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Isolation of Enteropathogenic *Escherichia coli* (EPEC) From Raw Milk in Kermanshah by Polymerase Chain Reaction (PCR)

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A B S T R A C T

Background: Infectious diarrhoeal diseases cause major problems throughout the world and are responsible for considerable morbidity and mortality. Enteropathogenic *Escherichia coli* (EPEC) may cause infantile diarrhoea among children in developing countries.
Objectives: The aim of this study was to define the prevalence of EPEC strains in raw milk samples.
Materials and Methods: Raw milk samples collected from various cow farms in Kermanshah, Iran, during the period of 22nd June to 22nd September 2009 and were examined for EPEC presence using PCR reactions targeting eaeA, and then stx1 and stx2.
Results: Of the 206 samples, 17 (8.25%) were contaminated with *E. coli* eaeA positive and stx1 and stx2 negative (EPEC).
Conclusions: Our results confirm that raw milk recovered in Kermanshah may be a source for gastrointestinal infections by EPEC and strict preventive measures should be adopted to decrease contamination of milk with EPEC and other bacteria originated from animals.

Keywords: Enteropathogenic *Escherichia coli*; Raw Milk

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Implication for health policy/practice/research/medical education:
According to high consumption of raw milk in defferent areas in Kermanshah province, detection and isolation of pathogenic bacteria in raw milk samples seems to be of utmost importance.

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1. Background

Infectious diarrhea is one of the world's leading causes of morbidity and mortality, resulting in about two million deaths per year (1, 2). Enteropathogenic *Escherichia coli* (EPEC) is a major cause of infantile diarrhea among children in developing countries (3, 4). The main mechanism of EPEC pathogenesis is a lesion called 'attaching and effacing' (A/E), which is characterized by intimate adherence of bacteria to the intestinal epithelium (5). The *eaeA* gene, which is located in the 'locus of enterocyte effacement' pathogenicity island, has been used for identification of EPEC.

In addition to EPEC, Shiga toxin-producing *E. coli* (STEC), the causative agent of gastroenteritis that produce one or two potent toxins called Shiga toxin (Stx1, Stx2),may be able to produce intimin protein (6, 7). For this reason, *E.coli* strains with the *eaeA* genotype which lack Shiga toxin gene (*stx1* and/or *stx2*) are classified as EPEC (8). As with many other *E.coli*, transmission of EPEC is through fecal-oral route, with contaminated hand or contaminated foods or infant formula as vehic (9-12). Studies showed that 1 – 5 % of food-infections were related to the consumption of milk and dairy products, and 53% of cases of food-borne infections caused byEPEC (13).

2. Objectives

Although some comprehensive studies have been conducted in Kermanshah to examine the contribution of EPEC as a cause of infectious diarrhea among children, unfortunately there is no relevant data about food contamination with EPEC. Thus, the objective of this study was the determination of the prevalence of EPEC in raw milk in Kermanshah Province.

3. Materials and Methods

3.1. Samples

From 22nd June to 22nd September 2009, a total of 206 bulk-tank milk samples were collected from 135 cow farms with a total of approximately 6,000 animals in Kermanshah. These farms ranged in size from 10 to 500 animals. The samples were placed on ice and transported immediately to the laboratory. The sample size was calculated according to the following formula: N= t 2 ×p× q/d 2

While n is the required sample size, t is the confidence level at 95% (standard value of 1.96), p is the estimated prevalence of malnutrition in the project area and d shows the margin of error at 5% (standard value of 0.05)

3.2. Bacterial Culture

25 mL of the milk sample (about 500 mL) was cultured in 225 ml of modified EC broth containing cefexime ($0.05 mgL^{-1}$, Daana Pharmaceutical Co.) and then incubated overnight at 37° C. A portion of EC broth was spread on MacConkey agar which was incubated overnight at 37° C.

3.3. DNA Extraction

According to the previously described protocol, bacteria were harvested from the primary streak of MacConkey agar, suspended in 250 µl of sterile water, incubated at 100 ◦C for five minutes to release the DNA and centrifuged (14). The supernatant was used in the PCR reactions targeting *eaeA*, and then *stx1* and *stx2* as described below.

3.4. PCR Primer and Reaction Conditions

Amplification of bacterial DNA was performed in thermal cycler (Bio Rad) using 25 ml volumes containing 5 µl of the prepared sample supernatant; 1x reaction buffer; 0.5 µM of each of the primers;0.2 mM of each dNTP; 1.5 mM MgCl2 and 1.2 U of Taq DNA polymerase (Cinnagen Co.).

Following amplification, 10 µl of each sample was analyzed by 1.5% agarose gel electrophoresis for detection of positive samples for *eaeA* gene. After identification of positive plate for *eaeA*, a number of colonies ranging from 30 - 90, located in third and fourth area of the culture, were tested in order to find the pure colony or colonies responsible for the positive results in the first PCR, and then DNA extracts from responsible colonies were examined for the following genes: *stx1* and *stx2*. The eaeA gene positive and *stx1* / *stx2* genes negative colonies which were confirmed as *E. coli* using biochemical tests were defined as EPEC (15).

Primers and cycling condition are listed in *Table 1*. For all amplification reactions, the mixture was heated at 96 °C for four minutes prior to thermocycling. The mixture was held at 72°C for six minutes after the final cycle before cooling at 4°C. The following *E.coli* strains were included as negative and positive controls in each PCR run: STEC, ATCC 43890 (*stx1*) and ATCC 43889 (*stx2*); and EPEC; ATCC 43887 (*eaeA*and *bfpA*). Agarose gel electrophoresis of *eaeA*, stx1, stx2, bfpA and PCR product is shown in *Figure 1*.

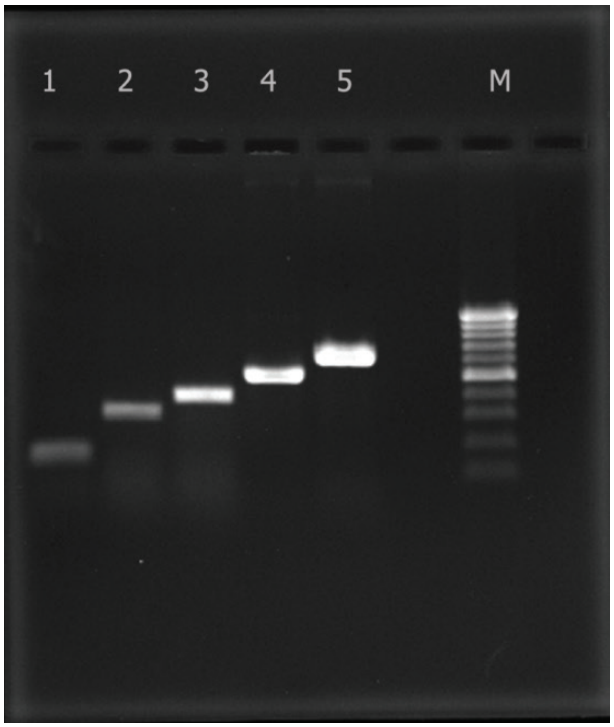
4. Results

Among the 206 milk samples, 17 (8.25%) were positive for EPEC, and 17 strains were isolated as described above.

Table 1. Primers and Condition

Target	Oligonuceotide Sequence (5´-3)	PCR Condition	No. of Cycles	Fragment Size (bp)	Reference
Stx1	GAAGAGTCCGTGGGATTACG AGCGATGCAGCTATTAATAA	94˚C, 20s 50˚C, 20s 70˚C, 12s	32	130	Pollard *et al.* 1990
Stx2	A C C G T T T T T C A G A T T T T - G A C A C A T A T A C A C A G G A G - C A G T T T C A G A C A G T	94˚C, 20s 61.3˚C, 20s 70˚C, 12s	32	298	Svenungsson *et al.* 2000
eaeA	CACACGAATAAACTGACTAAAAT-GAAAAACGCTGACCCGCACCTA-AAT	94˚C, 20s 61.3˚C, 20s 70˚C, 12s	32	376	Svenungsson *et al.* 2000

Figure 1. Agarose Gel Electrophoresis of EaeA PCR Product and 100 bp Molecular Size Marker



Lane 1-3:stx1, stx2 (Positive Controls) and eaeA , Lanes 4,5: rfo157 and fliCh7(data of the two latter are not shown in the text) respectively.

5. Discussion

For the rapid and sensitive detection of EPEC in clinical and food samples, PCR has proven to be of great diagnostic value (16). Cultivation of food and stool samples in liquid or solid media may increase the number of bacteria and may therefore assist in the detection of EPEC which might be present in lowquantities. Thus, PCR testwas carried out after the enrichment of milk samples in EC broth and cultivation of a portion ofthe EC broth on MacConkey agar. We showed that the EPEC prevalence in raw milk samples was 8.25%; however, the presence ofthis pathogene in milk proved to be variable in different regions. In Brazil, Aleixo and Aver (17) reported EPEC in 25% milk samples. In another report, of a total of 175 bulk milk samplesstudied, 83 (47.4%) contained *E.coli*, and 26 (27.7%) of 94 strains of *E.coli* isolated from bulkmilk were EPEC (18).

These variations may be due to geographical location, season, farm size, number of animals onthe farm, hygiene status, farm management practices, variation in sampling, variation in types ofsamples evaluated, and differences in detection methods. There was no report about prevalence of EPEC in raw milk in Iran, but there are several reportson presence of this pathogene in human fecal samples. For example, diarrhoeagenic *E.coli* was surveyed in 1087 children under the age of 5 with acute diarrhoea, and EPEC wasfound in 70 (12.6%) of samples (19). In another report from Alikhani *et al.* (2), 111 out of 247 fecal samples of children with acute diarrhea were positive for EPEC. In brief, our data revealed that strict preventive measures should be adopted to decreasecontamination of milk with EPEC and other bacteria originated from animals.

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Authors' Contribution

Ramin Abiri: Designed the study and protocols, analysed the data and revised the manuscript. Pantea Mohammadi: Wrote and submitted the manuscript.

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