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Occurrence, pathotypes, and antimicrobial resistance profiles of diarrheagenic *Escherichia coli* strains in animal source food products from public markets in Mashhad, Iran

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ABSTRACT

Diarrheagenic *Escherichia coli* (DEC) strains are common pathogens that transmitted through the consumption of contaminated foods, and cause acute intestinal diseases in human. The present study was conducted on 300 samples including raw milk, meat and some of their products from November 2016 to October 2017. Microbiological and molecular diagnostic techniques were used to identification of DEC. A total of 69% (207/300) *E. coli* was isolated and the frequency of DEC was 25.6% (53/207). The pathogenic groups of DEC recovered from the isolates had the following profile: Shiga-like toxin producing *E. coli* (STEC): 62.3%, enteropathogenic *E. coli* (EPEC): 24.5%, enteroaggregative *E. coli* (EAEC): 9.4%, and enterotoxigenic *E. coli* (ETEC): 3.8%. Neither enteroinvasive *E. coli* (EIEC) nor diffusely adherent *E. coli* (DAEC) were found. The highest incidence values were found in hamburgers (46.7%), and the highest rate of DEC positive prevalence was in March. Of the DEC strains, 90.6% (48/53) displayed resistance to at least one antibiotic and the highest level of resistance was found for tetracycline (69%). The obtained results revealed that the studied animal source food products may easily act as a reservoir of DEC with a potential ability to transfer antibiotic resistance and virulence genes to the gastrointestinal microbiota. Therefore, it is of paramount importance to develop effective strategies for improving food safety and updated guidelines for the prudent use of antimicrobial agents in Iran.

1. Introduction

Diarrheagenic *Escherichia coli* (DEC) strains are a main etiologic agent of moderate-to-severe diarrhea in humans (Canizalez-Roman, Gonzalez-Nuñez, Vidal, Flores-Villaseñor, & León-Sicairos, 2013). DECs are important foodborne pathogens that have been classified into six pathogenic types on the basis of their specific virulence traits. These types include Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), Diffusely adherent *E. coli* (DAEC) and Shiga-like toxin producing *E. coli* (STEC) (Amézquita-Montes et al., 2015) which constitute the subgroup of Enterohemorrhagic *E. coli* (EHEC). Outbreaks caused by DEC are closely linked to contaminated food chain worldwide.

EPEC is one of the major pathogens which is responsible for infantile diarrhea in developing countries. This pathotype is determined by the presence of the locus of enterocyte effacement (LEE) region encoding for the intimin (*eae* gene) and the lack of *stx* genes. This last trait is also used to distinguish strains of EPEC from STEC. EPEC adherence factor (EAF) plasmid-encoded bundle-forming pilus (bfp) gene is an index for the classification of EPEC. BFP-positive isolates are termed typical EPEC (tEPEC), whereas BFP-negative ones are classified as atypical EPEC (aEPEC) (Canizalez-Roman et al., 2013). ETEC strains are characterized by heat-labile (LT) and/or heat-stable (ST) enterotoxins which are plasmid-encoded. ETEC is recognized as the leading cause of traveler's diarrhea in developing countries and the most common pathotype of *E. coli* which causes infantile diarrhea. EAEC strains which are known as the cause of acute and persistent diarrhea are distinguished by their aggregative adherence pattern to cultured cells. This phenotype is associated with a plasmid which codes many virulence genes, including an anti-aggregation protein transporter (CVD432), aggregative

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Table 1
Sampling locations and times.

sampling sites	frequency of sampling	Number of samples				DEC positive isolates						
		red meat n = 71	ground meat n = 34	Hamburger n = 45	Cheese n = 39	red meat positives/month	n = 15	ground meat positives/month	n = 11	hamburger positives/month	n = 14	Cheese positives/month	n = 6	Total N = 46
A	Nov, Mar, Sep	7	6	11	0	0, 2, 1	3/7	3, 2, 1	6/6	0, 1, 1	2/11	–	–	**11/24**
B	Jan, Apr, May	9	0	5	14	0, 1, 2	3/9	–	–	0, 0, 0	0/5	0, 0, 0	0/14	**3/28**
C	Mar, Sep, Oct	24	0	8	5	3, 2, 1	6/24	–	–	2, 1, 1	4/8	0, 0, 1	1/5	**11/37**
D	Nov, Jan, May	8	9	0	4	0, 0, 2	2/8	3, 0, 0	3/9	–	–	0, 0, 0	0/4	**5/21**
E	Dec, Jun, Aug	8	8	14	0	0, 0, 0	0/8	0, 0, 0	0/8	0, 0, 4	4/14	–	–	**4/30**
F	Dec, Jun, Aug	8	8	0	5	0, 1, 0	1/8	0, 0, 0	0/8	–	–	0, 0, 1	1/5	**2/21**
G	Feb, Jul, Oct	0	3	7	2	–	–	0, 0, 2	2/3	0, 3, 1	4/7	0, 0, 2	2/2	**8/12**
H	Feb, Apr, Jul	7	0	0	9	0, 0, 0	0/7	–	–	–	–	0, 0, 2	2/9	**2/16**
milk collecting centers	frequency of sampling				raw milk n = 111	Positive samples/month						**n = 7**		
1	Dec, Dec, Jan, Mar, Mar, Jun, Jun, Aug, Aug, Sep				10	0, 0, 0, 0, 0, 0, 0, 0, 1						**1/10**		
2	Nov, Dec, Jan, Feb, Feb, May, May, Jul, Sep, Sep				10	1, 1, 0, 0, 0, 0, 0, 0, 0						**2/10**		
3	Nov, Nov, Jan, Mar, Mar, May, Jul, Aug, Aug, Oct, Oct				11	0, 0, 0, 0, 0, 0, 0, 0, 0						**0/11**		
4	Dec, Dec, Feb, Feb, Apr, Apr, Jul, Sep, Sep, Oct, Oct				11	0, 0, 0, 0, 0, 0, 0, 0, 0						**0/11**		
5	Nov, Dec, Mar, Apr, Apr, Jun, Jun, Aug, Aug				9	0, 0, 0, 0, 0, 0, 0, 0, 0						**0/9**		
6	Nov, Jan, Feb, Feb, May, May, Jul, Jul, Sep				9	0, 0, 0, 0, 0, 0, 0, 0, 1						**1/9**		
7	Jan, Jan, Mar, May, May, Jul, Sep, Oct				8	0, 0, 0, 0, 0, 0, 0, 0						**0/8**		
8	Nov, Nov, Feb, Feb, Apr, Apr, Jun, Aug, Aug, Oct				10	0, 1, 0, 0, 0, 0, 0, 0, 0						**1/10**		
9	Dec, Jan, Mar, Mar, Jun, Jun, Jul, Aug, Oct, Oct				10	0, 1, 0, 0, 0, 0, 0, 0, 0						**1/10**		
10	Nov, Nov, Jan, Mar, Mar, Apr, May, Jun, Jun, Sep, Sep				11	0, 0, 0, 0, 0, 0, 0, 0, 0						**0/11**		
11	Dec, Dec, Jan, Feb, Feb, Apr, Apr, May, Jul, Jul, Oct, Oct				12	0, 0, 1, 0, 0, 0, 0, 0, 0						**1/12**		

Abbreviation: DEC, Diarrheagenic *Escherichia coli*.

adherence fimbria (AAF), and the gene *aggR* that regulates the expression of fimbria (Aslani, Alikhani, Zavari, Yousefi, & Zamani, 2011; Canizalez-Roman et al., 2013). EIEC closely resembles *Shigella* and causes an invasive and dysenteric form of diarrhea in humans (Castro-Rosas et al., 2012) which is mediated by *ipaH* and *virF* genes (Canizalez-Roman et al., 2013). DAEC is a heterogeneous group that is defined by a distinct diffuse pattern of adherence (DA) to HeLa and HEp-2 cells (Bautista-De León, Gómez-Aldapa, Rangel-Vargas, Vázquez-Barrios, & Castro-Rosas, 2013) due to a fimbrial adhesion (F1845) encoded by *daaD*. STEC strains have been described by a range of symptoms in the human hosts, from mild diarrhea to severe hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). They produce one or two Shiga-like toxins encoded by *stx1* and *stx2* genes (Canizalez-Roman et al., 2013). The STEC isolates that contain LEE pathogenicity island are frequently termed EHEC (Perna et al., 2001).

Transmission of diarrheagenic pathotypes occurs through the fecal-oral route. Human beings constitute the main reservoir of non-STEC pathotypes, while the intestinal tracts of cattle and other animals are the primary reservoirs of STEC (Winstead, Hunter, & Griffin, 2015). That's why veterinary hygiene and food safety are strictly under control by the European Union to ensure consumer health (Bondoc, 2016a; 2016b). Animal-derived foods which are suspected to be the main source of antibiotic resistance in humans have emerged as a serious global problem (Campos, Gil, Mourão, Peixe, & Antunes, 2015). *E. coli* also has developed a high level of resistance due to excessive use of antibiotics. In addition, *E. coli* can transfer mobile resistance elements, such as plasmids, to other enteric pathogens leading to the spread of multi-drug resistance among different enterobacteria (Hannah et al., 2009; Uysal & Durak, 2012). Molecular biology techniques, including multiplex-PCR assays, have been reported in literature in recent decades for the rapid identification of DEC pathotypes. The present study aimed to investigate the animal source foods (raw milk, cheese, meat, and meat products) for the distinction of all six pathogenic groups of DEC and their antibiotic susceptibility profiles. Specific DNA amplification of virulence genes by a panel of PCR reactions has been used for the detection of pathotypes. To our knowledge, this was the first study of the prevalence of six DEC groups in foodstuffs in Iran.

2. Materials and methods

2.1. Sampling location

Mashhad is the second most populous city in Iran and the capital of Razavi Khorasan Province. It is located in the northeast of the country with a population of more than 3,000,000. In this city, food products of animal origin are available in eight municipality markets which are located in eight urban regions each entailing several butcheries and retail minimarkets. Moreover, there are 11 milk collecting centers in the suburbs and raw milk is delivered daily from dairy farms to the local milk collecting center.

2.2. Sample collection

From November 2016 to October 2017 a total of 300 food products of animal origin including meat and meat products (n = 150) and raw milk/cheese (n = 150) were randomly sampled twice a month. Cheese, meat, and meat products were sampled from different municipality markets, while raw milk samples were obtained from milk collecting centers. In the way that each market was sampled three times during the year. Moreover, bovine raw milk samples were collected from at least four milk collecting centers, at each sampling time. Therefore, milk collecting centers were sampled 8–12 times during the year (Table 1).

The food products that were analyzed consisted of red meat (n = 71) including beef and veal meat. Ground meat (n = 34) that is prepared at butcheries by mixing minced beef and fat of sheep flank. Hamburger (n = 45) that is packed (each pack includes 4 hamburgers) and sold in raw

frozen form contains 70–90% beef, and 10–30% flour, spices, and additives. Bovine raw milk samples (n = 111) were obtained from bulk tanks in milk collecting centers. Unpasteurized cheese (n = 39) which is derived from bovine or sheep raw milk and sold in bulk form. All five kinds of food samples were obtained almost within the year (Table 1). Approximately 100 g of meat, ground meat, and cheese samples, a pack of hamburger, and 250 ml of raw milk were collected using aseptic procedure into a sterile container. Thereafter, they were transferred to the Laboratory of Food Quality Control at a refrigerated temperature within 2 h. The bacterial analysis was started within 2 h. The history of the samples, including the origin and date of sampling, was also recorded.

2.3. Microbiological analysis

Samples were processed for the presence of *E. coli* following the FDA's Bacteriological Analytical Manual **(**BAM**).** A 25 gr or ml analytical unit of refrigerated samples was aseptically added to 225 ml Brain Heart Infusion (BHI) broth (Quelab, Canada) and homogenized in the stomacher for 1 min. The suspension was incubated at 35 °C for 3 h, and then 225 ml double strength Tryptone Phosphate (TP) broth (Quelab, Canada) was transferred to the contents and incubated at 44 °C for 20 h as the enrichment step. Subsequently, one loop from each stomacher bag was streaked first to the Mac-Conkey and then to the Levin-Eosin Methylene blue (L-EMB) agar (Quelab, Canada) (Feng, Weagant, & Jinneman, 2011). Both typical and atypical colonies (described by BAM) were picked for further characterization by conventional biochemical screening tests (IMVIC). The isolates suspected to be *E. coli* were preserved at −65 °C in BHI broth containing 25% (V/V) glycerol.

2.4. Bacterial strains

The reference strains used as control are listed in Table 2. The strains were grown on Nutrient agar (Merck, Germany) overnight at 37 °C. The genomic DNA of EPEC, ETEC, and EAEC were purchased from the National Laboratory of *E. coli*, Pasture Institute of Iran, and used as control templates. Since *Shigella flexneri* and EIEC share several common characteristics in invasion-associated genes, it was used as positive control for EIEC. Additionally, the detection of DAEC was set up based on previous studies (Guion, Ochoa, Walker, Barletta, & Cleary, 2008). The non-pathogenic *E. coli* ATTC 11775 was used as negative control.

2.5. DNA preparation

Genomic DNA was extracted from five typical colonies of a portion of bacterial cultures from each strain using the CinnaPure DNA isolation kit (CinnaGen Co., Iran) according to the manufacturer's protocol. Template DNA was stored at −20 °C for PCR tests.

Table 2
Reference strains.

Bacteria	strain/DNA	specific target	source
E. coli	ATCC 25922	*uidA*	CCFH[a]
EHEC	ATCC 35150	*eaeA, stx1, stx2*	CCFH
EPEC	E2348/69	*eaeA, bfpA*	PII[b]
ETEC	H10407	*LT*	PII
EAEC	O42	*pCVD432, aggR*	PII
Shigella flexneri	ATCC 12122	*ipaH*	CCFH
E. coli	ATCC 11775	–	IBRC[c]
E. coli	ATCC 35218	–	PTCC[d]

Note: The abbreviation in the source column indicates: a. Culture collection of Department of Food Hygiene; b. Pasteur Institute of Iran; c. Iranian biological resource center; d. Persian Type Culture Collection.

2.6. PCR

All the DNA templates of suspected isolates were analyzed following six PCR steps. The primers matched the corresponding sequences of the genes of DEC pathotypes in GenBank. Supplementary material is presented in Table 3. PCRs were performed in a 20 μl final volume mixture containing 3 μl of the template DNA, 10 μl of Taq 2X red master mix with 1.5 mM MgCl2 (Ampliqon, Denmark), and a 10 μM concentration of each primer (Macrogen, South Korea). Conditions for all PCR reactions were similar in the MWG-AG-BIOTECH Primus Gradient (California, US) thermal cycler as follows: 95 °C initial denaturation for 5 min, 35 cycles of 30 s at 95 °C, followed by different annealing temperatures for 40 s, 30 s extension at 72 °C with a final 10 min extension at 72 °C. PCR products were visualized under UV light after electrophoresis by 1.5% agarose gel in 0.5X Tris-borate EDTA buffer at DNA Green viewer staining. The amplicons showing expected bands were run twice in a single PCR to confirm the results.

2.7. Antimicrobial susceptibility testing

The standard Kirby-Bauer disk diffusion method was used to determine the antimicrobial sensitivity phenotype of DEC isolates for 12 different antimicrobial agents. According to the guidelines developed by Clinical and Laboratory Standard Institute (CLSI), these agents represent diverse classes of antibiotics as presented in Table 6. These antibiotics were chosen based on suggested grouping of U.S. FDA-approved antimicrobial agents that should be considered for routine testing of Enterobacteriaceae (Wayne, 2015). The protocol was performed as follows: A 10 cm diameter Mueller Hinton agar (Merck, Germany) medium plate was swabbed with Tryptic Soy Broth (Merck, Germany) inoculated with *E. coli* and incubated to turbidity standard of 0.5 McFarland. Antibiotic disks (Padtan Teb, Iran) were placed on the plates. *E. coli* ATCC 25922 and *E. coli* ATCC 35218 were used as control strains. After incubating aerobically at 35 °C for 16–18 h, the diameters of growth inhibition were measured in millimeters. The results were interpreted based on the criteria of CLSI (2015) and isolates were categorized as resistant, intermediate, or sensitive (Wayne, 2015). Furthermore, multiple-antibiotic-resistance (MAR) indexing of *E. coli* isolates was performed (Lima et al., 2017).

2.8. Data analysis

The data concerning the prevalence of *E. coli*, DEC strains and different pathotypes among five categories of samples, moreover, the multi-drug resistance rates among two main food products groups (dairy and meat) as well among pathotypes were analyzed in SPSS software (version 25) using Fisher's exact test. Additionally, statistical analysis of the data for prevalence of DEC positive isolates among months of the year was performed using chi-square test and *p*-value was calculated based on Monte-Carlo method. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Detection of E. coli

The sampling of the current study covered the geographical area of Mashhad in Khorasan Razavi province. Milk collecting centers for raw milk and different municipality markets for meat products and cheese samples were assayed to detect DEC contamination. The isolates were confirmed in molecular analyses by PCR targeting the *uidA* gene. At the first screening of 300 samples, *E. coli* was isolated from 207 items (69%), including 104 and 103 of meat and raw milk/cheese samples, respectively (Table 4). The frequencies of *E. coli*-contaminated products were similar to each other ($p > 0.05$).

Table 3
List of primers, their sequences and the size of the amplified products.

PCR sets	Target group	genes	location	sequence 5′→3′	amplicon size (bp)	Annealing temperature	references
I. Simplex	*E. coli*	*uidA*	chromosome	f ATGGAATTTCGCGGATTTTGC r ATTGTTTGCCTCCCTGCTGC	187	58	Heijnen and Medema (2006)
II. Multiplex	STEC	*stx1*	Lysogenic phage	f CAGTTAATGTGGTGGCGAAGG r CACCAGACAATGTAACCGCTG	348	61	Cebula, Payne, and Feng (1995)
		stx2	Lysogenic phage	f CAGTCGTCACTCACTGGTTTCATCA r GGATATTCTCCCCACTCTGACACC	283		Brian et al. (1992)
	EPEC	*eaeA*	chromosome	f TCAATGCAGTTCCGTTATCAGTT r GTAAAGTCCGTTACCCCAACCTG	482		(R. Vidal, Vidal, Lagos, Levine, & Prado, 2004)
		bfpA	Plasmid	f GGA AGT CAA ATT CAT GGG GGT AT r GGA ATC AGA CGC AGA CTG GTA GT	300		(M. Vidal et al., 2005)
III. Multiplex	ETEC	*ST*	Plasmid	f AAAGGAGAGCTTCGTCACATTTT r AATGTCCGTCTTGCGTTAGGAC	129	61	(R. Vidal et al., 2004)
		LT	Plasmid	f TCTCTATGTGCATACGGAGC r CCATACTGATTGCCGCAAT	322		Rappelli et al. (2001)
IV. Multiplex	EIEC	*ipaH*	Plasmid	f CTC GGC ACG TTT TAA TAG TCT GG GTG r GAG AGC TGA AGT TTC TCT GC	933	60.3	(M. Vidal et al., 2005)
	DAEC	*daaD*	Plasmid	f TGAACGGAGTATAAGGAAGATG r GTCCGCCATCACATCAAAA	371		Guion et al. (2008)
V. Simplex	EAEC	*pCVD432*	Plasmid	f CTGGCGAAAGACTGTATCAT r AATGTATAGAAATCCGCTGTT	630	55	Schmidt et al. (1995)
VI. Simplex	tEAEC	*aggR*	Plasmid	f GTATACACAAAAGAAGGAAGC r ACAGAATCGTCAGCATCAGC	254	59	Ratchtrachenchai, Subpasu, and Ito (1997)

Table 4
Presence of *E. coli* and diarrheagenic *E. coli* in food samples.

Food sample	*E. coli* (%)	DEC				
		total (%)[a]	STEC (%)[b]	EPEC (%)[b]	EAEC (%)[b]	ETEC (%)[b]
red meat n = 71	47 (66.2)	15 (31.9)	11 (73.3)	4 (26.7)	0	0
ground beef n = 34	27 (79.4)	11 (40.7)	6 (54.5)	3 (27.3)	1 (9.1)	1 (9.1)
hamburger n = 45	30 (66.7)	14 (46.7)	10 (71.4)	4 (28.6)	0	0
raw milk n = 111	76 (68.5)	7 (9.2)	1 (14.3)	2 (28.6)	4 (57.1)	0
cheese n = 39	27 (69.2)	6 (22.2)	5 (83.3)	0	0	1 (16.7)
Total N = 300	**207 (69)**	**53 (25.6)**	**33 (62.3)**	**13 (24.5)**	**5 (9.4)**	**2 (3.8)**

Note.
[a] Percentage according to the total *E. coli* detected in each food sample.
[b] Percentage according to the total diarrheagenic *E. coli* detected in each food sample.

3.2. Prevalence of DEC

DEC is defined as strains which possess virulence factors unique to each pathotype. To identify them, a protocol of three multiplex and two simplex PCR reactions was developed in the present study (Table 3). The optimization of PCRs was performed with positive control DNA extracted from reference strains. The frequency of contamination with DEC in 207 *E. coli* isolates was 25.6%. As 53 DEC strains were found in 52 samples. In other words, 13/103 (12.6%) of the raw milk and cheese samples, and also 40/104 (38.5%) of the meat and meat products were contaminated with DEC pathogenic groups. One ground meat sample demonstrated co-contamination with both EPEC and EAEC. The most frequently contaminated foodstuff with DEC was hamburger (14/30, 46.7%), followed by ground meat (11/27, 40.7%) and red meat (15/47, 31.9%). Cheese and raw milk, exhibited contamination in 6 (6/27, 22.2%) and 7 samples (7/76, 9.2%), respectively (Table 4). A highly significant correlation was observed between the kind of foodstuffs and frequencies of DEC strains ($P < 0.0001$).

Fig. 1 represents the prevalence of DEC positive isolates among months of the year. The percent of positive isolates in March (31%) was significantly higher than other months ($p = 0.004$).

Concerning DEC groups, the results of PCR reactions in the present study recognized the STEC as the most common pathotype (62.3%), whereas EAEC and ETEC were found in only 9.4% and 3.8% of DEC strains, respectively. Moreover, EPEC was detected in 24.5% of DEC isolates (Table 4). The arrangement of pathotypes prevalence in meat products was obtained as STEC > EPEC > EAEC = ETEC. Similarly, it was as STEC > EAEC > EPEC > ETEC in raw milk and cheese. No EIEC or DAEC strain was isolated from any of the food items that were evaluated. The differences in the frequencies of pathotypes in five food categories were statically significant ($P = 0.007$). Supplementary data in Table 5 demonstrates that 7 of 33 STEC strains contain both *eaeA* gene and shiga-like toxin genes which categorized them as EHEC subgroup. Furthermore, both ETEC strains which were isolated in the current study were *LT* positive and the gene *ST* encoding heat-stable enterotoxins were absent in them. The obtained results revealed that all of the recovered EPEC isolates belonged to atypical strains. It is due to the fact that they only contain the *eaeA* gene encoding intimin and lack EAF plasmid for bundle-forming pilus (*bpf*) genes. Among five EAEC strains, two isolates were positive in *aggR* gene and were classified as typical EAEC (Table 5).

3.3. Antimicrobial susceptibility testing

The antibiotic resistance profile of the strains is presented in Tables 6

Table 5
Prevalence of target genes in positive strains.

pathotype	gene	positive samples					
		red meat	ground meat	hamburger	raw milk	cheese	total
STEC	*stx1*	4	4	5	1	4	**18**
	stx2	2	0	0	0	1	**3**
	stx1, stx2	2	1	2	0	0	**5**
EHEC	*stx1, eaeA*	2	1	1	0	0	**4**
	stx2, eaeA	1	0	0	0	0	**1**
	stx1, stx2, eaeA	0	0	2	0	0	**2**
aEPEC	*eaeA*	4	3	4	2	0	**13**
aEAEC	*pCVD*	0	1	0	2	0	**3**
tEAEC	*pCVD, aggR*	0	0	0	2	0	**2**
ETEC	*LT*	0	1	0	0	1	**2**
Total		15	11	14	7	6	**53**

Table 6
Antimicrobial resistance among the diarrheagenic *E. coli* strains.

Antimicrobial agent	Disk drug concn. (μg)	DEC strains (%) N = 53	Phenotype and % of resistance			
			STEC (%) n = 33	EPEC (%) n = 13	EAEC (%) n = 5	ETEC (%) n = 2
Aminoglycosides						
Gentamicin	GM10	7/53 (13.2)	5/33 (15.2)	1/13 (7.7)	0/5	1/2 (50)
β-Lactam						
Ampicillin	AM10	23/53 (43.4)	12/33 (36.4)	8/13 (61.5)	3/5 (60)	0/2
Cefalosporins						
Cefotaxime	CTX30	32/53 (60.4)	18/33 (54.5)	11/13 (84.6)	2/5 (40)	1/2 (50)
Ceftazidime	CAZ30	19/53 (35.8)	12/33 (36.4)	6/13 (46.2)	1/5 (20)	0/2
Cefepime	FEP30	11/53 (20.8)	10/33 (30.3)	1/13 (7.7)	0/5	0/2
Cefoxitin	FOX30	7/53 (13.2)	6/33 (18.2)	1/13 (7.7)	0/5	0/2
Quinolones						
Ciprofloxacin	CP5	8/53 (15.1)	4/33 (12.1)	4/13 (30.8)	0/5	0/2
Sulfonamides						
Co-trimoxazole (Trimethoprim-Sulfametoxazole)	SXT1.25–23.75	20/53 (37.7)	13/33 (39.4)	6/13 (46.2)	1/5 (20)	0/2
Tetracyclines						
Tetracycline	TE30	35/53 (66)	21/33 (63.6)	11/13 (84.6)	2/5 (40)	1/2 (50)
Carbapenems						
Imipenem	IPM10	10/53 (18.9)	9/33 (27.3)	1/13 (7.7)	0/5	0/2
Monobactams						
Aztreonam	AZT30	8/53 (15.1)	7/33 (21.2)	1/13 (7.7)	0/5	0/2
Others						
Chloramphenicol	C30	11/53 (20.8)	10/33 (30.3)	1/13 (7.7)	0/5	0/2
Total resistance phenotype		**191**	**127/191 (66.5)**	**52/191 (27.2)**	**9/191 (4.7)**	**3/191 (1.6)**

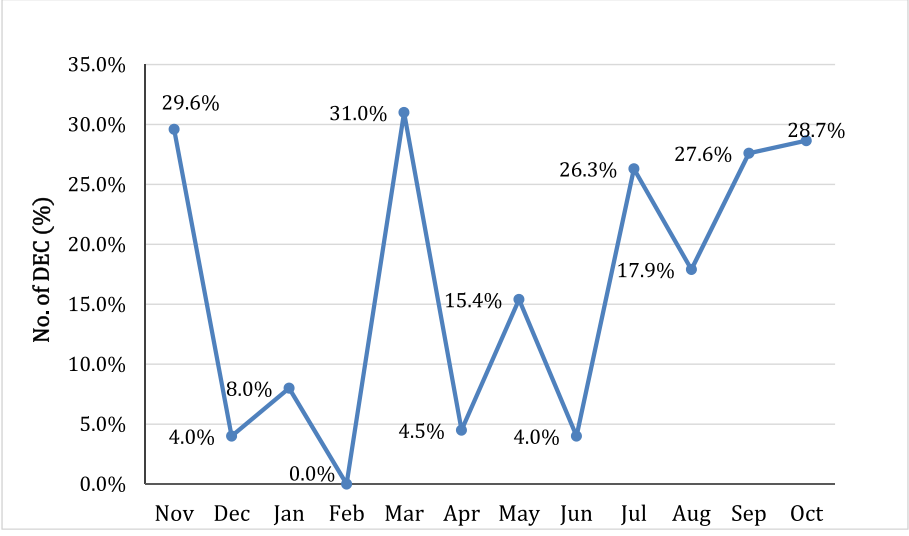

Fig. 1. Prevalence of DEC positive isolates in animal source food products between Nov. 2016 and Oct. 2017.

and 7. In brief, the lowest resistance rate (13.2%) was observed for gentamicin and cefoxitin, while 66% of the isolates were resistant to tetracycline (Table 6). In DEC groups, 5 of 33 STEC strains were found to be susceptible to all 12 antibiotics (Table 7). Tetracycline was also found to be the most frequent antibiotic in resistance profiles by 63.6% and 84.6% of STEC and EPEC strains, respectively. A similar percentage (84.6%) was found for cefotaxime in EPEC strains. Very low resistance (12.1%) was observed for ciprofloxacin in the STEC group. Moreover, 3 of 5 (60%) EAEC strains were found to be resistant to ampicillin (Table 6). It is worthy to note that both ETEC strains were resistant only to one or two drugs. Considering the MAR indexes, it was calculated zero for five susceptible STEC strains to all 12 antibiotics, as mentioned earlier. The MAR index of 3 isolates that were resistant to 9 antibiotics was found to be 0.75 and it was the highest value among the food isolates (Table 7). The difference in the number of resistant isolates between meat and dairy (raw milk and cheese) was not statically significant ($P > 0.05$), while this difference was significant among pathotypes ($P = 0.049$). STEC strains showed resistance to more antibiotics, in comparison with other pathotypes (Table 7).

4. Discussion

Foodborne diseases are a significant global public health concern and bacterial pathogens are the most common etiologic agent for this kind of disease (Amézquita-Montes et al., 2015). In this line, European Union has just cracked down the rules for strict control of food safety and public health to cope with developing certain infectious diseases (Bondoc, 2016c; 2016d). The present study investigated the frequencies of six pathogenic groups of DEC in some dairy and meat products in Mashhad, Iran. According our data, earned from one-year-sampling, of 300 food cases, 69% contamination with *E. coli* was observed based on detection of *uidA* gene (encoding beta-glucuronidase enzyme). Even *E. coli* strains that are unable to express the functional phenotype, possess this gene (de Lagarde et al., 2019). Notably, the ubiquity and analytical specificity of the primer set used in this assay have been verified by Maheux et al. (2009).

A panel of five sequential multiplex and single PCR reactions was developed to evaluate the presence of six DEC categories and their typical and atypical variants. Iranian studies conducted so far have investigated a limited number of DEC pathotypes in different foodstuffs (Dehkordi, Yazdani, Mozafari, & Valizadeh, 2014; Mohammadi & Abiri, 2012; Momtaz & Jamshidi, 2013; Sakhaie Shahreza, Rahimi, & Momtaz, 2017).

DEC contamination of meat (38.5%) and dairy (12.6%) is strongly suggestive of fecal contamination that may occur at any point along the food chain. These products are important DEC vectors as reported in previous studies in Iran (Bonyadian, Moshtaghi, & Taheri, 2014; Momtaz, Safarpoor Dehkordi, Rahimi, Ezadi, & Arab, 2013). The higher prevalence of DEC strains in hamburger and cheese samples in each food category indicates the probable contamination during processing or post-processing with intestinal pathogens (Amézquita-Montes et al., 2015). The strain combination (EAEC + EPEC) in the ground meat sample in the current study is in agreement with the results of the studies which investigated dairy, meat, and ready-to-eat salads (Bonyadian et al., 2014; Castro-Rosas et al., 2012; Comery et al., 2013).

In the present study, STEC was the most prevalent DEC which was identified in all food groups (62.3%) except the raw milk samples in which the EAEC strains had the highest percentage (57.1%). Similarly, STEC has been reported as the most frequent DEC pathotype in the studies conducted on ground meat, cheese (Amézquita-Montes et al., 2015; de la Rosa-Hernandez et al., 2018), and salads (Castro-Rosas et al., 2012; Gómez-Aldapa et al., 2016). Ground beef contamination with STEC has also been recorded in Europe and the United States (Robbins et al., 2014; Soborg et al., 2013). The high STEC contamination level in foodstuffs represents a high risk of foodborne outbreaks (Amézquita-Montes et al., 2015), as the STEC strains have commonly been isolated from cases with diarrhea in Iran (Aslani et al., 2008; Darbandi, Owlia, Bouzari, & Saderi, 2016; F; Jafari, Garcia-Gil, et al., 2009; Miri, Dashti, Mostaan, Kazemi, & Bouzari, 2017). Predominance of *stx1* in the present study is in confirmation with the observations of Dehkordi et al. (2014) in dairy products. Relatively high frequency of *stx2* positive strains (33.3%), may be a possible public health concern as *stx2* is most often associated with severe sequelae such as HUS in human (Perna et al., 2001).

The second most prevalent DEC pathotype was EPEC (24.5%). Our genotypic characterization demonstrated that all strains were negative for *bfpA* gene expressing the structural subunit of bundle-forming pili, so were the atypical variants of EPEC. This finding was in line with the results of a study conducted on food animals and retail meat in Canada in which none of the screened bacterial isolates were typical EPEC (Comery et al., 2013). Recent studies indicated that typical EPEC cases of diarrhea have been replaced with atypical EPEC in both developing and industrialized countries (Amézquita-Montes et al., 2015; Canizalez-Roman et al., 2013). A systematic review study which carried out in Iran, comprehensively searched databases in the last three decades and reported the prevalence of aEPEC and tEPEC as 11% and 3%, respectively (Alizade et al., 2019).

Another DEC pathotype inspected in our study was EAEC that further classified as typical or atypical. The *pCVD432* gene-positive strains harboring the *aggR* regulon are recognized as typical EAEC (Aslani et al., 2011). Of overall five EAEC, four strains isolated from raw milk and one from ground meat sample; thereafter, three and two of five strains were identified as typical and atypical EAEC, respectively. Some studies have reported typical EAEC-associated diarrheal cases (Estrada-Garcia et al., 2009). Although the pathogenicity of atypical EAEC has not been thoroughly clarified, it has been associated with foodborne outbreaks

Table 7
Comparative rates of antimicrobial resistance among diarrheagenic *E. coli* strains isolated from food sources.

No. of drugs resistant to:	MAR index	Total (%) N = 53	food isolates		DEC strains			
			meat n = 40	raw milk/cheese n = 13	STEC (%) n = 33	EPEC (%) n = 13	EAEC (%) n = 5	ETEC (%) n = 2
0	0	5 (9.4)	5	0	5 (15.2)	0	0	0
1	0.08	5 (9.4)	2	3	1 (3)	0	3 (60)	1 (50)
2	0.17	11 (20.8)	8	3	8 (24.2)	2 (15.4)	0	1 (50)
3	0.25	12 (22.6)	9	3	6 (18.2)	4 (30.8)	2 (40)	0
4	0.33	2 (3.8)	2	0	0	2 (15.4)	0	0
5	0.42	5 (9.4)	4	1	2 (6.1)	3 (23)	0	0
6	0.5	3 (5.7)	2	1	2 (6.1)	1 (7.7)	0	0
7	0.58	6 (11.3)	5	1	5 (15.2)	1 (7.7)	0	0
8	0.66	1 (1.9)	0	1	1 (3)	0	0	0
9	0.75	3 (5.7)	3	0	3 (9)	0	0	0

Abbreviation: MAR, multiple-antibiotic-resistance.

(Regua-Mangia, Gomes, Vieira, Irino, & Teixeira, 2009; Scavia et al., 2008). In the study carried out by Aslani et al. (2011) in Iran, 15 EAEC strains were isolated from 140 diarrheal cases, while the *aggR* gene was detected in 11/15 (73.3%) of the strains.

According to the results of the study conducted by Alizade et al. (2019), the ETEC has been identified as one of the most common etiological agents of diarrhea in Iran. The occurrence of ETEC among our samples was 3.8%. ETEC is known as the most important pathogen responsible for traveler's diarrhea and a leading cause of morbidity and mortality in children residing in developing countries, as well as in travelers visiting these destinations. As reported in earlier studies, ETEC also has been recognized as the most prevalent cause of diarrhea in under five-year-old children in Iran (Alizade, Ghanbarpour, & Aflatoonian, 2014; Haghi, Zeighami, Hajiahmadi, Khoshvaght, & Bayat, 2014; Pourakbari et al., 2013). It is worthy to note that the ETEC has been frequently recognized as a waterborne pathogenic agent, rather than a foodborne one (Amézquita-Montes et al., 2015; Daniels et al., 2000). However, same as the present study, the isolation of ETEC in food products has been previously reported in Iran, Canada, Colombia, and Mexico (Amézquita-Montes et al., 2015; Bonyadian et al., 2014; Comery et al., 2013; de la Rosa-Hernandez et al., 2018). Regarding the prevalence rate of ETEC in the current study, it is reasonable to speculate that ETEC-contaminated cheese and ground meat which are available at retail markets of Mashhad may contribute to the risk of travelers' diarrhea.

No EIEC or DAEC strains were detected from 300 samples evaluated in the current study. Similarly, Canizalez-Roman et al. (2013) who analyzed more than 5000 food and beverage samples could not isolate any EIEC and DAEC strains, whereas in Mexico, Castro-Rosas et al. (2012) identified the EIEC in ready-to-eat salads and Canizalez-Roman et al. (2019) reported 27.5% DEAC-contamination in surface water used to irrigate food products.

The highest rate of DEC positive prevalence was on March. This event might because of the coincidence of Norouz, the Persian New Year, with March, which demands more food supplies, subsequently the possibility of cross-contamination increases.

The surveillance of antibiotic resistance profiles revealed that 90.6% (48/53) of the DEC strains were resistant to at least one antimicrobial agent utilized in the present study. This worrying resistance rate might be attributed to the irregular and uncontrolled self-prescription of the antibiotics in developing countries in recent decades. Pathogenic *E. coli*-contaminated food can promote the dissemination of resistant bacteria or genes which are responsible for resistance (Lima et al., 2017). Tetracycline was the most frequent antibiotic in resistance profiles. The high bacterial resistance in food isolates against tetracycline has been previously reported (Mohammadi & Abiri, 2012; Momtaz & Jamshidi, 2013; Zhang, Wu, Zhang, & Zhu, 2016). In addition, based on surveys conducted on diarrhea in Iran, tetracycline has been one of the least effective antimicrobial agents since 64.3% of DEC strains were resistant to it (Bouzari, Jafari, & Zarepoor, 2007).

In the present study, the STEC strains exhibited resistance to more classes of antibiotics, as compared to other pathotypes. As it is well-known, infectious diseases caused by STEC are transmitted to humans primarily through the consumption of contaminated foodstuffs originated from cattle. In the last decades, antimicrobial agents are widely used in livestock for disease prevention or growth promotion. Therefore, it can lead to the emergence of resistance phenotypes in bacteria as a selective advantage (Aslani et al., 2008). Subsequently, humans became exposed to these bacteria via food. In support of our argument, Fereshteh Jafari, Hamidian, et al. (2009) performed a study on Iranian children with acute diarrhea. They found that among DEC categories, STEC had a significantly high resistance rate to commonly used antibiotics, such as amoxicillin and tetracycline.

5. Conclusion

The obtained results pointed to the high levels of fecal contamination in animal source foods. It is worthy to note that environmental pollution with human wastewater or manipulations during the process by people are the most probable source of pathotypes (Winstead et al., 2015). The obtained results suggested that raw milk and cheese made with unpasteurized milk, as well as undercooked red meat, ground meat and hamburgers, may be involved in the transmission of the foodborne infections caused by DEC strains. Furthermore, it is necessary to consider the health risks associated with contamination with multi-drug resistant DEC. This risk is described as the transfer of resistance genes to intestinal pathogens or commensal microfloral residents of the human gut.

The protocol is available for the evaluation of DEC strains in food products, as well as in *E. coli* strains isolated from cases of diarrhea, to monitor the presence of virulence factors. The results of these surveys can shed some light on the improvement of food safety and the prevention of foodborne outbreaks.

CRediT authorship contribution statement

Neda Fallah: Conceptualization, Investigation, Resources, Writing - original draft, Writing - review & editing. **Mehran Ghaemi:** Methodology, Validation. **Kiarash Ghazvini:** Validation. **Mehrnaz Rad:** Validation. **Abdollah Jamshidi:** Supervision, Writing - review & editing, Funding acquisition.

Declaration of competing interest

The authors have no competing interests to declare.

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Appendix A. Supplementary data

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References

Alizade, H., Ghanbarpour, R., & Aflatoonian, M. R. (2014). Molecular study on diarrheagenic *Escherichia coli* pathotypes isolated from under 5 years old children in southeast of Iran. *Asian Pacific Journal of Tropical Disease, 4*, S813–S817. https://doi.org/10.1016/S2222-1808(14)60733-7

Alizade, H., Teshnizi, S. H., Azad, M., Shojae, S., Gouklani, H., Davoodian, P., et al. (2019). An overview of diarrheagenic *Escherichia coli* in Iran: A systematic review and meta-analysis. *Journal of Research in Medical Sciences: The Official Journal of Isfahan University of Medical Sciences, 24*(23). https://doi.org/10.4103/jrms.JRMS_256_18

Amézquita-Montes, Z., Tamborski, M., Kopsombut, U. G., Zhang, C., Arzuza, O. S., & Gómez-Duarte, O. G. (2015). Genetic relatedness among *Escherichia coli* pathotypes isolated from food products for human consumption in Cartagena, Colombia. *Foodborne pathogens and disease, 12*(5), 454–461. https://doi.org/10.1089/fpd.2014.1881

Aslani, M. M., Alikhani, M. Y., Zavari, A., Yousefi, R., & Zamani, A. R. (2011). Characterization of enteroaggregative *Escherichia coli* (EAEC) clinical isolates and their antibiotic resistance pattern. *International Journal of Infectious Diseases, 15*(2), e136–e139. https://doi.org/10.1016/j.ijid.2010.10.002

Aslani, M. M., Salmanzadeh-Ahrabi, S., Alikhani, Y. M., Jafari, F., Zali, R. M., & Mani, M. (2008). Molecular detection and antimicrobial resistance of diarrheagenic *Escherichia coli* strains isolated from diarrheal cases. *Saudi Medical Journal, 29*(3), 388–392.

Bautista-De León, H., Gómez-Aldapa, C., Rangel-Vargas, E., Vázquez-Barrios, E., & Castro-Rosas, J. (2013). Frequency of indicator bacteria, *Salmonella* and diarrhoeagenic *Escherichia coli* pathotypes on ready-to-eat cooked vegetable salads from Mexican restaurants. *Letters in Applied Microbiology, 56*(6), 414–420. https://doi.org/10.1111/lam.12063

Bondoc, I. (2016a). European regulation in the veterinary sanitary and food safety area, a component of the European policies on the safety of food products and the protection of consumer interests: A 2007 retrospective Part One: The role of European institutions in laying down and passing laws specific to the veterinary sanitary and food safety area. *Review Universul Juridic(Supliment)*, 12–15.

Bondoc, I. (2016b). European regulation in the veterinary sanitary and food safety area, a component of the European policies on the safety of food products and the protection of consumer interests: A 2007 retrospective Part Two: Regulations. *Review Universul Juridic(Supliment)*, 16–19.

Bondoc, I. (2016c). European regulation in the veterinary sanitary and food safety area, a component of the European policies on the safety of food products and the protection of consumer interests: A 2007 retrospective. Part Four: Decisions. *Review Universul Juridic(Supliment)*, 24–27.

Bondoc, I. (2016d). European regulation in the veterinary sanitary and food safety area, a component of the European policies on the safety of food products and the protection of consumer interests: A 2007 retrospective. Part Three: Directives. *Rev. Universul Juridic(Supliment)*, 20–23.

Bonyadian, M., Moshtaghi, H., & Taheri, M. A. (2014). Molecular characterization and antibiotic resistance of enterotoxigenic and entero-aggregative *Escherichia coli* isolated from raw milk and unpasteurized cheeses. *Veterinary Research Forum, 5*(1), 29–34.

Bouzari, S., Jafari, A., & Zarepoor, M. (2007). Distribution of genes encoding toxins and antibiotic resistance patterns in diarrhoeagenic *Escherichia coli* isolates in Tehran. *Eastern Mediterranean Health Journal, 13*(2), 287–293.

Brian, M., Frosolono, M., Murray, B., Miranda, A., Lopez, E., Gomez, H., et al. (1992). Polymerase chain reaction for diagnosis of enterohemorrhagic *Escherichia coli* infection and hemolytic-uremic syndrome. *Journal of Clinical Microbiology, 30*(7), 1801–1806. https://doi.org/10.1128/JCM.30.7.1801-1806.1992

Campos, J., Gil, J., Mourão, J., Peixe, L., & Antunes, P. (2015). Ready-to-eat street-vended food as a potential vehicle of bacterial pathogens and antimicrobial resistance: An exploratory study in Porto region, Portugal. *International Journal of Food Microbiology, 206*, 1–6. https://doi.org/10.1016/j.ijfoodmicro.2015.04.016

Canizalez-Roman, A., Gonzalez-Nuñez, E., Vidal, J. E., Flores-Villaseñor, H., & León-Sicairos, N. (2013). Prevalence and antibiotic resistance profiles of diarrheagenic *Escherichia coli* strains isolated from food items in northwestern Mexico. *International Journal of Food Microbiology, 164*(1), 36–45. https://doi.org/10.1016/j.ijfoodmicro.2013.03.020

Canizalez-Roman, A., Velazquez-Roman, J., Valdez-Flores, M. A., Flores-Villaseñor, H., Vidal, J. E., Muro-Amador, S., … Tapia-Pastrana, G. (2019). Detection of antimicrobial-resistance diarrheagenic *Escherichia coli* strains in surface water used to irrigate food products in the northwest of Mexico. *International Journal of Food Microbiology, 304*, 1–10. https://doi.org/10.1016/j.ijfoodmicro.2019.05.017

Castro-Rosas, J., Cerna-Cortés, J. F., Méndez-Reyes, E., Lopez-Hernandez, D., Gómez-Aldapa, C. A., & Estrada-Garcia, T. (2012). Presence of faecal coliforms, *Escherichia coli* and diarrheagenic *E. coli* pathotypes in ready-to-eat salads, from an area where crops are irrigated with untreated sewage water. *International Journal of Food Microbiology, 156*(2), 176–180. https://doi.org/10.1016/j.ijfoodmicro.2012.03.025

Cebula, T. A., Payne, W. L., & Feng, P. (1995). Simultaneous identification of strains of *Escherichia coli* serotype O157: H7 and their shiga-like toxin type by mismatch amplification mutation assay-multiplex PCR. *Journal of Clinical Microbiology, 33*(1), 248–250. https://doi.org/10.1128/JCM.33.1.248-250.1995

Comery, R., Thanabalasuriar, A., Garneau, P., Portt, A., Boerlin, P., Reid-Smith, R. J., et al. (2013). Identification of potentially diarrheagenic atypical enteropathogenic *Escherichia coli* strains present in Canadian food animals at slaughter and in retail meats. *Applied and Environmental Microbiology, 79*(12), 3892–3896. https://doi.org/10.1128/AEM.00182-13

Daniels, N. A., Neimann, J., Karpati, A., Parashar, U. D., Greene, K. D., Wells, J. G., … Quick, R. (2000). Traveler's diarrhea at sea: Three outbreaks of waterborne enterotoxigenic *Escherichia coli* on cruise ships. *Journal of Infectious Diseases, 181*(4), 1491–1495. https://doi.org/10.1086/315397

Darbandi, A., Owlia, P., Bouzari, S., & Saderi, H. (2016). Diarrheagenic *Escherichia coli* pathotypes frequency in Khuzestan province of Iran. *Iranian Journal of Microbiology, 8*(6), 352–358.

Dehkordi, F. S., Yazdani, F., Mozafari, J., & Valizadeh, Y. (2014). Virulence factors, serogroups and antimicrobial resistance properties of *Escherichia coli* strains in fermented dairy products. *BMC Research Notes, 7*(1), 217. https://doi.org/10.1186/1756-0500-7-217

Estrada-Garcia, T., Lopez-Saucedo, C., Thompson-Bonilla, R., Abonce, M., Lopez-Hernandez, D., Santos, J. I., et al. (2009). Association of diarrheagenic *Escherichia coli* pathotypes with infection and diarrhea among Mexican children and association of atypical enteropathogenic *E. coli* with acute diarrhea. *Journal of Clinical Microbiology, 47*(1), 93–98. https://doi.org/10.1128/JCM.01166-08

Feng, P., Weagant, S. D., & Jinneman, K. (2011). BAM: Diarrheagenic *Escherichia coli. silver spring, MD: United States Food and drug administration*. Retrieved from https://www.fda.gov/food/laboratory-methods-food/bam-chapter-4a-diarrheagenic-escherichia-coli.

Gómez-Aldapa, C. A., Cerna-Cortes, J. F., Rangel-Vargas, E., Torres-Vitela, M. R., Villarruel-López, A., Gutiérrez-Alcántara, E. J., et al. (2016). Presence of multidrug-resistant Shiga toxin-producing *Escherichia coli*, enteropathogenic *E. coli* and enterotoxigenic *E. coli*, on raw nopalitos (Opuntia ficus-indica L.) and in nopalitos salads from local retail markets in Mexico. *Foodborne pathogens and disease, 13*(5), 269–274. https://doi.org/10.1089/fpd.2015.2065

Guion, C. E., Ochoa, T. J., Walker, C. M., Barletta, F., & Cleary, T. G. (2008). Detection of diarrheagenic *Escherichia coli* by use of melting-curve analysis and real-time multiplex PCR. *Journal of Clinical Microbiology, 46*(5), 1752–1757. https://doi.org/10.1128/JCM.02341-07

Haghi, F., Zeighami, H., Hajiahmadi, F., Khoshvaght, H., & Bayat, M. (2014). Frequency and antimicrobial resistance of diarrhoeagenic *Escherichia coli* from young children in Iran. *Journal of Medical Microbiology, 63*(3), 427–432. https://doi.org/10.1099/jmm.0.064600-0

Hannah, E. L., Johnson, J. R., Angulo, F., Haddadin, B., Williamson, J., & Samore, M. H. (2009). Molecular analysis of antimicrobial-susceptible and-resistant *Escherichia coli* from retail meats and human stool and clinical specimens in a rural community setting. *Foodborne pathogens and disease, 6*(3), 285–295. https://doi.org/10.1089/fpd.2008.0176

Heijnen, L., & Medema, G. (2006). Quantitative detection of *E. coli, E. coli* O157 and other shiga toxin producing *E. coli* in water samples using a culture method combined with real-time PCR. *Journal of Water and Health, 4*(4), 487–498. https://doi.org/10.2166/wh.2006.026

Jafari, F., Garcia-Gil, L., Salmanzadeh-Ahrabi, S., Shokrzadeh, L., Aslani, M., Pourhoseingholi, M., et al. (2009). Diagnosis and prevalence of enteropathogenic bacteria in children less than 5 years of age with acute diarrhea in Tehran children's hospitals. *Journal of Infection, 58*(1), 21–27. https://doi.org/10.1016/j.jinf.2008.10.013

Jafari, F., Hamidian, M., Rezadehbashi, M., Doyle, M., Salmanzadeh-ahrabi, S., Derakhshan, F., et al. (2009b). Prevalence and antimicrobial resistance of diarrheagenic *Escherichia coli* and *Shigella* species associated with acute diarrhea in Tehran, Iran. *The Canadian Journal of Infectious Diseases & Medical Microbiology, 20*(3), e56–e62. https://doi.org/10.1155/2009/341275

de Lagarde, M., Larrieu, C., Praud, K., Schouler, C., Doublet, B., Sallé, G., … Arsenault, J. (2019). Prevalence, risk factors, and characterization of multidrug resistant and extended spectrum β-lactamase/AmpC β-lactamase producing *Escherichia coli* in healthy horses in France in 2015. *Journal of Veterinary Internal Medicine, 33*(2), 902–911. https://doi.org/10.1111/jvim.15415

Lima, C. M., Souza, I. E. G. L., dos Santos Alves, T., Leite, C. C., Evangelista-Barreto, N. S., & de Castro Almeida, R. C. (2017). Antimicrobial resistance in diarrheagenic *Escherichia coli* from ready-to-eat foods. *Journal of Food Science and Technology, 54*(11), 3612–3619. https://doi.org/10.1007/s13197-017-2820-4

Maheux, A. F., Picard, F. J., Boissinot, M., Bissonnette, L., Paradis, S., & Bergeron, M. G. (2009). Analytical comparison of nine PCR primer sets designed to detect the presence of *Escherichia coli/Shigella* in water samples. *Water Research, 43*(12), 3019–3028. https://doi.org/10.1016/j.watres.2009.04.017

Miri, S. T., Dashti, A., Mostaan, S., Kazemi, F., & Bouzari, S. (2017). Identification of different *Escherichia coli* pathotypes in north and north-west provinces of Iran. *Iranian Journal of Microbiology, 9*(1), 33–37.

Mohammadi, P., & Abiri, R. (2012). Isolation of Enteropathogenic *Escherichia coli* (EPEC) from raw milk in Kermanshah by polymerase chain reaction (PCR). *Jundishapur Journal of Microbiology, 6*(4), e5439. https://doi.org/10.5812/jjm.5439

Momtaz, H., & Jamshidi, A. (2013). Shiga toxin-producing *Escherichia coli* isolated from chicken meat in Iran: Serogroups, virulence factors, and antimicrobial resistance properties. *Poultry Science, 92*(5), 1305–1313. https://doi.org/10.3382/ps.2012-02542

Momtaz, H., Safarpoor Dehkordi, F., Rahimi, E., Ezadi, H., & Arab, R. (2013). Incidence of Shiga toxin-producing *Escherichia coli* serogroups in ruminant's meat. *Meat Science, 95*(2), 381–388. https://doi.org/10.1016/j.meatsci.2013.04.051

Perna, N. T., Plunkett, G., 3rd, Burland, V., Mau, B., Glasner, J. D., Rose, D. J., … Kirkpatrick, H. A. (2001). Genome sequence of enterohaemorrhagic *Escherichia coli* O157: H7. *Nature, 409*(6819), 529–533. https://doi.org/10.1038/35054089

Pourakbari, B., Heydari, H., Mahmoudi, S., Sabouni, F., Teymuri, M., Ferdosian, F., … Mamishi, S. (2013). Diarrhoeagenic *E. coli* pathotypes in children with and without diarrhoea in an Iranian referral paediatrics centre. *Eastern Mediterranean Health Journal, 19*(7), 617–621.

Rappelli, P., Maddau, G., Mannu, F., Colombo, M., Fiori, P., & Cappuccinelli, P. (2001). Development of a set of multiplex PCR assays for the simultaneous identification of enterotoxigenic, enteropathogenic, enterohemorrhagic and enteroinvasive *Escherichia coli*. *New Microbiologica, 24*(1), 77–83.

Ratchtrachenchai, O.-A., Subpasu, S., & Ito, K. (1997). Investigation on enteroaggregative *Escherichia coli* infection by multiplex PCR. *Bulletin of the Department of Medical Science, 39*(4), 211–220.

Regua-Mangia, A. H., Gomes, T.n. A., Vieira, M.n. A., Irino, K., & Teixeira, L. M. (2009). Molecular typing and virulence of enteroaggregative *Escherichia coli* strains isolated from children with and without diarrhoea in Rio de Janeiro city, Brazil. *Journal of Medical Microbiology, 58*(4), 414–422. https://doi.org/10.1099/jmm.0.006502-0

Robbins, A., Anand, M., Nicholas, D. C., Egan, J. S., Musser, K. A., Giguere, S., et al. (2014). Ground beef recall associated with non-O157 Shiga toxin–producing *Escherichia coli*, United States. *Emerging Infectious Diseases, 20*(1), 165–167. https://doi.org/10.3201/eid2001.130915

de la Rosa-Hernandez, M. C., Cadena-Ramírez, A., Téllez-Jurado, A., Gomez-Aldapa, C. A., Rangel-Vargas, E., Chávez-Urbiola, E. A., et al. (2018). Presence of multidrug-resistant shiga toxin–producing *Escherichia coli*, Enteropathogenic *Escherichia coli*, and enterotoxigenic *Escherichia coli* on fresh cheeses from local retail markets in Mexico. *Journal of Food Protection, 81*(11), 1748–1754. https://doi.org/10.4315/0362-028X.JFP-18-166

Sakhaie Shahreza, M. H., Rahimi, E., & Momtaz, H. (2017). Shiga-toxigenic *Escherichia coli* in ready-to-eat food staffs: Prevalence and distribution of putative virulence factors. *Microbiology Research, 8*(2), 88–92. https://doi.org/10.4081/mr.2017.7244

Scavia, G., Staffolani, M., Fisichella, S., Striano, G., Colletta, S., Ferri, G., et al. (2008). Enteroaggregative *Escherichia coli* associated with a foodborne outbreak of gastroenteritis. *Journal of Medical Microbiology, 57*(9), 1141–1146. https://doi.org/10.1099/jmm.0.2008/001362-0

Schmidt, H., Knop, C., Franke, S., Aleksic, S., Heesemann, J., & Karch, H. (1995). Development of PCR for screening of enteroaggregative *Escherichia coli*. *Journal of*

Clinical Microbiology, 33(3), 701–705. https://doi.org/10.1128/JCM.33.3.701-705.1995

Soborg, B., Lassen, S. G., Muller, L., Jensen, T., Ethelberg, S., Mølbak, K., et al. (2013). A verocytotoxin-producing *E. coli* outbreak with a surprisingly high risk of haemolytic uraemic syndrome, Denmark, September-October 2012. *Euro Surveillance, 18*(2), 20350.

Uysal, A., & Durak, Y. (2012). Pulsed-field gel electrophoresis typing, antibiotic resistance, and plasmid profiles of *Escherichia coli* strains isolated from foods. *Canadian Journal of Microbiology, 58*(11), 1278–1287. https://doi.org/10.1139/w2012-108

Vidal, M., Kruger, E., Durán, C., Lagos, R., Levine, M., Prado, V., et al. (2005). Single multiplex PCR assay to identify simultaneously the six categories of diarrheagenic *Escherichia coli* associated with enteric infections. *Journal of Clinical Microbiology, 43*(10), 5362–5365. https://doi.org/10.1128/JCM.43.10.5362–5365.2005

Vidal, R., Vidal, M., Lagos, R., Levine, M., & Prado, V. (2004). Multiplex PCR for diagnosis of enteric infections associated with diarrheagenic *Escherichia coli*. *Journal of Clinical Microbiology, 42*(4), 1787–1789. https://doi.org/10.1128/JCM.42.4.1787–1789.2004

Wayne, P. (2015). *Clinical and laboratory standards Institute: Performance standards for antimicrobial susceptibility testing: 25th informational supplement*. CLSI. document M100-S25.

Winstead, A., Hunter, J. C., & Griffin, P. M. (2015). CDC yellow book: Chapter 4; travel-related infectious diseases CDC health information for international travel. Retrieved from https://wwwnc.cdc.gov/travel/yellowbook/2020/travel-related-infectious-diseases/escherichia-coli-diarrheagenic.

Zhang, S., Wu, Q., Zhang, J., & Zhu, X. (2016). Occurrence and characterization of enteropathogenic *Escherichia coli* (EPEC) in retail ready-to-eat foods in China. *Foodborne pathogens and disease, 13*(1), 49–55. https://doi.org/10.1089/fpd.2015.2020