Shiga-toxigenic *Escherichia coli* in ready-to-eat food staffs: Prevalence and distribution of putative virulence factors

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Abstract

Lack of proper hygiene and using from low quality raw materials cause high presence of food-borne pathogens in ready to eat foods. Shiga toxin producing *Escherichia coli* is one of the most common cause of food-borne diseases in the world. The present research was done to study the prevalence and distribution of virulence factors in the STEC strains isolated from various types of ready to eat food samples. Seven-hundred and twenty food samples were collected and cultured. Isolated *E. coli* bacteria were approved another time using the *16S rRNA*-based PCR amplification. Approved strains were subjected to multiplex PCR for identification of putative virulence factors. Twenty-six out of 720 food samples (5.20%) were positive for *E. coli*. Salad (15%), candy (12.50%) and barbecue (10%) were the most commonly contaminated. Prevalence of STEC strains was 2.63%. Prevalence of EHEC and AEEC subtypes were 36.84% and 52.63%, respectively. EHEC strains harbored all three *stx1*, *eae* and *ehly* genes. High presence of EHEC strains besides the considerable distribution of multiple virulence factors showed an important public health issue regarding the consumption of ready to eat foods.

Introduction

Food hygiene in restaurants and fast-foods are one of the most important critical issue. Using from low quality raw materials, cooking of foods more than daily requirement and their storage in unsuitable conditions and finally lack of the proper hygiene are the main factors causing enhancement of the microbial spoilage and growth of dangerous food-borne pathogens in foods.[1] Among all pathogenic agents causing food-borne diseases, *Escherichia coli* (*E. coli*) strains had a significant position.[2-8] *E. coli* is a Gram-negative, non-sporulating, flagellated, rod-shaped and facultative anaerobic bacterium of the *Enterobacteriaceae* family. Shiga (vero) toxin (*Stx*)-producing *E. coli* (STEC) is a subdivision of a significant pathogenic group of this bacterium named enterohemorrhagic *E. coli* (EHEC).[2-8] In the other hand, STEC strains are divided into two separate subtypes of EHEC and Attaching and Effacing *E. coli* (AEEC). EHEC strains are responsible for high morbidity and mortality. AEEC strains are described by their ability to occur attaching-and-effacing (A/E) lesions in the gastrointestinal tract of humans and animals.[2-8] STEC bacteria are responsible for severe clinical syndromes like Hemorrhagic Colitis (HC), Hemolytic Uremic Syndrome (HUS), bloody and non-bloody diarrhea and Thrombotic Thrombocytopenic Purpura (TTP).[2-8]

Presence of latent virulence factors including Shiga toxins (*stx1* and *stx2*), intimin (*eaeA*) and hemolysin (*hlyA*) in the STEC strains of food products make them dangerous pathogens for human health. These genes are responsible for bacterial adhesion, colonization and invasion into the gastric epithelial cells.[2-8]

According to the uncertain role of STEC strains in ready to eat foods and lack of epidemiological and microbiological investigations in this field in Iran, the present research was done to study the prevalence and distribution of virulence factors in the STEC strains isolated from various types of ready to eat food samples.

Materials and Methods

Ethical considerations

The research was permitted by the Ethical Board of Islamic Azad University, Shahrekjord Branch (Consent Ref Number IAU 2053). Confirmation of this project and the authorizations related to sampling process were given by the Prof. Ebrahim Rahimi (Approval Ref Number Food-Hygiene 952020).

Samples and *Escherichia coli* isolation

From September 2013 to September 2014, a total of 720 various types of ready-to-eat foods including sausage (n=70), salami (n=70), hamburger (n=60), roast mouthful (n=60), traditional dressing (n=65), traditional salad (n=60), traditional candy (n=60), traditional ice-cream (n=60), barbecue (n=70), soup (n=75) and spices (n=70) were randomly collected from various restaurants in the Isfahan province, Iran. Samples were immediately transferred to laboratory in cooler with ice-packs.

Totally, 10-g of crushed food samples were homogenized for 2 min in 90 mL of Peptone Water (PW, Merck, Germany). Then the samples were cultured on 5% sheep blood and MacConkey agar (Merck, Germany) and incubated for 18 to 24 h at 37°C. Colonies with the typical color and appearance of *E. coli* were picked and streaked again on blood agar plates and re-streaked on EMB agar (Merck, Germany). All plates were further incubated for 24 h at 37°C. The green metallic colonies were considered as *E. coli*. The presumptive colonies were biochemically tested for growth on triple sugar iron agar (TSI) and lysine iron agar (LIA), oxidative/fermentative degradation of glucose, citrate utilization, urease production, indol fermentation, tryptophan degradation, glucose degrada-

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Key words: Shiga toxin producing *Escherichia coli*, Virulence factors, Ready to eat foods, Iran.

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tion (methyl red test) and motility. Bacterial strains were sub-cultured overnight in Luria-Bertani broth (Merck, Germany) and further incubated for 48 h at 37°C. Genomic DNA was extracted from bacterial colonies using the DNA extraction kit (Fermentas, Germany) according to manufacturer's instruction. Bacterial colonies were further confirmed using the *16S rRNA*-based Polymerase Chain Reaction (PCR).[9] Set of primers used for this purpose were Forward: 5'-AGTTTGATCCTGGCTCAG-3' and Reverse: 5'-AGGCCCGGGAACG-TATTCAC-3' (1343 bp).

PCR amplification of virulence factors

Table 1 shows the list of primers and PCR program used for detection of virulence factors.[6-8] Programmable DNA thermo-cycler (Eppendorf Flexrcycler^2, Germany) was used in all PCR reactions. Those strains which were simultaneously positive for all *stx1*, *eaeA* and *ehly* genes were considered as EHEC subtype.[6-8] Others which were positive for *stx1*, *stx2* and *eaeA* genes were considered as AEEC subtype.[6-8] A multiplex PCR reaction was done in a final volume of 50 µL. Volume of each material is presented in Table 1. The PCR amplification products (15 µL) were subjected to electrophoresis in a 1.5% agarose gel in 1X TBE buffer at 80 V for 30 min, stained with SYBR Green (Fermentas, Germany). All runs included a negative DNA control consisting of PCR grade water and strains of *E. coli* O157:K88ac:H19, CAPM 5933 and *E. coli* O159:H20, CAPM 6006 were used as positive controls.

Statistical analysis

Statistical analysis was performed using SPSS/16.0 software for significant relationships. The prevalence of virulence factors in the *E. coli* strains isolated from various types of ready to eat food samples were statistically analyzed. Statistical significance was regarded at a P value <0.05.

Results

Table 2 indicates the prevalence of *E. coli* in various types of ready to eat food samples. Twenty-six out of 720 (5.20%) food samples were positive for *E. coli*. There were no positive results for sausage, salami, roast mouthful and soup samples. Salad (15%), candy (12.50%) and barbecue (10%) had the highest prevalence of *E. coli*. Statistically significant differences were seen between the types of samples and prevalence of *E. coli* (P<0.05).

Figures 1 and 2 represent the results of the gel electrophoresis for *stx1*, *eaeA* and also *stx2* and *ehly* virulence factors, respectively. Table 3 represents the distribution of virulence factors in the *E. coli* strains isolated from various types of ready to eat food samples. We found that 19 out of 26 *E. coli* strains (73.07%) had Shiga toxigenic virulence factors and were determined as STEC

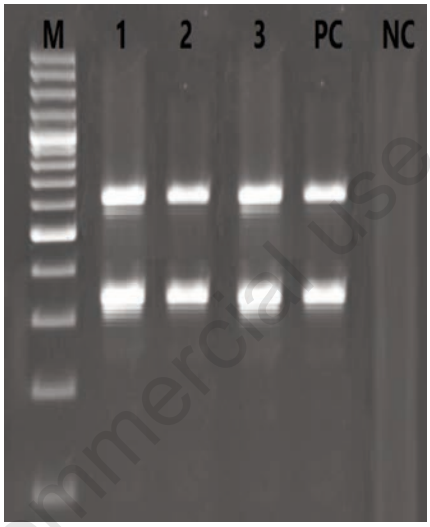

Figure 1. Results of the gel electrophoresis for virulence factors. M: 100 bp ladder, 1-3: Positive samples for *stx1* (366 bp) and *eaeA* (629 bp) genes, PC: Positive control and NC: Negative control.

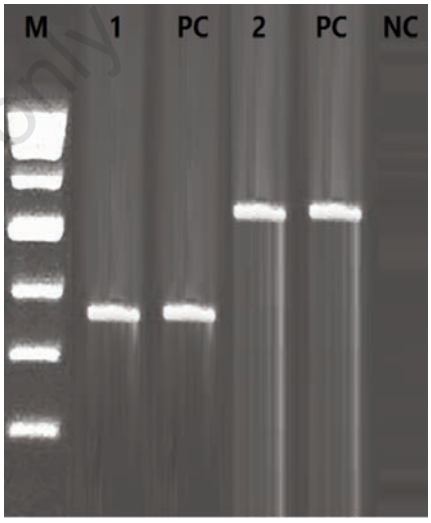

Figure 2. Results of the gel electrophoresis for virulence factors. M: 100 bp ladder, 1: Positive sample for *stx2* gene (282 bp), 2: Positive sample for *ehly* gene (432 bp), PC: Positive controls and NC: Negative control.

Table 1. Oligonucleotide primers and the PCR program used for amplification of virulence factors in the *Escherichia coli* isolates of ready to eat foods.[6-8]

Target gene	Primer sequence (5'-3')	PCR product (bp)	PCR programs	PCR volume (50 µL)
stx1	F: AAATCGCCATTCGTTGACTACTTCT R: TGCCATTCTGGCAACTCGCGATGCA	366	1 cycle: 95°C - 3 min. 34 cycle: 94°C - 60 s 56°C - 45 s 72°C - 60 s 1 cycle: 72°C - 10 min	5 µL PCR buffer 10X; 2 mM Mgcl2; 150 µM dNTP (Fermentas); 0.75 µM of each primers F&R; 1.5 U Taq DNA polymerase (Fermentas); 3 µL DNA template
stx2	F: CGATCGTCACTCACTGGTTTCATCA R: GGATATTCTCCCCACTCTGACACC	282		
eaeA	F: TGCGGCACAACAGGCGGCGA R: CGGTCGCCGCACCAGGATTC	629		
ehly	F: CAATGCAGATGCAGATACCG R: CAGAGATGTCGTTGCAGCAG	432		

strains. Total prevalence of STEC strains in various types of food samples were 2.63%. Results showed that the prevalence of EHEC and AEEC subtypes in the *E. coli* strains of ready to eat food samples were 36.84% and 52.63%, respectively. EHEC strains harbored all three *stx1*, *eae* and *ehly* genes. Statistically significant difference was seen between the prevalence of EHEC and AEEC subtypes (P<0.05).

Discussion

The present research showed that virulent STEC strains had a significant prevalence in various types of ready to eat food samples and especially Salad, candy and barbecue. We found that the prevalence of *E. coli* strains and also STEC strains in various types of ready to eat foods were 5.20% and 2.63%, respectively. Using from low quality and contaminated raw materials, application of contaminated dishes and equipment used for food preparation and finally lack of adequate time and temperature for well cooking of foods are the most important reasons for the high prevalence of *E. coli* and STEC strains in our study. However, the role of infected staffs as a sources of STEC strains could not be ignore.

The main source of human EHEC and AEEC contaminations are contaminated and undercooked ready to eat foods and especially foods with animal origin like raw and under-cooked meat.[9-12] All of the ready to eat food samples of our investigation were meat-based ready to eat foods. Some of them like sausage, salami and soup were

Table 2. Prevalence of *Escherichia coli* strains in various types of ready to eat food samples.

Types of samples	N. samples collected	N. positive strains (%)	PCR confirmation (%)
Sausage	70	-	-
Salami	70	-	-
Hamburger	60	1 (2.50)	1 (2.50)
Roast mouthful	60	-	-
Dressing	65	4 (8)	4 (8)
Salad	60	6 (15)	6 (15)
Candy	60	5 (12.50)	5 (12.50)
Ice cream	60	2 (5)	2 (5)
Barbecue	70	5 (10)	5 (10)
Soup	75	-	-
Spices	70	3 (5)	3 (5)
Total	720	26 (5.20)	26 (5.20)

Table 3. Distribution of virulence factors and subtypes in the *Escherichia coli* strains isolated from ready to eat food samples.

Samples (positive)		Subtypes	N. positive samples	Virulence genes
Hamburger (1)	Non detected	-		-
	EHEC	1 (100)		*stx1, eae, ehly:* 1 (100)
	AEEC	-		*stx1:* 0; *stx2:* 0; *eaeA:* 0; *stx1, eaeA:* 0; *stx2, eaeA:* 0; *stx1, stx2, eaeA:* 0
	Total	1 (100)		
Dressing (4)	Non detected	-		-
	EHEC	1 (33.33)		*stx1, eae, ehly:* 1 (100)
	AEEC	2 (66.66)		*stx1:* 2 (100); *stx2:* 1 (50); *eaeA:* 2 (100); *stx1, eaeA:* 1 (50); *stx2, eaeA:* 1 (50); *stx1, stx2, eaeA: 1 (50)*
	Total	3 (75)		-
Salad (6)	Non detected	1 (25)		-
	EHEC	1 (25)		*stx1, eae, ehly:* 1 (100)
	AEEC	2 (50)		*stx1:* 2 (100); *stx2:* 1 (50); *eaeA:* 2 (100); *stx1, eaeA:* 1 (50); *stx2, eaeA:* 1 (50); *stx1, stx2, eaeA:* 1 (50)
	Total	4 (66.66)		-
Candy (5)	Non detected	2 (50)		-
	EHEC	1 (25)		*stx1, eae, ehly:* 1 (100)
	AEEC	2 (50)		*stx1:* 2 (100); *stx2:* 1 (50); *eaeA:* 2 (100); *stx1, eaeA:* 1 (50); *stx2, eaeA:* 1 (50); *stx1, stx2, eaeA:* 1 (50)
	Total	4 (80)		-
Ice-cream (2)	Non detected	-		-
	EHEC	1 (50)		*stx1, eae, ehly:* 1 (100)
	AEEC	1 (50)		*stx1:* 1 (100); *stx2:* 1 (100); *eaeA:* 1 (100); *stx1, eaeA:* 1 (100); *stx2, eaeA:* 1 (100); *stx1, stx2, eaeA:* 1 (100)
	Total	2 (100)		-
Barbecue (5)	Non detected	-		-
	EHEC	1 (33.33)		*stx1, eae, ehly:* 1 (100)
	AEEC	2 (66.66)		*stx1:* 2 (100); *stx2:* 1 (50); *eaeA:* 2 (100); *stx1, eaeA:* 1 (50); *stx2, eaeA:* 1 (50); *stx1, stx2, eaeA:* 1 (50)
	Total	3 (60)		-
Spices (3)	Non detected	-		-
	EHEC	1 (50)		*stx1, eae, ehly:* 1 (100)
	AEEC	1 (50)		*stx1:* 1 (100); *stx2:* 1 (100); *eaeA:* 1 (100); *stx1, eaeA:* 1 (100); *stx2, eaeA:* 1 (100); *stx1, stx2, eaeA:* 1 (100)
	Total	2 (66.66)		-
Total (26)	Non detected	3 (15.78)		-
	EHEC	7 (36.84)		*stx1, eae, ehly:* 7 (100)
	AEEC	10 (52.63)		*stx1:* 10 (100); *stx2: 6 (60); eaeA:* 10 (100); *stx1, eaeA:* 6 (60); *stx2, eaeA:* 6 (60); *stx1, stx2, eaeA: 6 (60)*
	Total	19 (73.07)		-

produced in high temperature and therefore were free from any pathogenic bacteria. Barbecue is a meat-based Kebab produced by roasting of beef on the charcoal. This cooking style cause superficial roasting and the interior parts of the meat remains raw. This is the main reason for the high prevalence of STEC strains in this food product. Using from un-washed vegetables caused high prevalence of STEC strains in salad samples of our study.

Another part of our findings revealed the high prevalence of STEC virulence factors and especially *stx1* and *eaeA*. High presence of these factors in EHEC and AEEC subtypes showed their significant pathogenicity for people who consumed from these foods. Simultaneous presence of *stx1* and *eaeA* and *stx2* and *eaeA* virulence factors in some strains of *E. coli* of ready to eat foods represented an important public health issue regarding the consumption of ready to eat foods.

Several investigations have been conducted in this field al-around the world.[13-16] Momtaz *et al*.[13] reported that the prevalence of *E. coli* strains among meat samples was 29.02%. They showed a considerable prevalence of *E. coli* in sheep meat (35.45%). The results of their investigation revealed the higher prevalence of AEEC than EHEC subtypes and also considerable distribution of *stx1* and *eaeA* virulence factors. Hemmatinezhad *et al*.[14] reported that the prevalence of *E. coli* in various types of foods had a range of 9-28%. They showed that the prevalence of *stx1* gene in the AEEC strains was 100% and EHEC-positive strains harbored all three *stx1*, *eaeA* and *ehly* genes. Ranjbar *et al*.[15] showed that the prevalence of *E. coli* in various types of raw and cooked food samples was 6.72%. They showed that prevalence of *E. coli* in raw meat, raw chicken and raw fish, cooked meat, cooked chicken, cooked fish and soup samples were 20%, 16.66%, 1.42%, 6%, 3%, 2.72% and 5%, respectively. They showed that prevalence of EHEC and AEEC subtypes in raw meat and chicken, cooked meat, chicken and fish and soup samples were 16.66% and 50%, 20% and 60%, 25% and 50%, 0% and 33.33%,0% and 50% and 33.33% and 66.66%, respectively. Their findings showed that EHEC strains harbored all three *stx1*, *eaeA* and *ehly* genes together (100%), while AEEC strains harbored different percent of these genes. Shayegh *et al*.[16] reported that 22 out of 200 samples studied (11%) were positive for *E. coli*. They represented that 54.54% of *E. coli* isolates were STEC. In addition to our results and also finding of Momtaz *et al*.,[13] Hemmatinezhad *et al*.[14] and Ranjbar *et al*.,[15] simultaneous presences of *stx1*, *stx2*, *eaeA* and *ehly* virulence factors have also been reported by Franz *et al*.,[17] Jay-Russell *et al*.[18] and Kabiru *et al*.[19]

Conclusions

In conclusions, we identified a large numbers of virulence factors in the STEC strains isolated from ready to eat foods. High contamination rate of salad, candy and barbecue and considerable prevalence of *stx1* and *eaeA* virulence factors were the most commonly determined properties of STEC strains of ready to eat foods. Presence of EHEC strains in majority of food samples represents inadequacy of time and temperature used for cooking of food samples and also transmission of pathogenic agents from animal sources to them. It seems that there were no firm managements on the principles of food hygiene in Iranian ready to eat foods. However, further studies are required to determine the distribution of serotypes and antibiotic resistance properties of STEC strains and also their sequencing to found important epidemiological data about the presence of STEC strains in ready to eat foods.

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