

Clonal Relatedness of Enterotoxigenic and Enteropathogenic *Escherichia coli* Isolates from Diverse Human, Foods and Calf Sources

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Abstract

Background: Foodborne infection caused by Enterotoxigenic *Escherichia coli* (ETEC) and Enteropathogenic *Escherichia coli* (EPEC) is one of the major health problems, particularly in the developing countries. Therefore, it is vital to identify the origin of food contamination to plan control strategies efficiently.

Method: A total of 219 *E. coli* isolates from human and calf feces, raw meat, and dairy product samples were screened for virulence genes of ETEC and EPEC pathotypes by duplex-PCR assay. Then, rep-PCR was performed for the pathotypes. DNA fingerprints were analyzed with NTSYS-pc program, and dendrogram was generated.

Results: Among the *E. coli* isolates, ETEC (6.4%), typical-EPEC (3.2%) and atypical-EPEC (5.5%) were detected. Dendrogram analysis showed two clusters; all human ETEC isolates and one meat ETEC isolate were grouped under cluster A, and all EPEC isolates collected from the four sources along with two animal fecal ETEC isolates and one ETEC isolate from meat products were grouped under cluster B. According to Jackknife analysis, the average percentage of ETEC and EPEC strains that were accurately clustered were 98% and 93.75%, respectively.

Conclusion: Animal source food (ASF) isolates were placed in the same phylogenetic group as calf isolates. Moreover, the positioning of human and animal isolates in two separate groups suggested the genetic diversity between these two groups. Thus, it could be argued that *E. coli* isolates from animals may be transmitted via meat and dairy products, emphasizing the necessity of applying more accurate standards in the processing of ASFs.

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Introduction

Infections and foodborne diseases caused by bacterial contamination are some of the major health problems in different countries (1-4). In this regard, animal source foods (ASFs), including raw dairy and meat products, which fulfill a large part of human nutritional needs, play a crucial role in disease transmission to humans (5). Many studies have reported the prevalence of microbial contamination of food products, particularly by pathogenic *Escherichia coli*, suggesting that such contamination significantly endangers food quality (1,6). *E. coli* is a commensal bacterium in the intestinal tract of humans and animals and is one of the main causes of diarrhea, particularly in developing countries (1). Moreover, this bacterium is an indicator of fecal contamination, and its presence in food could imply the possibility of the presence of other gastrointestinal pathogens (e.g., *Salmonella*, *Shigella*, Hepatitis A, and Norwalk group viruses) in the food, which may threaten public health (5,7). Enterotoxigenic *E. coli* (ETEC), one of the pathotypes of *E. coli*, causes pediatric and traveler's diarrhea leading to various diseases through heat-stable (ST) and heat-labile (LT) enterotoxins (8,9). Another important pathotype of the species is enteropathogenic *E. coli* (EPEC), which is the primary cause of diarrhea in infants, particularly in developing countries. EPEC strains exist in two forms: typical EPEC (harboring *eae* and *bfp* genes) and atypical EPEC (lacking *bfp* gene) (2,8). Both pathotypes are highly prevalent in developing and poor countries and are transmitted through contaminated food. Cattle and sheep are the main sources of pathogenic *E. coli*, and contaminated foods from these sources are important factors of disease transmission to humans (10,11). Contamination of raw meat products is primarily caused by cattle feces during slaughter, de-hiding

process, and removal of viscera (12). Dairy products may also be contaminated as a result of using unpasteurized milk or milking by hand. Moreover, contamination can occur because of failure to comply with personal and environmental hygiene standards at manufacturing and processing centers (5,12). Therefore, identifying the source of fecal contamination to prevent food poisoning and food-borne diseases and determining strategies to control these infections will be an effective step in promoting health and food safety in the community (13,14). Several methods have been proposed for DNA fingerprinting and detecting fecal contamination and clonal relationships between various pathogenic *E. coli* strains (15). These methods include pulsed field gel electrophoresis (PFGE), ribotyping, ribosomal DNA heterogeneity, random amplified polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR) and repetitive extragenic palindromic-PCR (rep-PCR) (7,15-17). Among these methods rep-PCR has been proposed as an analytical tool for nationwide or worldwide epidemiology studies (18). The aim of this study was to investigate the clonal relationship of *E. coli* isolates from fecal samples of human and cattle as well as food samples using rep-PCR to identify the risk of ETEC and EPEC transmission to human through consumption of contaminated foods.

Materials and Methods

Sample collection

In this study, a total of 506 samples, including human (n=224) and calf (n=60) feces, raw meat products including meat, ground meat, and hamburgers (n=120), and raw dairy products including milk, traditional cheese, buttermilk, and yogurt (n=102) were collected. All of the food samples were raw and made traditionally. Human fecal specimens were

collected from in- and out-patients with diarrhea who were recently not taking any antibiotics prior to sampling. Moreover, calf fecal specimens were obtained from different farms in Northwest of Iran. Stool specimens were collected by rectal swabs and placed in Carry-Blair transport medium and were immediately processed.

Bacterial isolation

Stool and food samples were examined as follows: for the enrichment of the *E. coli* strains, all specimens cultured in Lauryl sulphate broth (Merck, Germany) overnight at 37°C were subsequently streaked onto MacConkey agar (Merck, Germany) and incubated at 37°C for 24 h. As a rule, 2-3 lactose-fermenting colonies were identified by standard biochemical tests including oxidase negative, indole positive, Simon's citrate negative, urease negative and hydrogen sulfide negative (1,19).

PCR detection of virulence factors in ETEC and EPEC strains

DNA was prepared by using the Promega DNA extraction kit (A1125, USA), following the instructions given by the manufacturer. The DNA templates were examined for the presence of the virulence factors by two separate duplex PCRs

with specific primers (Table 1) for detection of *eae* and *bfp* genes for EPEC, and *lt* and *st* genes for ETEC (20,21). The PCR assays were accomplished in a 25 µl reaction mixture, consisting of 2X PCR Master Mix (2X concentrated solutions of *Taq* DNA polymerase, reaction buffer, MgCl₂ and dNTPs) (CinnaGen Inc., Iran), 1 µl of DNA template and a 0.5 µM concentration (each) primers with a BioRad T100™ thermal cycler. Primers were provided by GeNetBio Inc. (Korea). Duplex PCR assays were subjected to two plans of amplification. First plan (for detection of ETEC) consisted an initial denaturation at 95°C for 5 min for one cycle followed by 35 cycles of 95°C for 45 sec, 49°C for 45 sec, 72°C for 45 sec and final extension at 72°C for 7 min. The second plan (for detection of EPEC) consisted an initial denaturation at 95°C for 3 min for one cycle followed by 38 cycles of 95°C for 1 min, 53°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. Amplified PCR products were observed after electrophoresis on 1% agarose and staining with safe dye (CinnaGen Inc., Iran). The PCR products were visualized under UV transilluminator and photographed. In this study the reference strains including ETEC (H10407) and EPEC (2348/69) were used as positive controls.

Table 1. Primers used for detection of ETEC and EPEC virulence genes

Target organism	Target genes	Gene location	Primer sequences (5'→3')	Product size (bp)
ETEC	<i>st</i>	Plasmid	F: ATT TTT MTT TCT GTA TTR TCT T R: CAC CCG GTA CAT GCA GGA TT	190
	<i>lt</i>	Plasmid	F: GGC GAC AGA TTA TAC CGT GC R: CGG TCT CTA TAT TCC CTG TT	450
EPEC	<i>eae</i>	Chromosome	F: AGG CTT CGT CAC AGT TG R: CCA TCG TCA CCA GAG GA	570
	<i>bfp</i>	Chromosome	F: AAT GGT GCT TGC GCT TGC TGC R: GCC GCT TTA TCC AAC CTG GTA	326

Repetitive extragenic palindromic-PCR

The putative rep-like elements in the isolated *E. coli* chromosomes were amplified. The rep-PCR assay was carried out with 50ng template DNA and 2 μ M BOX A1R primer (5'-CTA CGG CAA GGC GAC GCT GAC G-3') (22). The rep-PCR amplifications were performed with initial denaturation at 95°C for 2 min, followed by 35 cycles including of denaturation at 94°C for 30 sec and annealing at 55°C for 1 min, with a final single step extension at 65°C for 8 min. The separation of amplified DNA fragments was achieved by electrophoresis on 1% agarose gel with 1 kb and 100 bp DNA ladders (GeNetBio Inc. Korea). The gels were stained with safe dye, and imaged under UV illumination.

Statistical analysis

The rep-PCR fingerprints of isolates were analyzed with NTSYS-pc program (Ver. 2.2). The patterns obtained were compared for similarity by visual study of band patterns. A dendrogram was obtained using Jaccard similarity coefficient and UPGMA algorithm. Jackknife algorithm was also performed with the cluster analysis results to find out the Rate of Correct Classification (RCC). In all categories of pathotypes, the Average Rate of Correct Classification was defined as percentage of isolates correctly classified. In order to determine the compliance of cluster analysis with data, we obtained the cophenetic matrix and compared it with the similarity matrix. Subsequently, cophenetic correlation coefficient (r) was calculated by Dice similarity coefficient (13,23).

Results

Among the 506 samples examined in this study, 219 (43.3%) *E. coli* strains were isolated that 60 (27.4%) and 50

(22.8%) of them were recovered from specimens of human and calf feces respectively. While, 63 (28.7%) and 46 (21%) of them were isolated from meat and dairy products, respectively.

Duplex-PCR test for detection of ETEC and EPEC

According to the PCR results, 33 (15%) pathogenic isolates were detected from the 219 strains of *E. coli* with 14 ETEC (6.4%), 7 typical-EPEC (3.2%), and 12 atypical-EPEC (5.5%) among *E. coli* strains isolated from humans, 10 (16.6%), 1 (1.6%), and 2 (3.3%) were found to be ETEC, typical-EPEC, and atypical-EPEC, respectively. Also, among *E. coli* samples isolated from calves, 2 (4%), 4 (8%), and 6 (12%) were identified as ETEC, typical-EPEC, and atypical-EPEC, respectively. Furthermore, no ETEC was observed among dairy products, whereas typical-EPEC and atypical-EPEC each showed 1 case (2.17%). As for meat products, 2 (3.17%), 1 (1.58%), and 3 (4.76%) cases were reported for ETEC, typical-EPEC, and atypical-EPEC, respectively.

Analyzing the genetic diversity of pathotypes using rep-PCR

The similarity between strains, based on the presence or absence of bands, was assessed using the Jaccard index and UPGMA cluster analysis (Fig. 1). The strains with similarity coefficient ≥ 70 were considered interdependent. The clonal relationship between the strains of pathogens in question was determined between 70 and 100 percent. Moreover, based on the similarity matrix, the highest and the lowest genetic similarities were 100% and 20%, respectively. Because cluster analysis alone could not classify all pathotypes separately, Jackknife analysis was also implemented. Jackknife analysis indicated that the average percentages of ETEC and EPEC

strains that were accurately clustered were 98% and 93.75%, respectively. It is worth noting that one of the ETEC strains isolated from meat had 100% similarity with a human EPEC

strain. The rates of correct classification (RCC) of ETEC and EPEC pathotypes clustered with a high criterion are given in Tables 2 and 3.

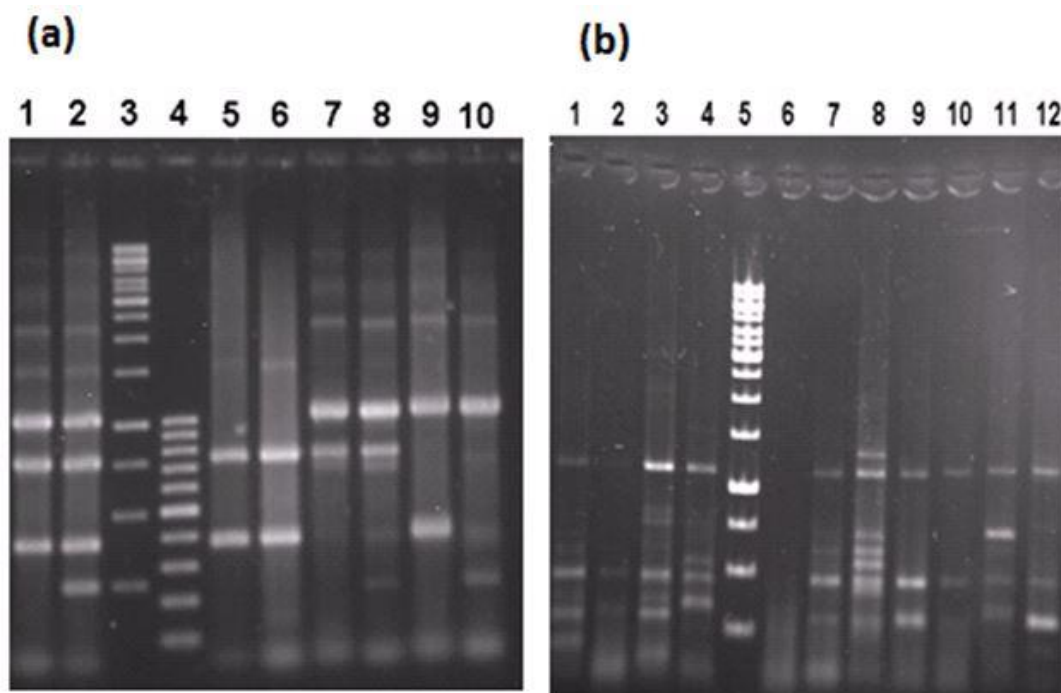


Figure 1. rep-PCR fingerprint patterns:(a) ETEC isolates of humans, calves, and raw meat products. Lanes 1 and 2: calf isolates; Lane 3: 1 Kb DNA ladder; Lane 4: 100 bp DNA ladder; Lanes 5 and 6: raw meat products isolates; Lanes 7-10: human isolates. (b) EPEC isolates of humans, calves, raw meat products and dairy products. Lanes 1 and 2: isolates of raw meat products; Lanes 3 and 4: human isolates; Lane 5: 1 Kb DNA ladder; Lane 6: negative control; Lanes 7 and 8: dairy products isolates; Lanes 9-12: isolates of calves

Table 2. Assignment of fecal ETEC isolates to source groups by using DNA fingerprints and Jackknife analysis

Host sources of ETEC isolates	%of fecal ETEC isolates clustered as(a):		
	Human (ET1-ET10)	Calf (ET11, ET12)	Raw meat products (ET13, ET14)
Human (ET1-ET10)	96	4.2	0
Calf (ET11, ET12)	4.2	98	0
Raw meat products (ET13, ET14)	1.6	5.3	100

a) Values in bold show the rate of correct classification (RCC). The ARCC was 98%.

ET= ETEC

Table 3. Assignment of fecal EPEC isolates to source groups by using DNA fingerprints and Jackknife analysis

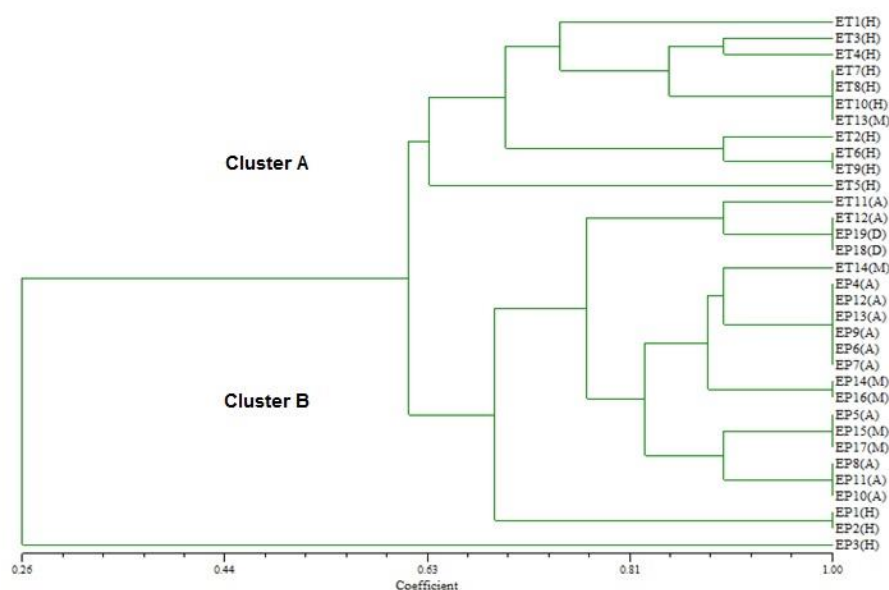
Host sources of EPEC isolates	% of fecal EPEC isolates clustered as a):			
	Human (EP1-EP3)	Calf (EP4-EP12)	Raw meat products (EP14-EP17)	Raw dairy products (EP18-EP19)
Human (EP1-EP3)	94	2.18	0	1.08
Calf (EP4-EP12)	2.18	95	0	4.14
Raw meat products (EP13-EP17)	1.23	1.08	100	0
Dairy products (EP18-EP19)	1.08	4.14	0	86

a) Values in bold show the rate of correct classification (RCC). The ARCC was 93.75%.

EP= EPEC

Dendrogram analysis demonstrated that when the similarity coefficient was 70–80%, comprising distinct groups, *E. coli* pathotypes were primarily situated in the A and B clusters, with each cluster having its own sub-clusters. In this study, all human ETEC isolates and one ETEC isolated from

meat were grouped in cluster A, and all EPEC isolates collected from the four sources along with two animal fecal ETEC isolates and one meat ETEC isolate were placed in cluster B (Fig. 2).

**Figure 2.** Dendrogram analysis of ETEC and EPEC isolates

Abbreviations: ET= ETEC, EP= EPEC, H= Human isolate, A= Calves isolate, M= Meat isolate, D=Dairy

Determination of cophenetic correlation coefficient

To determine the compliance of cluster analysis with the data, we compared the obtained cophenetic matrix with the similarity matrix. Subsequently, the cophenetic correlation

coefficient (r) was calculated by Dice similarity coefficient.

One of the methods for choosing the best dendrogram is to obtain the r -value. If the r -value is ≥ 0.6 – 0.9 , it is considered as an appropriate dendrogram. In this study, the cophenetic

correlation coefficient was 0.875, indicating a high correlation between the similarity and dendrogram matrices (13,23).

Discussion

Given the necessity of providing microbiological safety of food and its role in public health, it is vital to identify the origin of food contaminations in order to plan efficient control strategies (12,13,24). By the same token, detecting the source of *E. coli* as an indicator of fecal contamination and food hygiene is an invaluable aim (5). Among *E. coli* pathotypes, ETEC and EPEC strains (two major causes of diarrhea in developing countries transmitted usually through contaminated foods) are important health challenges which, in addition to economic losses, annually result in significant morbidity and mortality (12). Today, several molecular techniques are deployed to determine similarities and differences of *E. coli* strains isolated from different sources. The analysis of electrophoresis pattern of rep-PCR obtained from human and calf feces, raw meat, and dairy products revealed that each strain has its specific genetic fingerprinting pattern. In addition, dendrogram analysis demonstrated that all *E. coli* pathotypes can be divided into two main clusters A and B, with each cluster having its own sub-clusters. Thus, from this clustering, we concluded that strains isolated from different sources exhibited a high similarity and clonal relationship and were most likely constituted by a single clone. Moreover, the positioning of ETEC and EPEC pathotypes in two separate groups suggested the genetic diversity between these two groups. Jackknife analysis was performed to verify the accuracy of clustering (13). Based on previous studies, this analysis is a suitable method for genotyping different bacteria (7,25). In the present study, the average percentage of properly clustered strains was

high, confirming the validity of clustering. A similar study in Canada by Mohapatra *et al* used rep-PCR to genotype human and animal fecal *E. coli* isolates. The authors examined a total of 625 human fecal samples, three samples from domestic animals (a cow, horse, and dog), and seven samples obtained from wild animals. In the dendrogram obtained for all the samples, isolates were divided into two main groups; one group comprised mostly human samples (81%) and the other primarily comprised animal samples (79%) (7). Thus, DNA fingerprinting could be a good complex molecular technique to correctly identify the source of infection in humans and animals. Other similar studies by Dombek *et al.* and Johnson *et al.* recommended using rep-PCR for performing molecular analysis and exploring the genetic diversity of *E. coli* strains isolated from humans and animals (13,23). Furthermore, we noted that RCC of each pathotype isolated from humans was compatible with RCC obtained by Carson *et al.* (26). However, the RCC obtained in the present study was higher than that obtained by Dombek *et al.* (13) and Mohapatra *et al.* (7). Similarly, the RCC obtained from calf samples in the present study was higher than that observed by Carson *et al.* (26), Mohapatra *et al.* (7), and Seurink *et al.* (27). The inconsistency in the rates of correct classification may be because of the differences in the hosts, genetic diversity of various *E. coli* isolates, and geographical differences. It should be noted that we could not compare the results of *E. coli* strains isolated from dairy and meat products in this study because no previous study has reported the isolation of the above-mentioned pathotypes in raw meat and dairy products and there is no precise knowledge about the pattern of bacterial genome isolated from these products. According to the results of this study, the *E. coli* isolates from food products were in the same phylogenetic

group as calf isolates. Hence, it could be argued that most *E. coli* isolates from animals may be transmitted via meat and dairy products. Thus, owing to people's tendency to use traditional dairy products because of their lack of trust in pasteurized dairy products and supervisory organization, particularly in the developing countries, *E. coli* contamination is still a serious problem. This finding stresses the necessity of applying strict standards in the processing of ASFs. In addition, the results of the present study reaffirm previous studies by highlighting the power of rep-PCR technique to separate different *E. coli* strains. Furthermore, its simple operation and low-cost implementation turn it into an advisable technique to be used by other epidemiological studies as an efficient genotyping tool. In this regard Bae *et al* previously reported that rep-PCR is suitable for investigating in nationwide or worldwide epidemiology studies (18). Therefore in the present study, rep-PCR has been applied as a bacterial source tracking method because it was conducted in a wide geographical area (Northwestern Iran). Moreover, our investigation is the first report on phylogenetic analysis and genetic differences existing

between pathogenic *E. coli* strains isolated from ASFs and human and calf fecal samples. This study, in effect, provides a new perspective concerning the necessity of identifying the source of food microbial contaminations and efficient strategies for controlling infections by detecting the main sources of *E. coli* pathotypes and their transmission channels. Furthermore, because animal manure is used in many agricultural fields for cultivation of vegetables, the risk of vegetable contamination by animal *E. coli* pathotypes and, consequently, the possibility of transmission of pathogenic pathotypes to humans increases. Further studies is suggested to investigate the prevalence of *E. coli* pathotypes isolated from plant-based foods and the similarity in the fingerprinting patterns of these bacteria with human isolates. In addition, future researches should address other *E. coli* pathotypes and should examine the similarity between human and nonhuman strains and their etiology.

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Conflicts of Interest: Authors have no conflict of interest.

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