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ORIGINAL ARTICLE

Occurrence of diarrheagenic *Escherichia coli* pathotypes from raw milk and unpasteurized buttermilk by culture and multiplex polymerase chain reaction in southwest Iran

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Abstract

Background In developing countries including Iran, there are limited data on diarrheagenic *Escherichia coli* (DEC) contamination in milk and unpasteurized buttermilks. This study aimed to determine the occurrence of DEC pathotypes by culture and multiplex polymerase chain reaction (M-PCR) in some dairy products from southwest Iran.

Methods and results In this cross-sectional study (September to October 2021), 197 samples (87 unpasteurized buttermilk and 110 raw cow milk) were collected from dairy stores of Ahvaz, southwest Iran. The presumptive *E. coli* isolates were primarily identified using biochemical tests and then confirmed by PCR of *uidA* gene. The occurrence of 5 DEC pathotypes: enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), and enteroinvasive *E. coli* (EIEC) were investigated using M-PCR. Overall, 76 (76/197, 38.6%) presumptive *E. coli* isolates were identified by biochemical tests. Using *uidA* gene, only 50 isolates (50/76, 65.8%) were confirmed as *E. coli*. DEC pathotypes were detected in 27 of 50 (54.0%) *E. coli* isolates (74.1%, 20/27 from raw cow milk and 25.9%, 7/27 from unpasteurized buttermilk). The frequency of DEC pathotypes was as follows: 1 (3.7%) EAEC, 2 (7.4%) EHEC, 4 (14.8%) EPEC, 6 (22.2%) ETEC, and 14 (51.9%) EIEC. However, 23 (46.0%) *E. coli* isolates had only the *uidA* gene and were not considered DEC pathotypes.

Conclusion Possible health risks for Iranian consumers can be attributed to the presence of DEC pathotypes in dairy products. Hence, serious control and prevention efforts are needed to stop the spread of these pathogens.

Keywords Diarrheagenic *Escherichia coli* · Milk · M-PCR · Iran

Introduction

Escherichia coli is a facultative anaerobic bacterium and an important species of the microbial flora of the human and animal digestive tract, belonging to the family *Enterobacteriaceae* [1]. Because these bacteria are found in the intestines of animals, they contaminate cow's milk during the milking process due to inadequate hygiene practices [2, 3]. Therefore, *E. coli* is often considered a reliable indicator and good marker for direct or indirect fecal contamination, as well as for the presence of enteric pathogens in raw milk and raw milk products [2].

There is a great deal of global concern regarding the safety of dairy products and dairy by-products in relation to food-borne diseases [4]. According to the World Health Organization (WHO), food-borne pathogens are responsible

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for 600,652,361 disease cases and 418,608 deaths globally. While most *E. coli* strains are commensal, certain pathogenic strains contribute to various infections due to the presence of specific virulence factors [5]. Several dairy products, including cheese, milk, and yogurt may contribute to the transmission of the pathogenic bacteria harboring antibiotic resistant genes to human. Therefore, dairy products serves as a very efficient means of transferring antimicrobials resistance (AMR) factors into consumers' intestinal tracts [4]. Among the 31 major food-borne pathogens, *E. coli* is one of the main causes of adverse health effects [6].

Pathogenic *E. coli* are divided into two groups: those responsible for gastrointestinal diseases, known as intestinal pathogenic *E. coli* (IPEC) or diarrheagenic *E. coli* (DEC), and those responsible for extraintestinal diseases, caused by extraintestinal pathogenic *E. coli* (ExPEC) [7]. IPEC (DEC) strains are classified into the following pathotypes: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), Shiga-toxin producing (enterohemorrhagic) *E. coli* (STEC/EHEC) *E. coli*, enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), a subclass of EAEC, and adherent invasive *E. coli* (AIEC) [7–10]. *E. coli* strains possess a plethora of mobile genetic elements that enable them to cause a variety of gastrointestinal diseases, as well as septicemia, meningitis, and urinary tract infections [1].

ETEC is responsible for 380,000 deaths in children under five years of age per year and 840 million gastrointestinal illnesses that is characterized by its ability to produce heat-labile (LT) and/or heat-stable (ST) enterotoxins [1, 11]. EPEC is considered to be responsible for 5–10% of all pediatric diarrheal diseases in countries such as Iran. EPEC strains can be classified into two types: typical EPEC (tEPEC) pathotypes, which carry the EPEC adherence factor (EAF) plasmid, and atypical EPEC (aEPEC) pathotypes, which lack this plasmid. Furthermore, aEPEC strains do not express or synthesize the bundle-forming pilus (BFP), a type-4 fimbriae [12]. EHEC or Shiga (Vero) toxin-producing *E. coli* (STEC/VTEC) strains are a significant pathogenic group of *E. coli*, capable of causing serious infection in humans. According to epidemiological studies, cattle typically contain VTEC in their feces and hence may be a significant source of infection. Contamination of raw and unpasteurized milk and dairy products with VTEC has the potential to enter the human food chain [13]. EAEC appears to be an important cause of diarrheal disease in children which is potentially fatal if untreated [14]. EIEC, like *Shigella* species, is associated with community diarrhea and has the potential to cause foodborne epidemics. Also, DAEC strains have been associated with diarrhea [14, 15].

The multiplex polymerase chain reaction (M-PCR), is a quick and efficient gene detection tool that is becoming increasingly popular in the identification of bacteria [16, 17]. M-PCR uses more than one pair of primers in the same reaction mixture to amplify several target sequences at the same time [17]. It has been frequently employed to detect *E. coli* pathotypes, and numerous multiplexes have been designed for this purpose. Each *E. coli* subgroup has its own set of genes that code for virulence proteins that affect host physiology [18]. The most important genes for detection of each pathotype are as follows: *stx* for STEC; enterotoxin encoding genes *elt* and *est* for ETEC; *bfpA* and intimin encoding gene (*eaeA*) for EPEC; *stx1, stx2*, and *eaeA* genes for EHEC; plasmid *pCVD432* gene for EAEC; and invasion gene (*invE*), *ial* and *ipaH* genes for EIEC [2, 19–21].

This study aimed to determine the occurrence of DEC pathotypes by culture and M-PCR in raw milk and unpasteurized buttermilk samples in Ahvaz, southwest Iran.

Materials and methods

Ethics approval

This study was approved by the Research Ethics Committee (REC) of the Ahvaz Jundishapur University of Medical Sciences (No: IR.AJUMS.REC.1397.438), Ahvaz, Iran following the Declaration of Helsinki.

Sample collection

In this cross-sectional study from September to October 2021, 197 samples, including 87 unpasteurized buttermilk (Persian yogurt drink or doogh) and 110 raw cow milk (at least 250 ml each) were collected from dairy stores in different parts of Ahvaz, southwest Iran. Each region was randomly sampled, with a minimum of 9 milk and 9 buttermilk samples. The samples were placed in a cold box with dry ice and immediately delivered to the microbiology laboratory of the Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

Isolation and identification of *E. coli* pathotypes

Nearly 10 µL of the collected milk and buttermilk samples were aseptically cultured on blood agar and McConkey agar (Merck, Germany). The cultured plates were then incubated at 37 °C for 24 h. Lactose-positive colonies (pink) were isolated and purified from bacteria. Standard biochemical tests such as oxidase, sulfide indole motility (SIM), triple sugar iron (TSI), Methyl Red/Voges-Proskauer (MR-VP), indole, and citrate (IMViC) were used to identify presumptive *E. coli* isolates [6, 22]. After primary identification, *E. coli* isolates were frozen at -80 °C in trypticase soy broth

Table 1 The primers sequences and the products sizes of the studied genes used in this study

Target organism	Target gene	Primer sequence (5′-3′)	Size of product (bp)	Reference
Escherichia coli	*uidA*	F: AGCCAGTCCAGCGTTTTTGCT R: AAAGTGTGGGTCAATAATCAGGAAGTG	1487	[19]
ETEC	*est*	F: TGTCTTTTTCACCTTTCGCTC R: CGGTACAAGCAGGATTACAACAC	171	[19]
ETEC	*elt*	F: TCTATGTGCATACGGAGC R: ATACTGATTGCCGCAAT	322	[24]
EPEC	*eaeA*	F: TTATGGAACGGCAGAGGT R: CTTCTGCGTACTGCGTTCA	790	[24]
EAEC	*pCVD432*	F: CTGGCGAAAGACTGTATCAT R: CAATGTATAGAAATCCGCTGTT	630	[24]
EHEC	*stx*	F: ACGAAATAATTTATATGT R: TGATTGTTACAGTCAT	900	[24]
EIEC	*invE*	F: ATATCTCTATTTCCAATCGCGT R: GATGGCGAGAAATTATATCCCG	382	[25]

(TSB) with 15% glycerol for long-term storage. The presumptive *E. coli* isolates were further confirmed with the PCR method in next step. Positive control strains were purchased from the Iranian Biological Resources Center (Tehran, Iran). Before the PCR stage, the isolates were thawed at room temperature and cultured on nutrient broth (Merck, Germany) and incubated at 37 °C for 24 h. The grown colonies were used for DNA extraction.

DNA extraction

The boiling method was used to extract DNA from *E. coli* isolates. For this, 3–5 pure colonies of each isolate were dissolved in a microtube containing 500 µL of sterile distilled water (DW) and shaken with a shaker to completely dissolve the samples. The microtubes were placed in a thermoblock at 95 °C for 10 to 15 min. To induce a thermal shock, the microtubes were immediately placed in a freezer at -20 °C for 10 min. The microtubes were then centrifuged for 10 min at 14,000 rpm. Subsequently, the supernatant solution was transferred to new sterile microtubes and used as template DNA for PCR [23].

PCR amplification

First, to confirm the identified *E. coli* isolates, all of them were examined for the presence of *uidA* gene (*E. coli* control gene) by singleplex PCR. Then, the presence of *elt, est, pCVD432, stx, invE*, and *eaeA* genes were investigated by M-PCR to detect different DEC pathotypes using previously published primers (Bioneer Corporation, Korea) (Table 1) [19, 24, 25]. The singleplex PCR reaction of the *uidA* gene was prepared in a final volume of 25 µL, so that each of primers (1 µL) F and R was mixed at a concentration of 10 µM with 12.5 µL of master mix (Pishgam, Iran), 5 µL of extracted DNA, and 5.5 µL of DW. Amplification temperature program includes: initial denaturation at 94 °C for 5 min, 30 cycles including: denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s and extension at 72 °C for 1 min, with a final cycle of extension at 72 °C for 5 min.

Two M-PCR series including A for *elt, estb, pCVD432* and B for *stx, invE*, and *eaeA* genes were designed to amplify the desired target. The reactions were carried out in a final volume of 25 µL, and the primer concentration in the reactions was determined to be 0.4–0.8 µL. The M-PCR was performed in an Eppendorf thermocycler (Germany) with the following temperature program: initial denaturation at 94 °C for 5 min, 35 cycles including denaturation at 94 °C for 30 s, annealing 30 s (for set A at 55 °C and for set B at 60 °C), extension at 72 °C for 1 min, and a final cycle of extension at 72 °C for 5 min. Positive control strains harboring studied genes were purchased from the Pasteur Institute of Iran (Tehran, Iran). All PCR products were isolated by 1.5% agarose gel (Sinacolon, Iran) electrophoresis (80 V, 40 min) in TAE (Tris/Acetate/EDTA) buffer 1X containing DNA safe stain (Yektatajhiz, Iran) and visualized under UV light.

Statistical analysis

The descriptive statistical analysis was carried out using SPSS version 22.0 software (IBM Analytics, USA). Significant correlations (P-value < 0.05) was determined using Fisher's exact test or Chi-square test.

Results

Detection of *E. coli* isolates by biochemical tests and PCR

Of the 197 samples, 144 (144/197, 73.1%) bacterial isolates were identified and 53 (53/197, 26.9%) samples lack any bacterial growth. Out of 144 bacterial isolates, 28 (28/144,

Table 2 Frequency of diarrheagenic *Escherichia coli* (DEC) pathotypes based on sample type

DEC pathotypes	Total (n = 197) n (%)	Raw cow milk (n = 110) n (%)	Unpasteurized buttermilk (n = 87) n (%)	*P*-value
ETEC	6 (3.0)	3 (2.7)	3 (3.4)	> 0.999
EAEC	1 (0.5)	1 (0.9)	0 (0.0)	> 0.999
EPEC	4 (2.0)	3 (2.7)	1 (1.1)	0.632
EHEC	2 (1.0)	2 (1.8)	0 (0.0)	0.504
EIEC	14 (7.1)	11 (10.0)	3 (3.4)	0.096
Total	27 (13.7)	20 (18.2)	7 (8.0)	0.059

DEC: Diarrheagenic *Escherichia coli*, ETEC: Enterotoxigenic *Escherichia coli*, EAEC: Enteroaggregative *Escherichia coli*, EPEC: Enteropathogenic *Escherichia coli*, EHEC: Enterohemorrhagic *Escherichia coli*, EIEC: Enteroinvasive *Escherichia coli*

19.4%) Gram-positive staphylococci and 116 (116/144, 80.6%) coliforms bacteria were identified. In total, 76 (76/197, 38.6%) presumptive *E. coli* isolates were identified by culture and standard biochemical tests. Using PCR method for *uidA* gene, only 50 presumptive *E. coli* isolates (50/76, 65.8%) showed positive amplicon and confirmed as *E. coli* strains. Thus, the true prevalence of *E. coli* isolates was 25.4% (50/197). Out of the 50 *E. coli* isolates, 34 (34/50, 68.0%) isolates were from raw cow milk and 16 (16/50, 32.0%) isolates were from unpasteurized buttermilk. The prevalence rate of *E. coli* isolates was significantly higher in raw cow milk samples than in unpasteurized buttermilk samples (*P*-value = 0.049).

Detection of DEC pathotypes by M-PCR

In total, 54.0% (27/50) of *E. coli* isolates were identified as DEC pathotypes that accounted for 13.7% (27/197) frequency rate of these pathotypes in dairy samples. Of these DEC pathotypes, 20 (74.1%) and 7 (25.9%) isolates were detected from raw cow milk and unpasteurized buttermilk samples, respectively. The prevalence rate of DEC pathotypes was not significantly different in raw cow milk and unpasteurized buttermilk samples (*P*-value = 0.059). ETEC isolates accounted for 22.2% (6/27) of the DEC pathotypes. Each of *est* and *elt* genes were detected in 3 (50.0%) ETEC isolates. The occurrence of other DEC pathotypes was as follows: 1 (3.7%) EAEC, 4 (14.8%) EPEC, 2 (7.4%) EHEC, and 14 (51.9%) EIEC isolates. The most and the least frequent pathotypes were EIEC and EAEC, respectively. However, 23 (46.0%) *E. coli* isolates had only the *uidA* gene and were not categorized as DEC pathotypes. These isolates may be related to other DEC strains that were not investigated or may be non-pathogenic or normal flora strains.

Distribution of DEC pathotypes in dairy products

The distribution of different DEC pathotype in unpasteurized buttermilk and raw cow milk samples were shown in Table 2. In total, 8.0% (7/87) of unpasteurized buttermilk and 18.2% (20/110) of raw cow milk samples were contaminated with DEC pathotypes. EIEC with frequency rate of 10.0% (11/110) was the most prevalent DEC pathotype in row cow milk samples, whereas ETEC and EIEC with frequency rate of 3.4% (3/87) were the most predominant DEC isolates in unpasteurized buttermilk. All 5 DEC pathotypes were identified in raw cow milk samples, whereas EAEC and EHEC were not detected in unpasteurized buttermilk samples.

Discussion

To the best of our knowledge, studies that evaluate all *E. coli* pathotypes in Iranian dairy products are very few and most of them have studied a specific strain or have not determined the type of strain [26, 27]. This study showed the prevalence rate of 25.4% (50/197) for *E. coli* isolates in raw cow milk and unpasteurized buttermilk samples from southwest Iran that was almost similar to previous report from Iran (27.0%, 54/200) [6]. In a previous study from Romania, a higher isolation rate (81.1%) of *E. coli* was reported in raw milk cheese [28]. Also, higher prevalence rates of *E. coli* isolates were reported by Fallah et al. [29] from Iran (68.7%, raw milk/cheese samples), Zhang et al. [30] from China (52.4%, retail fresh milk), and Ribeiro et al. [31] (66.67%, raw milk samples) from Brazil. High frequency rates of *E. coli* isolates may be due to unsanitary and poor hygiene conditions during preparation of dairy products. Moreover, the discrepancies among different studies can be explained by the seasonal variations in the area, and the differences in study design, sample sources, and the bacterial isolation methods [28, 32].

In this study, the presence of 5 DEC pathotypes were investigated using M-PCR. To date, there has been a limited number of Iranian studies investigating all DEC pathotypes in various dairy products [6, 29]. In total, 54.0% (27/50) of *E. coli* isolates were identified as DEC pathotypes that accounted for 13.7% (27/197) frequency rate of these pathotypes in dairy samples. In previous study from Iraq, no DEC pathotypes were detected in any of the raw milk samples [32]. Previous studies from Iran by Madani et al. [6] (19.0%, 38/200) and Fallah et al. [29] (12.6%, 13/103) reported different prevalence rates of DEC pathotypes than the current study. This indicated the probable fecal contamination of raw milk and unpasteurized buttermilk during the preparation of dairy products in our region. These

inconsistencies among various studies can be explained by the differences in origin of samples, studied DEC pathotypes, and the sample size.

In this study, EIEC with frequency rate of 7.1% (14/197) was the most prevalent DEC pathotype. In contrast to this study, Madani et al. [6] and Fallah et al. [29] from Iran, reported EPEC and STEC as the most frequent DEC pathotypes in dairy products, respectively. EIEC was detected in 3.5% (7/200) of dairy products by Madani et al. [6], whereas, no EIEC was found in the study of Fallah et al. [29]. In another study by Liu et al. [33] from China, 34.4% (67/195) of raw milk samples were positive for *E. coli* pathotypes, among which EIEC accounted for 1.5% (1/67). In a study by Dell'Orco et al. [34] from Italy, EIEC isolates were the second most prevalent pathotypes (11.3%, 6/53) in bulk tank milk and raw milk filters. Since EIEC isolates were relatively abundant in dairy products of our region, more attention should be given to these pathotypes to prevent enteric infections in humans and animals [34].

ETEC with frequency rate of 3.0% (6/197) was the second most widespread DEC pathotype in our study. This incidence rate was almost similar to a previous report by Fallah et al. [29] from Iran (3.8%). In another study from Iran, no ETEC isolate was detected in dairy products [35].

In this study, EPEC pathotypes were detected in 2.0% (4/197) of dairy samples that was lower than previous studies from Iran [29], China [33], and Brazil [36]. Also, Eldesoukey et al. [37] from Egypt reported a higher incidence rate (5.3%, 8/150) of EPEC pathotypes in milk samples than the current study.

Other findings of this study were the frequency rate of 1.0% (2/197) for EHEC and 0.5% (1/197) for EAEC pathotypes which were only detected in raw cow milk samples. No unpasteurized buttermilk samples were positive for these two DEC pathotypes. In a previous study from Iran, the EAEC pathotypes were found in 4 (3.6%) of 111 of raw milk samples and no one of 39 cheese samples were positive for these pathotypes [29]. However, in contrast to the current study, no EAEC isolate was found in previous study by Taha et al. [32] from Iraq. Vanitha et al. [38] from India reported EHEC in 8.8% (11/125) of raw milk samples which was higher than the current results.

In this study, the prevalence of DEC pathotypes in raw cow milk samples was not significantly different with their incidence in unpasteurized buttermilk samples. This observation was similar to previous study from Iran [29]. The differences in the prevalence of DEC pathotypes in this study and previous studies from different countries may be explained by several reasons including the difference in the type and origin of sample examined, the sample size studied, and most importantly, the DEC pathotype detection method. To date, researchers use different virulence factors to detect DEC pathotypes, which may cause differences in results. DEC pathotypes are widely detected and studied with PCR-based molecular methods because they are sensitive, specific, and relatively fast. However, there are issues with defining molecular targets for EAEC and DAEC that make molecular diagnostics ineffective for all DEC pathotypes [39].

In this study, 46.0% (23/50) of *E. coli* isolates had only the *uidA* gene. Hence, we could not assign them to any DEC pathotype. These isolates may belong to commensal strains of *E. coli* or may be DEC pathotypes that contain other virulence genes that were not investigated in this study. In a previous study from Iran, 74.4% (154/207) of *E. coli* isolates from meat and dairy products were not classified into any DEC pathotype [29].

This study had some limitations. Because all virulence genes were not investigated in this study, a majority of isolates were not classified as DEC pathotype. It is recommended to assay other DEC-associated virulence factors with a larger sample size. Also, in this study, the antibiotic resistance patterns and other drug resistance genes were not investigated. Hence, a more in depth study is needed to reveal further epidemiological features of DEC pathotypes in dairy products in Iran.

Conclusion

This study revealed the potential health risk of dairy products for their customers. Based on the results of this study, it is imperative that operators and personnel involved in the production of traditional dairy products receive appropriate hygiene measures, including regular hygienic supervision and training.

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Authors' contributions Amanollah Zarei Ahmady, Raziyeh Aliyan Aliabadi, and Mansour Amin: Conceptualization, Methodology, Writing-Original draft preparation, Writing-Reviewing and Editing, Formal analysis. Abdoghani Ameri and Effat Abbasi Montazeri: Methodology, Data curation, Formal analysis, Writing- Original draft preparation. All authors read and approved the final manuscript.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The authors have no conflict of interest.

Ethics approval This study was approved by the Research Ethics Committee (REC) of the Ahvaz Jundishapur University of Medical Sciences (No: IR.AJUMS.REC.1397.438), Ahvaz, Iran following the Declaration of Helsinki.

Consent to participate Not applicable.

Consent for publication Not applicable.

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