

Detection of cytolethal distending toxin (*cdt*) Genes in *Campylobacter jejuni* and *Campylobacter coli* isolated from the intestinal of commercial broiler chickens, turkey and quail of Iran

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Abstract:

BACKGROUND: *Campylobacter jejuni* and *Campylobacter coli* are zoonotic bacteria which are frequently associated with human diarrhea. Sharing of the cytolethal distending toxin (*cdt*) genes in *Campylobacter* is common and is considered species specific. **OBJECTIVES:** In this study we focused on detecting the presence of *cdt* gene in *C. jejuni* and *C. coli* isolated from broilers, turkeys and quails of Iran. **METHODS:** Cecal samples were randomly collected from 240 broiler chickens, 100 meat type turkeys and 100 quails after slaughtering. We used PCR as a method for detecting *cdt* genes. **RESULTS:** In broilers, 93% of 58 *C. jejuni* positive samples possessed *cdt* gene and in all cases the three different subunits of *cdt* genes were present. However, only 56% of 14 *C. coli* isolates in broilers had contained *cdt* genes, while one fourth having all three subunits present. In turkeys, around 65% of 34 *C. jejuni* positive samples had *cdt* gene present with 38% possessing all three subunits of *cdt* genes. But all 5 *C. coli* isolates had all three subunits *cdt* gene. In quails, 67% of 30 *C. jejuni* positive samples were identified by *cdt* gene, 20% of those possessed all three gene subunits. On the other hand, all 28 *C. coli* isolates of quails had *cdt* gene present while 36% of those held all three gene subunits. **CONCLUSIONS:** Our data is indicating the isolation, culture and *cdt* PCR amplification approaches in this study seemed to be efficient. However, the presence of different variation of *Campylobacter cdt* gene types in our sample isolates signifies the necessity of further functional gene studies to elucidate which gene type combinations result in encoding effective toxins.

Introduction

Campylobacter jejuni and *C. coli* are zoonotic bacteria that are frequently associated

with human diarrhea in both developing and in desterilized countries. Among many animal species housing *Campylobacter* species in their tract, wild birds and poultry are the most

important reservoirs (Blaser et al., 1983). Typing of bacterial isolates from different sources provides epidemiological information needed for infection control and contributes to risk assessment of *Campylobacter* transmission. In *Campylobacter*, specific virulence mechanisms are not fully elucidated, although flagella mediated-motility, adhesion to intestinal mucosa, invasion and production of enterotoxin and cytotoxin have been identified as possible virulence determinates (Konkel et al., 1997; Wassenaar, 1997). A number of putative virulence and toxin genes have been studied, including *fla A* and *cad F* genes which are involved in adhesion and colonization of the host's intestine (Konkel, 1999). *Ceu E* genes seem to encode a binding-protein transport system for the siderophore enterochelin (Gonzales, 1997; Park and Richardson, 1995). Cytolethal distending toxin (*cdt*) cluster consists of three adjacent genes of *cdt A*, *B* and *C*. *Cdt* protein is composed of *cdt B* as the enzymatically active subunit, and the two heterodimeric subunit *cdt A* and *cdt C*, responsible for the holotoxin binding to cell membrane (Lara-Tejero and Galàn, 2001, 2002). *Campylobacter* species cytotoxin cause DNA lesions, chromatin fragmentation, and cytoplasm distension which arrest cell cycle in G2/M transition phase, and leads to progressive cellular distension and ultimately cell death (Lara-Tejero and Galàn, 2002; Nesic and Stebbins, 2005; Pickett and Whitehouse, 1999).

The aim of this study was to examine the presence of subunit *cdt A*, *B*, and *C* genes in *Campylobacter jejuni* and *Campylobacter coli* strains isolated from intestinal of commercial broiler chickens, turkeys and quails by conventional microbiological culture followed by PCR amplification.

Materials and Methods

Sample collection: Intestine samples (cecal samples) were randomly collected from 240

broiler chickens of 8 flocks, 100 meat type turkeys, and 100 meat type quails, each from 5 flocks at the slaughter houses. The ceca of birds were placed in sterile plastic bags and transported to laboratory on ice for processing in the same day.

Isolation and identification of *C. jejuni* and *C. coli*: We used *Campylobacter* Charcoal Differential agar (CCDA) plate as direct culture (Funbac media, 23122, Tehran, Iran). The ceca was aseptically opened and a loop full of contents was plated on to be cultured. The plate was incubated in micro-aerobic conditions (Co₂ 8%) at 42 C° for 24-48 hour. Colonies suspected as being *Campylobacter* species were examined for cell morphology by optical microscope. Single colonies were picked and characterized to species level by their catalase reaction, ability to hydrolyze hippurate and indoxyl acetate according to standard procedures (On and Holmes 1992). Isolates were stored with glycerol 15% at 70 C° until required for further investigations.

DNA extraction: We transferred a loop full of colonies from the CCDA into an Eppendorf tube containing 250 µl phosphate-buffered saline (PBS). After the tubes were vortexed, the suspension were kept in 95 C° Ban Mary for 15 min and then centrifuged at 9000xg for 10 min. Consequently, we were able to disassociate DNA in the upper phase of centrifuged tube.

Identification of *Campylobacter* species using multiplex PCR: For species identification, PCR was performed initially with the universal *Campylobacter* 16s rRNA primer (Linton et al., 1996). All PCR positive samples were then subjected to a second PCR for differentiation of *C. jejuni* from *C. coli* (Table 1). For CampF/CampR primer (Sina colon, Tehran, Iran), the reaction was performed in a 25 µl solution, containing 0.5 ng DNA, 0.1 µM each of CampF and CampR primers, 2 units of Tag DNA polymerase (Sina colon, Tehran, Iran), 0.2 mM of each dNTP, 10 mM Tris-HCl

and 2.5 mM MgCl₂. The amplification reactions were carried out using a Perkin Elmer 9600 thermo cycler with the following program: one cycle of 10 min at 95 °C, 35 cycles of 30 s at 95 °C, 1.5 min at 59 °C, 1 min at 72 °C and a final extension step of 10 min at 72 °C. For *Campylobacter* species used 0.2 μM of MDmapA1/MDmapA2 and COL3/ MDCOL2 primers. The amplification reactions were carried out using a Perkin Elmer 9600 thermo cycler with the same program described above. For the visualization of PCR products, 10 μl aliquots were subjected to electrophoresis in 1.5% agarose gel stained with ethidium bromide for 1.5 hour at 100 V, and viewed under UV light.

Detection of *cdt* genes from *Campylobacter* isolates by multiplex PCR: The multiplex PCR was used to detect the *cdt* A, B and C genes of *C. jejuni* and the *cdt* A, B and C of *C. coli* (Asakura et al., 2008) are summarized in table 2. The PCR protocol of Findik et al., (2011) was modified as described below. Our result prove that the concentration of the primer sets (Sina colon, Tehran, Iran) in the multiplex PCR reactions was appropriate, 40 μl of PCR reaction mix contained 0.2 mM dNTP mix and 1.0 U of Ex Taq DNA polymerase. PCR products were analyzed by 1.5% agarose gel electrophoresis. Bands were visualized and images were captured.

Results

In this study, PCR method was used not only for confirmation of genus and species but also for detecting of *cdt* subunits genes using three different primer sets in two different combinations (Tables 1 and 2). In first step, for genus detection of *Campylobacter* isolates, *Campylobacter jejuni* ATCC 29428 and *Campylobacter coli* ATCC 43478 was used as our positive control (Figures 1 to 3).

From 240 samples of broiler chickens, 72 (30%) thermophilic *Campylobacter* were iso-

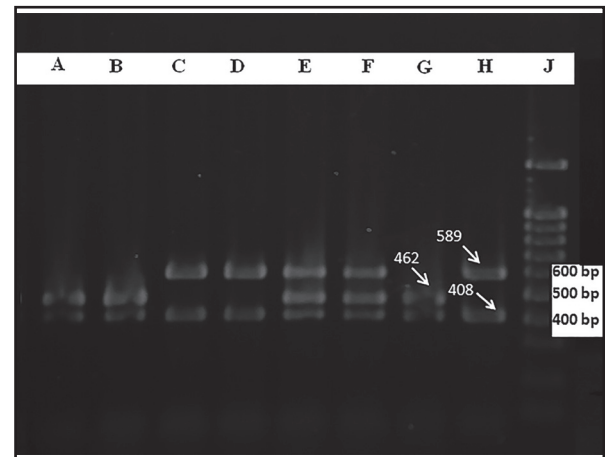


Figure 1. Multiplex polymerase chain reaction for detection of *Campylobacter jejuni* (H) and *coli* (G). A & C: broiler; B & E: turkey; D & F: quail and J: Gene Ruler, 408 bp: fragment represents *Campylobacter* genus, 462 bp: fragment corresponds to *C. coli* species and 589 bp: to *C. jejuni* species.

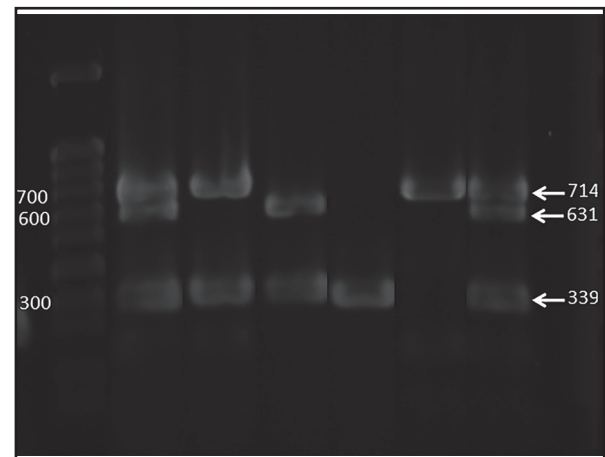


Figure 2. Amplification of *cdt* A (631 bp), *cdt* B (714 bp) and *cdt* C (339 bp) genes of *Campylobacter jejuni* in cecal samples of three different birds, using by multiplex PCR.

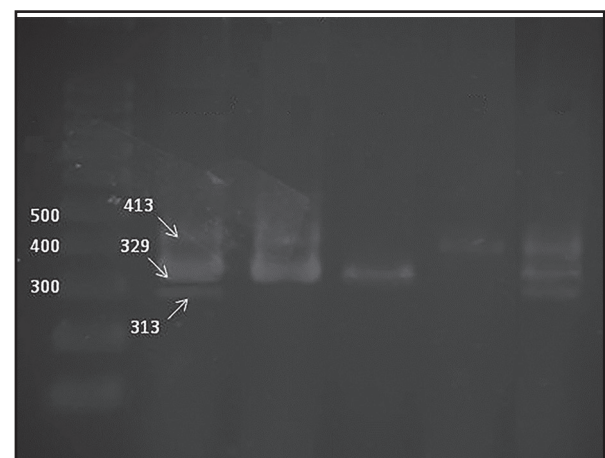


Figure 3. Amplification of *cdt* A (329 bp), *cdt* B (413 bp) and *cdt* C (313 bp) genes of *Campylobacter coli* in cecal samples of three different birds, using by multiplex PCR.

Table 1. Primers used for identification genus and species of *Campylobacter* by polymerase chain and multiplex polymerase chain reaction.

Target	Primer Name	Sequence (5'-3')	PCR Product Size	Specificity	References
16S rRNA	CampF CampR	AGTCTTGGCAGTAATGCACCTAACG ATATGCCATTGTAGCACGTGTGTCG	408	Genus	Wangroongsarb et al, 2011
MapA	MDmapA1 MDmapA2	CTATTTTATTTTGTAGTGCTTGIG GCTTTATTTGCCATTTGTTTTATTA	589	<i>C. jejuni</i>	Stucki et al, 1995
CeuE	COL3 MDCOL2	AATTGAAAATGCTCCAACTATG TGATTTTATTATTTGTAGCAGCG	462	<i>C. coli</i>	Gonzalez et al, 1997

Table 2. Characteristics of PCR primers used for *cdt* genes in the study. (*= *C.jejuni* ; **= *C.coli*).

Primer	Sequence (5'-3')	Target	PCR condition			Amplicon (bp)
			Denaturing	Annealing	Extension	
*Cj- <i>Cdt</i> AU2	AGGACTTGAACCTACTTTTC	Cj- <i>cdtA</i>	94 °C, 30s	55 °C, 30s	72 °C, 30s	631
Cj- <i>Cdt</i> AR2	AGGTGGAGTAGTTAAAAACC					
Cj- <i>Cdt</i> BU5	ATCTTTTAAACCTTGCTTTTGC	Cj- <i>cdtB</i>	94 °C, 30s	56 °C, 30s	72 °C, 30s	714
Cj- <i>Cdt</i> BR6	GCAAGCATTAAAATCGCAGC					
Cj-CDTC-F	ACTCCTACTGGAGATTTGAAAG	Cj- <i>cdtC</i>	94 °C, 60s	57 °C, 60s	72 °C, 60s	339
Cj-CDTC-R	CACAGCTGAAGTTGTTGTTGTTGGC					
**Cc- <i>Cdt</i> AU1	ATTGCCAAGGCTAAAATCTC	Cc- <i>cdtA</i>	94 °C, 30s	55 °C, 30s	72 °C, 30s	329
Cc- <i>Cdt</i> AR1	GATAAAGTCTCCAAAACCTGC					
Cc- <i>Cdt</i> BU5	TTTAATGTATTATTTGCCGC	Cc-B	94 °C, 30s	56 °C, 30s	72 °C, 30s	413
Cc- <i>Cdt</i> BR5	TCATTGCCTATGCGTATG					
Cc- <i>Cdt</i> CU1	TAGGGATATGCACGCAAAAG	Cc- <i>cdtC</i>	94 °C, 30s	55 °C, 30s	72 °C, 30s	313
Cc- <i>Cdt</i> CR1	GCTTAATACAGTTACGATAG					

Table 3. The occurrence of *cdt* gene subunits in *Campylobacter* species isolated from commercial broiler chickens, turkeys and quails. *= number / percentage of characters *cdt* gene subunits

Birds	Number isolates (%)	Number / percentage of characters <i>cdt</i> gene subunits in thermophilic <i>Campylobacter</i>							
		ABC	AB	AC	BC	A	B	C	non
<i>Campylobacter jejuni</i>									
Broilers	58 (81%)	54 (93%)	-	-	-	-	-	-	4 (7%)
Turkeys	34 (87%)	13 (38%)	1 (3%)	1 (3%)	1 (3%)	1 (3%)	3 (9%)	2 (6%)	12 (35%)
Quails	30 (52%)	6 (20%)	-	-	10 (33%)	-	4 (14%)	-	10 (33%)
Total	122 (72%)	73 (59%)	1	1	11 (9%)	1	7 (6%)	2 (6%)	26 (21%)
<i>Campylobacter coli</i>									
Broilers	14 (19%)	2 (14%)	2 (14%)	-	-	2 (14%)	-	2 (14%)	6 (44%)
Turkeys	5 (13%)	5 (100%)	-	-	-	-	-	-	-
Quails	28 (48%)	10 (36%)	4 (14%)	-	-	10 (36%)	2 (7%)	2 (7%)	-
Total	47 (28%)	17 (36%)	6 (13%)	-	-	12 (26%)	2 (4%)	4 (9%)	6 (13%)

lated. 58 of these isolates (81%) were *C. jejuni* and 14 (19%) were *C. coli*. Out of 58 *C. jejuni* isolates, 54 (93%) were *cdt* positive, which all of them had all three types of *cdt* A, B and C genes, but 4 isolates had no any of those subunit genes (Table 3). Between the 14 *C. coli*

isolates, 8 (56%) isolates indicated at least one of those *cdt* A, B or C genes and 6 (44%) had no *cdt* gene. Out of 8 isolates 2 (25%) had all three types of *cdt* genes, 2 (25%) isolates had both *cdt* A and B, 2 (25%) isolates had only *cdt* A and 2 (25%) isolates had only *cdt* C (Table

3).

Of the 100 intestinal samples collected from turkeys, 39 were thermophilic *Campylobacter* positive. Among these positive, 34 isolates (87%) were *C. jejuni* and 5 (13%) were *C. coli*. Out of 34 isolated *C. jejuni*, 22 (65%) had at least one of the three subunit *cdt* genes. Between these, 13 (38%) isolates had all types of *cdt* A, B and C genes, 1 isolate had both *cdt* A and B, 1 isolate had *cdt* A and C, 1 isolate had *cdt* B and C, 1 isolate had only *cdt* A, 3 isolates (9%) had only *cdt* B and 2 isolates (6%) had only *cdt* C. Furthermore, all 5 *C. coli* isolates in turkeys had all types of *cdt* A, B and C genes (Table 3).

Among 100 samples collected from quails, 58 thermophilic *Campylobacter* were isolated. Out of those, 30 (52%) were *C. jejuni* positive and 28 (48%) were *C. coli*. Between 30 *C. jejuni*, 20 isolates were *cdt* positive, in which 6 of them (20%) had all three types of *cdt* genes, 10 isolates (33%) had *cdt* B and C simultaneously and 4 isolates (14%) had only *cdt* B. Out of 28 *C. coli* positive, 10 isolates (36%) had all three types *cdt* A, B and C genes, 4 (14%) had *cdt* A and B together, 10 (36%) had *cdt* A, 2 (7%) had *cdt* B and 2 (7%) also had *cdt* C (Table 3). Interestingly that both *Campylobacter jejuni* and *Campylobacter coli* were frequently recovered at the same time from individual examined- birds.

Discussion

According to epidemiological studies taken place in Iran, the reported prevalence of *Campylobacter* infection in poultry of Isfahan was 56 % (Rahimi and Tajbakhsh, 2008). These reports in Shahrekord was 47 % (Rahimi and Ameri, 2011; Rahimi, 2013) but in Tehran, was 50 to 63 % (Taremi et al., 2006). In the other study in Tehran, the prevalence of *Campylobacter* on turkeys was 55 % and quails was 22 % respectively (Mirzaie et al., 2011). The *Campylobacter* infection of poultry meat

in other places of the world is also considerably high, for instance 92 % in turkey (Yildirim et al., 2005), 87 % in Poland (Wieczorek et al., 2012) and 64 % in Italy (Di Giannatale et al., 2012). Also in an Irish study on turkey and poultry meat, *Campylobacter* infection was reported 38 % and 50 % respectively (Whyte et al., 2004). In our study, the number of samples infected with *Campylobacter jejuni* is considerably higher than the ones infected with the *Campylobacter coli* (73 and 27 percent respectively). This figures are confirmed by similar earlier studies (Rahimi and Tajbakhsh, 2008; Son et al., 2007; Dipineto et al., 2010), however they differ with some reports (Di Giannatale et al., 2012). The proportion of the infection with these two bacterial species seems to be highly variable in different studies. Such difference might be a result of various avian infection in different area, sampling methods, different slaughter and processing approaches, seasonal of sample collection or sensitivity of statistical methods in these studies (Stern and Line, 1992; Wallace et al., 1997).

In the present study, regardless of avian sample type, 59% of all *C. jejuni* isolates and 36% *C. coli* isolates had all three sub-types of *cdt* genes (A, B and C). However in the study done by Dipineto et al. (2010) in Italy, 100 % *C. jejuni* isolates had all three types of *cdt* gene present. Authors also pointed that 100% of *C. coli* isolates had *cdt* B, 99% isolates showed *cdt* A and 99% showed *cdt* C positive. In the another report on Danish broilers, Bang et al. (2001) mentioned all *cdt* gene types were present in 99% isolates of *C. jejuni* and 100% isolates of *C. coli*. In the study performed by Van Deun et a., (2007) four pathogenicity factors of *C. jejuni* were compared between 24 human and 20 poultry isolates. The result of their study confirmed the presence of *cdt* genes in all studied isolates. Study done by Findik et al. (2011) on human, cattle, sheep, dog and poultry samples indicated that 76% of *C. jejuni* isolates were *cdt* A, B and C positive. While,

0.6%, 11% and 6% of their isolates were respectively *cdt B*, *cdt C* and *cdt A* negative. Among their isolates 4% showed only *cdt B* and 3% did not have any type of *cdt* genes present. According to Findik et al. (2011) prevalence of *cdt* gene types can be vary between 69 and 100% for *cdt A*, 92 and 100% for *cdt B* and 39 and 98% for *cdt C*.

Recently, Ehsannejad et al. (2015) reported the virulence markers of *cdt* genes in fecal samples of pet birds of Iran. In their study, 20 thermophilic *Campylobacter*s were detected from 8 different avian species. From 20 confirmed *Campylobacter* spp. 16 samples (80%) were *C. jejuni* positive and 4 (20%) were *C. coli* by species-specific PCR test. Furthermore, out of 20 detected *Campylobacter*, 13 (65%) harbored the various subunits of *cdt A*, *cdt B* and *cdt C* genes, and 7 (35%) were negative for all tested *cdt* genes. They concluded that the carriage rate of *Campylobacter* in different species of cage and/or in zoo birds was also high and confirmed that *cdt* genes may frequently be present in *Campylobacter* spp.

The present study is indicated that *cdt* genes may frequently be present in *Campylobacter* spp, isolated from commercial birds, however the role of those in campylobacteriosis is still in question. Further study is needed to improve the understanding of the role of CDT in the pathogenesis of campylobacteriosis. Considering the importance of presence of *cdt* gene types in construction of an active *cdt* holotoxin, it is also recommended to take more gene functional approaches for future exploration of pathogenicity mechanism of thermophilic *Campylobacter*.

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شناسایی ژن‌های سم‌کشنده تورمی سلولی (*cdt*) در کمپیلوباکتر ژژوئی و کمپیلوباکتر کلی جدا شده از روده جوجه‌های گوشتی، بوقلمون و بلدرچین تجاری در ایران

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چکیده

زمینه مطالعه: کمپیلوباکتر ژژوئی و کمپیلوباکتر کلی، باکتری‌های زئونوزی هستند که در بسیاری از موارد سبب اسهال در انسان می‌شوند. ژن سم‌کشنده تورمی سلولی *cdt* در کمپیلوباکترها شایع و اختصاصی می‌باشد. هدف: در این مطالعه ما به بررسی حضور ژن‌های *cdt* در کمپیلوباکتر ژژوئی و کمپیلوباکتر کلی که از گله‌های جوجه گوشتی، بوقلمون و بلدرچین پرورشی جدا نمودیم، پرداخته ایم. روش کار: نمونه‌های سکومی را بطور تصادفی بعد از کشتار، از ۲۴۰ قطعه جوجه گوشتی، ۱۰۰ قطعه بوقلمون و ۱۰۰ قطعه بلدرچین تهیه و پس از جداسازی باکتری با استفاده از PCR به آنالیز ژن *cdt* آن مبادرت نمودیم. نتایج: در جوجه‌های گوشتی، ۹۳٪ از ۵۸ نمونه کمپیلوباکتر ژژوئی جدا شده، واجد هر سه قطعه ژن *cdt* بودند ولی فقط ۵۶٪ از ۱۴ نمونه کمپیلوباکتر کلی جدا شده واجد قطعاتی از ژن *cdt* بودند. در بوقلمون، حدود ۶۵٪ از ۳۴ نمونه کمپیلوباکتر ژژوئی جدا شده، واجد قطعاتی از این ژن بودند و ۳۵٪ آنها فاقد هیچیک از سه بخش این ژن نبودند. در حالی که هر ۵ نمونه کمپیلوباکتر کلی جدا شده از این پرنده واجد هر سه قطعه این ژن بودند. در بلدرچین، ۶۷٪ از ۳۰ نمونه مثبت کمپیلوباکتر ژژوئی، دارای قطعاتی از این ژن بودن که ۳۰٪ آنها هر سه بخش این ژن را داشتند. از طرفی هر ۲۸ نمونه کمپیلوباکتر کلی مثبت در این پرنده دارای قطعاتی از این ژن بودند. نتیجه‌گیری نهایی: با توجه به اطلاعات بدست آمده در این مطالعه، جداسازی، کشت و PCR روش‌های مناسبی جهت بررسی ژن *cdt* می‌باشند. تفاوت‌های موجود در حضور قطعات این ژن در نمونه‌های بدست آمده هم، اهمیت عملکرد این ژن در کنار سایر ژن‌ها، برای تولید سم فعال این باکتری را نشان می‌دهد.

واژه‌های کلیدی: جوجه گوشتی، بوقلمون، بلدرچین، سم‌کشنده سلولی، کمپیلوباکتر ژژوئی

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