Original Article

Journal of Food Safety and Hygiene

Journal homepage: http://jfsh.tums.ac.ir

Detection of cytolethal distending toxin and other virulent factors in *Escherichia coli* samples from animal livestock, retail foods and gastroenteritis human cases in Qatar

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ARTICLE INFO

Article history:
Received 06 Apr. 2019
Received in revised form 22 Jul. 2019
Accepted 02 Aug. 2019

Keywords:
Cytolethal distending toxin;
E. coli

ABSTRACT

Cytolethal distending toxin (CDT) is a heterotrimeric AB-type genotoxin produced by several clinically important bacterial pathogens. To better understand the risk of CDT within the food supply and human gastroenteritis patients in Qatar, we investigated the frequency of the CDT gene (cdtB) among *Escherichia coli* (*E. coli*) strains recovered from food products, animal livestock, and human gastroenteritis patients. In this cross-sectional study, *E. coli* isolates were screened for cdtB using polymerase chain reaction (PCR). cdtB positive strains were further examined for *E. coli* cdtB gene types (cdt I, cdt II, cdt III, cdt IV and cdtV), serotypes O157: H7, and non-O157 Shiga toxin-producing *E. coli* O26, O45, O103, O111, O121, and O145. Screening for other virulent factors, stx (Shiga toxin gene) and eae (gene that encodes intimin) genes were also performed. The cdtB gene was detected in *E. coli* isolates sourced from all three groups; animal livestock (17%), retail foods (8%), and human gastroenteritis patients (3%). Although the incidence of cdtB gene harboring *E. coli* is relatively low among gastroenteritis patients, there is still a risk of infection from animal reservoirs as well as retail food products. Among the three groups, *E. coli* isolates from humans had the lowest occurrence of cdtB, stx, eae, and O157: H7. Furthermore, we advise implementing monitoring at the food production and preparation level.

Citation: Gómez AM, Valenzuela NN, Peters KE, Salem A, Sultan A, Doiphode S, Ibrahim EE, Mohammed HO. **Detection of cytolethal distending toxin and other virulent factors in *Escherichia coli* samples from animal livestock, retail foods and gastroenteritis human cases in Qatar.** J Food Safe & Hyg 2019; 5(2): 112-119

1. Introduction

Cytolethal distending toxin (CDT) is an emerging virulence factor of gram-negative bacteria with the unique capacity to cause double-stranded DNA breaks, irreversible cell cycle arrest, and subsequent apoptosis in human T-cells, Chinese Hamster Ovary (CHO), Vero (kidney epithelial cells derived from African green monkey), HeLa (human cell line derived from cervix carcinoma), and human epithelial type 2 cells (Hep-2) (1). Previous studies have concluded that CDT acts as a holotoxin, where cdtB is the active toxic unit while cdtA and cdtC are the protein units required for proper delivery and binding of CDT into cells (2).

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CDT acts as a virulence factor in promoting diseases by toxin-producing pathogens, including foodborne-pathogens (3,4).

CDT occurrence has been reported in *Escherichia coli* (*E. coli*), *Salmonella* and *Campylobacter spp.* isolates from food supply chain and from patients (5-9). Even though all CDTs cause similar effects (cell cycle arrest and apoptotic effects that eventually cause cell death) their genomic sequences differentiate among various bacterial species (2). For cells to produce CDT, all three linked genes, cdtA, cdtB and cdtC must be present (1).

Due to higher DNA variability in the cdtA and cdtC subunits, cdtB sequences which are more conserved among CDT producing bacteria have been more Sequence variability within the cdtB gene have been characterized in *E. coli spp.* (2,11). Studies have reported five E. coli cdtB genetic variants cdt I, cdt II, cdt III, cdt IV and cdt V (4, 12-15).

Given the pathogenic risk of CDT, it is important to understand its epidemiological burden associated with the contamination of the food system with foodborne pathogens and the potential risk to humans. In this study, the occurrence of CDT in *E. coli* recovered from animal, retail and human sources collected in Qatar was investigated. In addition, cdtB typing on samples positive for the cdtB gene and possible co-expression of other common *E. coli* virulent factors, stx and eae, was examined .

2. Materials and Methods

2.1. Target and Study Sample Size

We carried out a cross-sectional study to investigate the prevalence of positive cdtB gene in *E. coli* isolates. Samples recovered from two target populations, human and non-human, were collected and examined. Human samples consisted of fecal samples obtained from gastroenteritis patients admitted to the Hamad Medical Corporation Hospitals in Qatar (7). Non-human samples were collected from animal livestock and retail foods. Details on the target population and study population are provided in previous studies (16). Briefly, fecal samples were obtained from animals, food processing plants, retail, and humans in a cross-sectional study aimed at investigating the occurrence of *E. coli* O157:H7 and non-O157 Shiga toxin-producing *E. coli*, along the food supply chain. Retail samples were collected from different types of produce that were processed and packaged in different sites. Sterile gauze pads (4x4 inches) were used to swab surfaces and utensils and were placed into sterile vials until testing. Cuts of meat, cheese, and samples of ready-to-eat food were collected aseptically and placed directly into sterile tubes until testing. All samples were transported to Weill Cornell Medical College of Qatar in ice boxes for processing. Various types of foods were sampled, including beef, chicken, lamb, goat, camel, seafood (fish, shrimp, crab, cuttlefish, squid), cheese, and salads. Surfaces including tables, cutting boards, knives, containers, refrigerators, serving plates, gloves, balances, and mincing machines. Human isolates of E. coli were retrieved from the bacteriology laboratory at Hamad Medical Corporation hospital in Qatar.

2.2. Sample Processing for Pathogen Detection

E. coli O157:H7, non-O157 shiga toxin-producing *E. coli* and virulence factors were detected using the BAX® Automated System (Dupont, USA). The BAX® Automated System is a real-time polymerase chain reaction (PCR) process which consists of bacterial enrichment and molecular detection. For all sources, each sample was processed using bacterial enrichment, which consisted of two steps: primary (bacterial repair) and secondary (bacterial growth).In the primary enrichment all samples were inoculated into Modified *E. coli* broth (MEC broth) supplemented with novobiocin (16 mg/l) at a ratio of 1:10. The inoculum was incubated for 24 h at 37°C. A total of 20 µl of the incubated enriched inoculum was transferred into 1 ml of the secondary enrichment medium (Brain Heart Infusion (BHI) without antibiotics) and incubated for three h at 37°C before being processed for the real-time PCR. The bacterial lysate from the secondary enrichment was tested using the *E. coli* O157:H7 and the Shiga toxin-producing *E. coli* (STEC) Suite kit on the BAX PCR system. The STEC Suite kit targets stx (Shiga toxin gene) and eae (gene that encodes for the intimin protein, a protein that allows bacterial attachment to the gut mucosa) genes. Samples positive for both or either stx and eae genes were further screened for non-O157:H7 Shiga toxin-producing *E. coli* serotypes, namely O26, O45, O103, O111, O121, and O145. Non-0157 Shiga toxin-producing *E. coli* serotypes O26, O111 and O121 were detected using the STEC Suite Panel 1 Assay, and the Non-0157 Shiga toxin-producing *E. coli* serotypes O45, O103 and O145 were detected using STEC Suite Panel 2 Assay. *E. coli* isolates were stored at -80°C, with a 1:1 ratio of 30% glycerol and secondary enrichment bacteria until cdtB gene detection.

For our cdtB+ samples the occurrence of both virulence factors (stx and eae) tested was seen in 11:23 (48%), only 2:23 (8%) of samples tested negative for both virulence factors, 5:23 (22%) were positive for only stx and 5:23 (22%) were positive for only eae. In addition to cdtB PCR detection, cdtB+ samples were further screened for cdtB typing. The cdtB type I, III, IV and V were seen throughout all the sources of samples (Table 4). The majority of the samples from animals had cdtB type III and V. Most of the cdtB types were not common in samples tested from human and only type IV was detected in the two samples that were tested. The cdtB types III and V were common among the retail samples tested followed by type I. cdtB II was not detected in any of the samples that were tested.

cdtB gene detection Genomic DNA (gDNA) was extracted from *E. coli* strains isolates using the Wizard Genomic DNA Purification Kit (Promega®). Multiplex primers previously designed for detecting the presence of cdtB (14) were used as an initial screening. cdtB positive (cdtB+) isolates were further investigated for cdtB typing (cdt I, cdt II, cdt III, cdt IV, and cdtV) using primers from Kim et al., 2009. PCR and amplification conditions for all samples were as follows: 0.2 µM of dNTP Mix, 1X Taq Buffer with 2 mM $MgCl_2$, 0.2 µM concentrations for each primer, and 0.75 U Taq polymerase; amplification protocol was performed as stated on (14). cdtB+ samples were sent to the Cornell Biotechnology Resource Center (BRC) Genomics Facility for sequencing. Sequences obtain from the BRC were later compared with other sequences published on the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) database to confirm cdtB typing.

2.3. Data Analysis

The occurrence of a particular serogroup in each sample was computed as the proportion of the samples that tested positive out of all samples tested from that particular food. The odds of a specific serotype within each category was evaluated using logistic regression and quantified using the odds ratio (OR). All statistical tests were performed using the SPSS 24 (IBM-statistical software, White Plains, NY) and p-values were evaluated at $p < 0.05$ unless indicated otherwise.

3. Results

Random selection was used to select a subset from the total samples by source (animal, human, or retail). Table 1 shows the distribution of the *E. coli* serotypes and samples selected by source.

A total of 249 samples were used in this study—69 animal, 72 human, and 108 retail. Among the selected samples the virulence factors stx and eae genes were detected at an approximately equal proportion. The retail samples had the highest proportion of both genes. The presence of the *E. coli* serotypes varied among the selected samples, with O45 being the most common followed by O103 and O26 (Table 2). All the six targeted serotypes were detected in animal and retail samples at different proportions while only serotype O157:H7 was detected in samples from humans.

Table 3 shows the results of statistical analyses of the association between the source of samples and a particular *E. coli* serotype. It was twice more likely to detect serotype O157:H7 in samples recovered from animals in comparison to samples selected from retail (OR = 2.1, p-value = 0.094). There was no significant difference in the likelihood of finding O157:H7 between human or retail samples. The likelihood of recovering serotype O26 was twice more likely from samples recovered from animals in comparison to samples from retail (OR = 2.1). Serotype O26 was not detected in the 72 human samples that were examined. It was ten times more likely to detect serotype O103 in samples from animals than that from retail (OR= 9.9) (Table 3).

The stx gene was detected in all the different sources of samples: animal (52%), human (11%), and retail (57%). Similarly, the eae gene was detected in animal (49%), human (29%), and retail (50%) samples (Table 1). The results of evaluation of association between the two genes and the source of the samples are shown in Table 3. It was less likely to detect stx in samples recovered from humans in comparison to samples from retail (OR = 0.1). There was no significant difference in the detection of stx in samples selected from animal or retail. Similarly, it was less likely to detect eae in samples from humans in comparison to retail (OR = 0.4) (Table 3). There was no significant difference in the detection of eae in samples recovered from animals in comparison to retail.

Table 4 shows the results of the screening of all the selected samples (n = 249) for the cdtB gene. The cdtB virulence factor was present in 9% of the samples. Furthermore, the virulence factor was detected in samples recovered from different sources: animal (17%), human (3%) and retail (8%). The virulence gene was detected in five of the serotypes targeted in this study among animal samples, while it was also detected in two of the six serotypes identified among retail samples. There was an association between the presence of the cdtB virulence gene and the source of the samples. It was twice more likely to detect the cdtB gene in samples selected from animals in comparison to samples from retail (OR = 2.3, P-value = 0.069).

Table 1. *E. coli* serotypes and genotypes of all samples (animal, retail and human) collected in Qatar.

		Serotypes							Genotype	
Source	**No.**	**O157:H7**	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**	***stx***	***eae***
Animal	69	10(3) [a]	12 (2) [a]	16(5) [a]	19(4) [a]	1(1) [a]	7(1) [a]	4	36(10) [a]	33(9) [a]
Retail	108	8	10(1) [a]	37(1) [a]	4	9	2	4	62(4) [a]	54 (5) [a]
Human	72	3	0	0	0	0	0	0	8 (2) [a]	21 (2) [a]
Total	249	21(3) [a]	22(3) [a]	53(6) [a]	23(4) [a]	10(1) [a]	9(1) [a]	8	106	108

Footnote: Serotype and genotype numbers represent the amount of positive samples for each given criteria.
a Number of samples positive for cdtB for serotype or genotype indicated.

Table 2. Detailed report of genotypes and serotypes of *E. coli* animal, retail and human samples collected in Qatar.

Samples	**Total No.**	**O157:H7**	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**
Animal	**69** [a]	**10** [c]	**12** [c]	**16** [c]	**19** [c]	**1** [c]	**7** [c]	**4** [c]
stx	36 [b]	8 [d]	11 [d]	13 [d]	14 [d]	1 [d]	6 [d]	4 [d]
eae	33 [b]	9 [d]	12 [d]	14 [d]	17 [d]	1 [d]	7 [d]	4 [d]
cdtB	12 [b]	2 [d]	2 [d]	5 [d]	4 [d]	1 [d]	1 [d]	4 [d]
Retail	**108** [a]	**8** [c]	**10** [c]	**37** [c]	**4** [c]	**9** [c]	**2** [c]	**4** [c]
stx	62 [b]	7 [d]	4 [d]	11 [d]	2 [d]	0 [d]	2 [d]	2 [d]
eae	54 [b]	7 [d]	10 [d]	13 [d]	4 [d]	3 [d]	2 [d]	3 [d]
cdtB	9 [b]	0 [d]	1 [d]	2 [d]	0 [d]	0 [d]	0 [d]	0 [d]

Footnote: Numbers represent the amount of positive samples for each given criteria: a Total number of samples tested.
b Total number of samples positive for genes.
c Total number of samples positive for serotype.
d Total number of samples positive for both gene and serotype indicated

Table 3. Results of significance of association between *E. coli* serotypes and the source of the samples as analyzed by logistic regression analysis.

Source	Regression coefficient	Standard error	Odds ratio and 95% confidence interval	*P-value*
O157:H7				
Animal	0.751	0.502	2.1 (0.8, 5.7)	*0.094*
Retail	0	0	1.0	*0.135*
Human	-0.610	0.695		
Constant	-2.526	0.367	0.5 (0.1, 2.1)	
O26				
Animal	0.724	0.459	2.1 (0.8, 5.1)	*0.115*
Retail	0	0	1.0	
Constant	-2.282	0.332		
O45				
Animal	-0.546	0.350	0.6 (0.3, 1.1)	*0.119*
Retail	0	0	1.0	
Constant	-0.652	0.203		
O103				
Animal	2.291	0.576	9.9 (3.2, 30.5)	*0.001*
Retail	0	0	1.0	
Constant	-3.258	0.510		
stx				
Animal	-0.211	0.310	0.8 (0.4, 1.5)	*0.495*
Retail	-2.378	0.442		
Human	0	0		
Constant	0.298	0.195	0.1 (0.04,0.2)	*0.001*
eae				
Animal	-0.087	0.308	0.9 (0.5, 1.7)	*0.495*
Retail	0	0		
Human	-2.378	0.442	0.4 (0.2,0.8)	*0.001*
Constant	0	0.192		

Table 4. Analysis of serotypes and genotypes of animal, retail and human *E. coli* semples possession cdtB gene.

Source of sample	Serotype		Genotype			
	O157:H	O Type	stx	eae	cdtB	Cdt type
Animal			+	-		
S2- Sheep swab	-	-	+	+	+	I
F12-Animal feces	-	-	+	+	+	I
F11- Animal feces	+	-	+	+	+	V
F11- Animal feces	+	O45, O103	-	-	+	V
F11- Animal feces	+	O45, O103	+	+	+	III, V
F11- Animal feces	-	-	-	+	+	III, V
F11- Animal feces	-	O45	-	+	+	III, V
F11- Animal feces	-	O45, O103	+	+	+	III, V
F11- Animal feces	-	O103	+	+	+	III, V
F11- Animal feces	-	-	+	-	+	III, V
F12- Animal feces	-	O26	+	+	+	III, V
S8- Camel feces	-	O26, O45, O111, O121	+	+	+	III, V
Retail						
Chicken gizzard	-	-	-	+	+	I
Chicken gizzard	-	O26	-	+	+	I
Shish –Yogurt	-	-	-	-	+	IV
Mincing Machine	-	-	+	-	+	I, IV
Chopped goat	-	-	-	+	+	III, V
Minced Beef	-	-	+	-	+	III, V
Local beef	-	-	-	+	+	III, V
Local beef	-	-	-	-	+	III, V
Lamb Kidney	-	045	-	+	+	I, III, V
Human						
Human	-	-	-	+	+	IV
Human	-	-	-	+	+	IV

Footnote: Abbreviations: cdtB = cytolethal distending toxin B gene; stx = Shiga toxin gene, eae = gene that encodes intimin protein; F11= Farm 11; F12 = Farm 12; S2 = Slaughterhouse 2; S8 = Slaughterhouse 8.

4. Discussion

This manuscript is a part of our long-term objective to investigate the risk of foodborne pathogens to humans and to shed light on the possible virulence factors along the food supply chain in Qatar (16,17).

The focus of this study was on *E. coli* and the potential presence of the CDT virulence factor in hopes of identifying the source(s) of disease to humans along the food supply chain.

To our knowledge this is the first effort that attempted to track the occurrence of cdtB producing *E. coli* along the supply chain (animal livestock and retails foods) and in hosts (gastroenteritis human cases). Former research concentrates on the understanding of how CDT affects cells, but not on the occurrence of the toxin (1). Since CDT is not specific to bacteria cells, its sequence variability within the cdtB gene is the best way to identify cdt gene prevalence in *E. coli* (1,2).

Identifying which sources could potentially produce CDT in the future will help us recognize points of intervention along the food supply chain, if there is outbreak.

Our results revealed a low incidence of CDT-producing *E. coli* among human gastroenteritis patients. These findings are similar to larger studies on patients in Korea and Mexico, which yielded CDT *E. coli* prevalence of 2.7% of 336 patients and 1% of 1060 patients, respectively (4,18). Despite the low proportion of human cases of CDT-producing *E. coli* among these studies, our results indicate risk of CDT-producing *E. coli* from retail meats, food processing areas, and livestock. A study in Nara, Japan found much higher percentages of CDT-producing *E. coli* from animal livestock, with 88.23% of 102 cattle stool specimens from a single farm and 31.11% out of 45 swine stool specimens from another farm (13). The difference

between that study and ours could be attributed to the fact that all the samples in the former study were from a single farm. A similar study of gastroenteritis patients noted much higher proportions of CDT-producing Campylobacter jejuni and *Campylobacter coli* (7). In our study this may indicate that although a low prevalence of CDT-producing *E. coli* exists among gastroenteritis patients in Qatar, there is still high risk of CDT intoxication via other microbial vehicles.

STEC O157:H7 has been known worldwide for producing foodborne illness, yet current research has mainly been done on these rather than non-O157 STEC even though both have been the cause of major outbreaks (19). Non-O157 STEC are increasingly being reported due to their ability to cause other acute diarrheal diseases (20,21). Our results show that cdtB+ is present in both STEC O157:H7 and non-O157 STEC (serotypes O26, O45, O103, O111 and O121). Our samples from livestock and retail foods are shown to have higher proportions of non-O157 STEC serotypes, rather than STEC O157:H7. Other studies have also shown similar reports in animals, food, surface, water and clinical samples (22,23). Even though only 13% of our cdtB+ samples tested positive for STEC O157:H7 serotype we saw the presence of all three stx, eae and cdtB in non-O157 STEC samples. The presence of multiple virulence factors could increase pathogenic potential, in both non-O157 STEC and STEC O157:H7 serotypes (4,21). The presence of CDT and its association to severe human infection is scarce and therefore it is still being studied, since previous studies on rats have linked CDT toxicity to Bowel disease and potential risk to chronic gastroenteritis (24).

The use of type-specific primers for cdtB in E. coli suggested the existence of CDT variance among the samples examined. Previous studies demonstrated similar variability among the cdtB recovered from different animals and humans (13,25). Furthermore, in vitro studies have proposed that cdtB I could potentially express the highest levels of toxicity in cells, followed by cdtB III and cdtB V (14). The same authors found cdtB IV shares 84% nucleotide sequence identity with cdtB I, suggesting that in theory, cdtB IV could also produce high levels of toxicity (14).

5. Conclusion

Our study demonstrated a relatively low proportion of samples with the cdtB gene, which was consistent with other studies around the world. Samples from animals and retail were detected with the presence of cdtB I, III, IV and V and these samples could pose risk to human health. Lending credence to this statement was the fact that our study samples from humans demonstrated the presence of cdtB IV.

Conflict of interest

The authors declare no competing or potential conflicts of interest.

Acknowledgements

None.

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