coli* are conserved. The high level of genomic plasticity, including gaining and losing genes, in the rest of these genes contributed to developing a simple and inexpensive assay of genomic classification of *E. coli* strains to assign these isolates to specific phylogenetic groups [11]. *E. coli* strains of the same phylogenetic group share similar phenotypic and genotypic characterizations, disease causing ability, life history features and ecological attributes [37]. Different phylogenetic groups of *E. coli* have been found in specific hosts and demonstrated the same level of adaptability to the environmental conditions [11]. Of the 24 *E. coli* isolates from food samples in this study, phylogenetic group A was the most prevalent (46%; 11 out of 24) and phylogroup D was the least common. The results of our study differ from another study has previously been conducted by Higgins et al. (2007) in the USA and they show that 26, 25 and 17% of the *E. coli* isolates from animal, humans, and water were belonging to the phylogroups B1, A and D, respectively [38]. Most *E. coli* isolates belonging to the phylogroups B2 and lesser to D carry more virulence encoding gene factors than other phylogroups members and contribute to extra-intestinal diseases; however, some strains included in other phylogroups (A and B1) have been identified as causes of diarrheal diseases in humans [37]. Also, several studies showed the phylogroup B1 strains were more resistant to inappropriate environmental conditions [39]. In our study, we have not isolated any *E. coli* strain belonging to the phylogroup B2 indicating low extra intestinal pathogenic potentiality of the *E. coli* isolates from food products.

Many DNA fingerprinting assays such as ERIC, BOX and RAPD-PCR methods have widely been used to evaluate the clonal relatedness and genetic diversity among foodborne pathogens isolated from different food sources [40]. In this study, we used RAPD-PCR to assess the genetic diversity and clonality of *E. coli* strains isolated from vegetable salad, raw milk and ground meat samples as previous studies showed for clinical and foodborne pathogen isolates [41]. The results of RAPD-PCR with the primer UBC245 on *E. coli* isolates demonstrated a major cluster (cluster R5) with 8 members including 33.3% of total *E. coli* isolates. In this study, a high level of genetic diversity was found among the isolates. Isolates from ground meat samples were grouped in the same clusters (R3 and R5) and the *E. coli* isolates belonging to the phylogenetic group B1 were also grouped in the same clusters (R1 and R5). Our RAPD-PCR analysis suggested that antibiotic resistance patterns are randomly distributed among all 11 genetic clusters. Vidovic and Korber (2006) used RAPD-PCR assay to evaluate genetic diversity among the *E. coli* serotype O157: H7 isolates from feedlot cattle. They found a high level of genetic diversity and wide distribution of antibiotic resistance patterns among the isolates [42]. The greater level of diversity of genotypic and phenotypic properties among the *E. coli* isolates in this and previous studies could be due to some important factors including inter-species transfer of *E. coli* strains from domestic or farm animals to human and vice versa, consuming foods and drinks contaminated with foodborne pathogens, long duration of sample collection period and international travels [43,44].

#### 4. Materials and Methods

##### 4.1. Collection of food samples

A total number of 345 food samples including vegetable salad (n=115), raw cow milk (n=115), and ground meat samples (n=115) were purchased and collected from 27 restaurants and 45 local markets located in different areas throughout the Qazvin province, Iran, between August 2018 and February 2019. All samples were aseptically collected in sterile tubes and containers, immediately transported to the food microbiology laboratory in cool boxes with ice packs for further analysis.

##### 4.2. Isolation and identification of *E. coli*

*E. coli* was isolated and identified in food samples according to the method previously described by Ombarak et al. (2016) [30]. 10 mL of raw milk, 25 gr of vegetable salad and 25 gr of ground meat samples were mixed with either 90, or 225 mL of tryptic soy broth (TSB, Promedia, Spain) yielding a 1:10 sample dilution, then homogenized at 400 rpm for 10 min using a Stomacher-blender BagMixer (InterScience, France) and incubated at 37 °C for 16 h. 100 µL of the enrichment cultures were streaked onto eosin methylene blue agar (EMB, Promedia, Spain) plates and incubated at 37 °C for 24 h. Presumptive *E. coli* colonies on each EMB agar plate (blue-black colonies with a metallic green sheen) were selected, picked and subjected to Gram-staining and biochemical tests including motility, oxidase, indole production, citrate utilization, methyl red, Voges-Proskauer, triple sugar iron, urease, lysine and recommended sugar fermentation tests (Promedia, Spain). All confirmed *E. coli* isolates were stocked in TSB (Promedia, Spain) medium containing 18% (v/v) glycerol, incubated at 37 °C for 24 h and kept at -80 °C until further analysis. *Escherichia coli* ATCC 25922 (Serotype O6) a recommended reference strain for antibiotic susceptibility testing was used as positive control [45]. The control strain was activated by inoculation into TSB medium and incubation at 37 °C for 24 h.

##### 4.3. Antimicrobial resistance testing

Antimicrobial susceptibility testing for *E. coli* isolates was performed using a Kirby-Bauer disk diffusion assay based on the standards and interpretive criteria previously established and developed by Clinical and Laboratory Standards Institute [46]. Nineteen commercial antibiotic disks (Oxoid, UK) used in this study included cefepime (FEP), 30 µg; cefoxitin (FOX), 30 µg; kanamycin (KAN), 30 µg; ampicillin (AMP), 10 µg; imipenem (IPM), 10 µg; amoxicillin (AMX), 25 µg; amoxicillin-clavulanic acid (AMC), 20/10 µg; streptomycin (SPT), 10 µg; amikacin (AMK), 30 µg; norfloxacin (NOR), 10 µg; gentamicin (GEN), 10 µg; nalidixic acid (NAL), 30 µg; levofloxacin (LVX), 5 µg; colistin (CST) 10

$\mu$ g; azithromycin (AZM), 15  $\mu$ g; tetracycline (TET), 30  $\mu$ g; chloramphenicol (CHL), 30  $\mu$ g; trimethoprim-sulfamethoxazole (SXT), 1.25/23.75  $\mu$ g, and nitrofurantoin (NIT), 300  $\mu$ g. The results of antibiotic resistance phenotypes were recorded and interpreted according to CLSI guidelines [46]. *Klebsiella pneumoniae* ATCC 700603, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as the reference strains for quality control.

#### 4.4. DNA extraction

All isolates and the control strain (*E. coli* ATCC 25922) were grown on bovine heart infusion (BHI, Promedia, Spain) broth overnight at 37 °C. 1 mL of Phosphate Buffered Saline (PBS, Promedia Spain) was mixed with 1 mL of the bacterial suspension and centrifuged at 8 000 g for 4 min. The supernatant was removed, and the bacterial sediment was subjected to genome extraction using Sinaclon bacterial DNA extraction kit (Sinaclon Co., Iran) according to the manufacturer's instruction. Quantity and quality of the extracted DNA were measured using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The concentrations of the extracted DNA were adjusted to 50  $\mu$ g/mL with PBS prior to PCR reactions.

#### 4.5. Phylogroups determination

To determine phylogroups in the *E. coli* isolates, a triplex PCR method described by Clermont et al. (2000) was used [10]. PCR was carried out in an ABI PCR thermal cycler model 9092 (Applied Biosystems, USA). Specific primers which have previously been described by Clermont et al. (2000) were used to amplify TSPE4, yjaA and chuA genes [10]. PCRs were performed in 20- $\mu$ L reaction volumes containing 10  $\mu$ L of PCR Master Mix kit (Ampliqon, Denmark), 0.5  $\mu$ L of each primer (2  $\mu$ M/ $\mu$ L), 2  $\mu$ L of DNA template and nuclease-free deionized water to reach the final reaction volume. The PCR reaction was performed as follows: initial denaturation step at 95 °C for 5 min, following by 35 cycles comprised 95 °C for 30 s, 59 °C for 30 s, 72 °C for 40 s; and a final extension at 72 °C for 5 min. Amplified PCR products were separated and characterized using gel electrophoresis on a 1.5% (w/v) agarose gel containing DNA safe stain (Invitrogen, UK) at 110 v for 1 h. Gels were visualized and the phylogroup patterns were recorded using Novin-Pars Gel Documentation system (NovinPars Co., Iran). Non-pathogenic *E. coli* ATCC 25922 containing all three genes was used as the control strain.

#### 4.6. RAPD-PCR genotyping

In this study, the genetic diversity among the *E. coli* isolates from food products was evaluated using RAPD-PCR assay as previously described. The primer UBC245: 5'-CGCGTGCCAG -3', was used for RAPD-PCR [47]. The PCR was performed in 25- $\mu$ L reaction volumes containing 12.5  $\mu$ L of PCR Master Mix (Ampliqon, Denmark), 1  $\mu$ L of UBC245 primer (5 pM/ $\mu$ L), 2  $\mu$ L of DNA template and sterilized deionized water to reach the final reaction volume. The PCR was carried out as follows: 6 min at 95 °C, 1 min at 36 °C, 4 min at 72 °C and 35 cycles of 95 °C for 1 min, 36 °C for 1 min, and 72 °C for 4 min. The amplicons were separated and characterized using gel electrophoresis on a 1% (w/v) agarose gel containing DNA safe stain (Invitrogen, UK) at 90 v for 2 h. Gels were visualized, and the RAPD patterns were recorded by Novin-Pars Gel Documentation system (NovinPars Co., Iran). PyElph software version 1.4 [48] was used for analysis of RAPD genetic markers. The RAPD-PCR dendrogram was constructed based on UPGMA method and Dice coefficient using NTSYS-pc version 2.1 [49] software. The RAPD-PCR patterns of the *E. coli* isolates with a similarity index higher than 0.59 were regarded as the closely related RAPD-PCR pattern type.

#### 4.7. Statistical analysis

Fisher's exact and Chi-square tests were used to evaluate significant differences ( $P < 0.05$ ) between the incidence rates using SPSS version 21.0.1 (IBM Corp., NY, USA) software. All experimental and measurements were performed in triplicates.

### 5. Conclusions

In conclusion, the study investigates the prevalence rate, antimicrobial susceptibility, phylogenetic groups and genetic diversity of *E. coli* strains isolated from food prod-

ucts including raw milk, vegetable salad, and ground meat samples. Our results demonstrated that prevalence the rate of *E. coli* was higher in raw milk samples than in vegetable salad and ground meat samples. This indicates poor hygiene and sanitation practices and fecal contamination during raw milk production and distribution. This study showed that *E. coli* isolates were highly resistant to amoxicillin, trimethoprim-sulfamethoxazole, amoxicillin-clavulanic acid, tetracycline, chloramphenicol, nitrofurantoin, ampicillin, streptomycin, and kanamycin as well as completely sensitive to norfloxacin and azithromycin antibiotics. We found that 70.83% of *E. coli* isolates were MDR to at least three classes of antimicrobial agents. Irrational and indiscriminate use of clinical and veterinary antibiotics in livestock feeds and water fed may contribute to multi-drug resistance in *E. coli* strains of food origin which is becoming a serious concern of public health. Most *E. coli* isolates (46%) belonged to phylogroup A. High level of genetic diversity was found among the isolates. RAPD-PCR with UBC245 primer discriminated the *E. coli* isolates into 11 genetic clusters. 33.3% of all isolates were grouped in a major cluster (R5). Isolates from ground meat samples were grouped in the same clusters. Also, the *E. coli* isolates belonging to the phylogenetic group B1 were grouped in the same clusters. Antimicrobial resistance patterns are randomly distributed among the clusters. A cognitive scientific approach is strongly necessary to improve public health and prevent foodborne diseases. Novel and efficient food safety control and surveillance systems of foodborne pathogens especially MDR strains in developing and low-income countries is strongly required to control and prevent foodborne pathogen contamination and diseases.

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