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Brief report

Study of frequency of *eaeA*, *stx1* and *stx2* genes in *Escherichia coli* isolated from local cheeses in Maragheh city by multiplex PCR

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Abstract

Pathogens can be transmitted to the humans through the consumption of contaminated local dairy products such as cheese and, thus, cause pathogenic diseases. Shiga toxin produced by *Escherichia coli* can cause mild watery diarrhea as well as serious complications such as hemorrhagic colitis, and hemolytic uremic syndrome and may even lead to death. The present study was conducted to investigate the frequency of *eaeA*, *stx1* and *stx2* genes in *Escherichia coli* isolated from local cheese in Maragheh city through multiplex PCR. Thirty two *Escherichia coli* isolates from local cheese in Maragheh city were studied with regard to the frequency of *stx1*, *stx2* and *eaeA* genes through multiplex PCR. The frequency of *eaeA* gene in *Escherichia coli* isolates was 15.62% (5:32). *Stx1* and *stx2* genes were not found in any isolate. It was concluded that shiga toxin produced by *E.coli* exists in local cheeses and can pose risks to the human health in this region.

Keywords: *Escherichia coli*, Cheese, Multiplex PCR , Virulence genes

Introduction

Escherichia coli as an indicatoris used for determining the fecal contamination of water and food and presence of intestinal pathogens. This bacterium has different strains that its pathogen types are involved indifferent diseases in human and animals and play important role in public health. Shiga toxin producing *Escherichia coli* (STEC)serotypes arean important group of zoonotic and food borne pathogens that causes different diseases such as hemorrhagic diarrhea, hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura(TTP) (Robinson &Batt Carl, 2000). STEC family is serologically diverse and more than 200 serotypes have been reported; out of which more than 100 are linked to human infections (Rammurthy, 2008). STEC contains different toxins the production of which is controlled by specific genes. *Stx1*,*stx2* and *eaeA* genes are the most important virulence factors in STEC. Shiga toxin 1 is 98% homologous to the stx produced by *Shigella dysenteriae* type 1, while *stx2* is about 60% homologous with *stx1* and is different from antigenic point of view (Tahamtan *et al*., 2010). The types of producing *stx1* and *stx2* toxins of *Escherichia coli* cause the mentioned syndromes. Shiga toxin genes in *Escherichia coli* generally are in specific phages. Various types of these toxins have been known such as: stx, *stx1*, *stx1*c, *stx2*, *stx2*c, *stx2*d, *stx2*e,*stx2*f (Friedrich *et al*., 2003). Intimin, a protein which is responsible for attachment of bacterium to intestine,is causing specific lesions calling attaching-effacing lesions in intestine epithelial cells. For this reason, the coding gene of this protein called eae (*E.coli* attaching and effacing) (Wales *et al*., 2005).Researches indicated that Shiga toxin-producing *Escherichia coli* strains are in ruminants that 40% of them are pathogen for human. This fact considers the ruminants as the important reservoir for these strains (Montenegro *et al*., 1990). Animal products such as unpasteurized dairy products such as domestic cheese are one of the most important transmission ways of STEC to human (Feng*et al*., 2011). According to

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different studies, 1-5% of food borne intoxications are associated with consumption milk and dairy products that 53% of food borne infections are due to consumption of contaminated cheese (Schrade&Yager, 2001). Since the Multiplex PCR has suitable sensitivity and specificity and it has high capability of identification of shiga toxin producing *E.coli* strains and possibility of direct application on clinical and food samples, it considers this method can be used as a common method in laboratories for the detection of suspicious samples.The aim of this study is to determine offrequency of *eaeA*, *stx1* and *stx2* genes in *Escherichia coli* isolated from domestic cheeses in Maragheh city by multiplex PCR

Materials and methods

Samples

Thirty two strains of isolated *E.coli* from local cheese in villages of Maragheh city were examined for occurrence of *stx1*, *stx2* and *eaeA* genes.

DNA extraction

DNA extraction was performed on 32 cultured isolates of*E.coli* in Brain Heart Infusion Agar (BHI) (Merck,Germany) medium. One ml of bacterial culture centrifuged in 5000g for 5 minutes and supernatant was poured off. Subsequent to addition of 1ml lysis buffer (Tris 1 M[pH=7.5], NaCl 5 M, EDTA 0.5 M and C-TAB 2%) on mixed pellet,it was put into 85ºc for 30 minutes(in water bath). In the next step, the supernatant was separated and 0.5 µl Rnase was added to it and ,later, it was kept at 37ºc for 30 minutes. Then an equal volume of isopropanol was added to the same and it was kept at -20ºc for 15 minutes and, later,it was centrifuged in 12000 g resulting in some DNA samples to be sedimented. Later, DNA samples were dried in lab temperature. Finally, dried DNA samples were dissolved in 50µl of double distilled water (Atashpaz *et al*., 2010).

Multiplex PCR

This reaction was performed with the help of selected primers of the bacteria under experimentation for 25µl reaction volume (Table 1).The mixed reaction contained master kit of PCR 12.5µl, specific primers (0.5M) and extracted DNA(1µl). Cycling condition for all were the following: 1 cycle at 95ºc for 4 min, 32 cycles at 95ºc for 1 min, 50ºc for 1 min and 72ºc for 1 min,with a final extension at 72ºc for 1 min. Electrophoresis of PCR product was conducted in 1.5% agarose gel. Indicator bacterial strain *E.coli* PTCC 1270 was used as positive control in PCR. Double distilled water was used as negative control.

Table1: Primers used in PCR for the detection of *stx1*,*stx2* and *eaeA* genes (Vidal *et al*., 2004).

Product size (bp)	Primer & Sequence	Gene
348bp	*Stx1* F; 5'CAG TTA ATG TGG TGG CGA AGG 3' *Stx1* R; 5' CAC CAG ACA ATG TAA CCG CTG 3'	*Stx1*
584bp	*Stx2* F; 5' ATC CTA TTC CCG GGA GTT TAC G 3' *Stx2* R; 5' GCG TCA TCG TAT ACA CAG GAG C 3'	*Stx2*
482bp	*eaeA* F; 5' TCA ATG CAG TTC CGT TAT CAG TT 3' *eaeA* R; 5' GTA AAG TCC GTT ACC CCA ACC TG 3'	*eaeA*

Results and Discussion

Among 32 isolated and biochemically characterized *E.coli*, none of the samples had *stx1* and *stx2* genes. Only standard bacterium (PTCC 1270) showed *stx1* gene. Five samples(15.62%) had only *eaeA* gene. None of the samples had 2 or 3 studied genes simultaneously. Standard bacterium (PTCC 1270) did not show both *stx2* and *eaeA* genes (Figure 1).

The present study, based on the literature, was the first study describing the detection and frequency of major virulence genes of STEC isolated from domestic cheese in Maragheh, Iran. *E.coli* is the normal gut flora of the human beings, but certain subsets of this

species have acquired virulence genes that enabled them to cause diarrhea and other extra-intestinal infections. Such is shiga toxigenic *E.coli* containing stx genes, which has direct enterotoxic properties resulting from selective targeting of Gb3 containing absorptive villus epithelial cells in the ileum (Feng*et al*., 2011). Among the *stx1* and *stx2*genes, *stx2* is considered to be the most important virulence factor associated with the human disease. It is about 400 fold more toxic to mice than *stx1* and also been shown to induce feto-placental re-absorption, intra-uterine haematoma, fibrin deposition and neutrophil infiltration (Islam *et al*., 2008). The spread of STEC infection among the humans could have been from contamination of food with water and sewage signifying poor level of hygiene maintained. One of the chief ways of transferring shiga toxin producing *E.coli* to human is food habits such as consuming half cooked or raw meats and unpasteurized dairy products that have close relationship with rural life (Mehrabiyan*et al*., 2013). Except of *E.coli O157*, other serogroups of STEC are the causative agent of 60% of shiga toxigenic *E.coli* that are widespread in many countries such as Argentina, Australia, Spain, Denmark, Chile and Germany (Johnson *et al*., 2006). The results of the presentstudy showed that the frequency of *eaeA* gene in *Escherichia coli* isolates were 15.62% (5/32). *Stx1* and *stx2* genes were not found in any isolate.Bonyadian*et al*., (2011) showed that among 14 *E.coli* strains isolated from unpasteurized cheese samples, none of them harbored*stx1*, *stx2* and *eaeA* genes. Also they showed that among 38 *E.coli* strains isolated from raw milk samples, none of them had *stx1* gene, but 3 isolates(7.89%) and 2 isolates(5.26%) had *stx2* and *eaeA* genes, respectively. In a study on shiga toxin producing *E.coli* strains isolated from milk tanks and new cheese samples in Spain, it was identified that only *E.coli* O157:H7 contained *eaeA*gene(Rey *et al*., 2006). In a study on 42 raw milk samples for occurrence of verotoxigenic *E.coli* in northern Ireland in 2003, only 4 cases carried both *stx2* and *eaeA* genes(McKee*et al*., 2003).

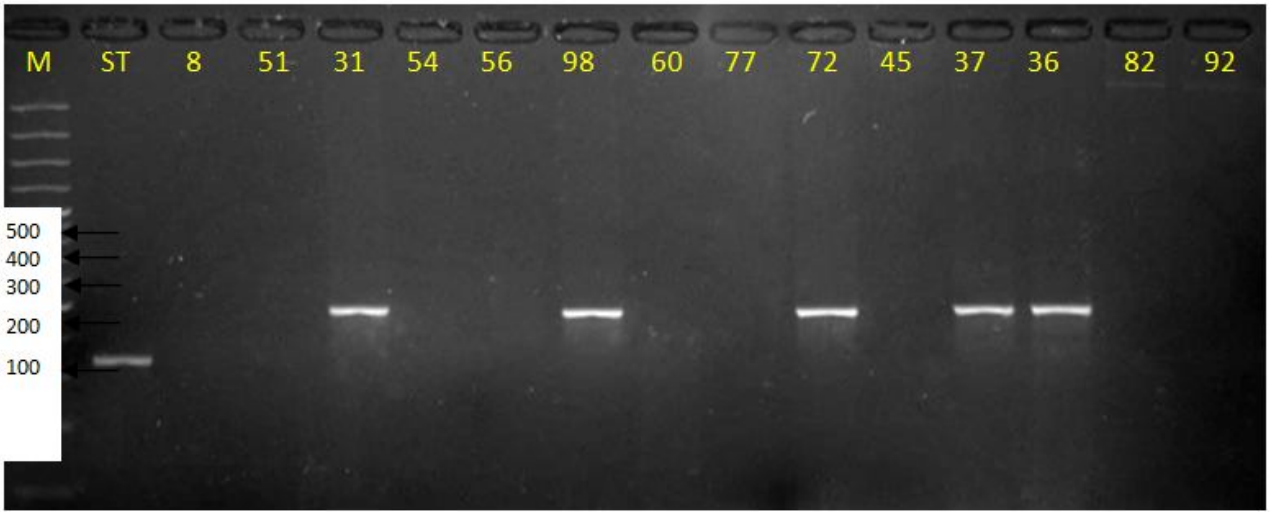

Fig 1: Lanes 31,98,72,37 and 36 are associated with *eaeA* gene(482bp). Lane ST indicates *E.coli* PTCC 1270 that bands within 348bp and is associated to *stx1* gene. The standard bacterium(ST) shows negative result for *eaeA* and *stx2* genes. Lane 8 is negative control (Double distilled water). Lane M is marker (100bp).

Mehrabiyan *et al*., (2013) reported that prevalence of *stx1*,*stx2* and *eaeA* genes in *Escherichia coli* isolated from sheep meat in Chaharmahal va Bakhtiari province were 11.1%,8.8% and 0, respectively. The reported investigations on contamination to virulence genes of *E.coli* in food in numerous areas of the world indicated the different rates (from low to up) of contamination to these genes. Shah Illi*et al*., (2010) reported that prevalence of *stx1* and *stx2* genes of shiga toxin-producing *Escherichia coli* from juice

purchase and vegetables in Shiraz city were 3.37% and 0, respectively. Moreover, 1.12% of isolated *E.coli* carried both *stx1* and *eaeA* genes. Simultaneous molecular investigations on environmental samples (such as water and soil), dairy products and ruminants fecal samples may indicate genetic association of strains and etiology of causative agents of disease. The results of present study, comparing to the previous studies, indicated the difference in dispersion of effective genes in *E.coli* virulence. This may be due to the geographical diversities and also difference in ecologic origin of isolated strains (Milk, human and different animals).

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مقاله کوتاه پژوهشی

بررسی فراوانی ژن‌های *eaeA*، *stx1* و *stx2* در *اشریشیاکلی‌های* جداسازی شده از پنیرهای محلی شهر مراغه به روش Multiplex PCR

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چکیده

عوامل بیماری‌زا قادرند که از راه مصرف محصولات لبنی سنتی آلوده مثل پنیر به انسان منتقل شده و در نتیجه بیماری ایجاد کنند. *اشریشیاکلی* تولید کننده شیگاتوکسین، اسهال آبکی ملایم تا مشکلات جدی‌تر مثل التهاب کولون خونریزی‌دهنده و سندرم اورمی همولیتیک تا حتی مرگ را باعث می‌شود. مطالعه حاضر برای بررسی فراوانی ژنهای *eaeA*، *stx1* و *stx2* در *اشریشیاکلی*های جداسازی شده از پنیرهای محلی شهر مراغه به روش Multiplex PCR انجام شد. ۳۲ جدایه *اشریشیاکلی* از پنیرهای محلی شهر مراغه برای جستجوی ژنهای *eaeA* ، *stx1* و *stx2* به روش Multiplex PCR مورد بررسی قرار گرفتند. فراوانی ژن *eaeA* در جدایه‌های *اشریشیاکلی* ۱۵/۶۲٪ (۵/۳۲) بود. ژنهای *stx1* و *stx2* در هیچ جدایه‌ای مشاهده نشد. *اشریشیاکلی* تولیدکننده شیگاتوکسین در پنیرهای محلی این منطقه وجود داشته و برای سلامت انسان می‌تواند خطرناک باشد.

واژه‌های کلیدی: *اشریشیاکلی*، پنیر، مولتی‌پلکسPCR ، ژنهای حدت

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