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Investigation of Virulence-Associated Genes and Cytolethal Distending Toxin Production in *Campylobacter* spp. Isolated from Broilers

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ABSTRACT

The aim of this study was to investigate the prevalence of virulence and Cytolethal Distending Toxin (CDT) genes in the *Campylobacter* isolates from intestinal contents and gall bladders of broilers and, to evaluate their cytotoxic effects on HeLa cell cultures. These genes play important roles in bacterial adherence to intestinal mucosa, flagella-mediated motility, invasive capability and the ability to produce toxins in *Campylobacter* pathogenesis. A total of 121 *Campylobacter* isolates (106 *C. jejuni*, 11 *C. coli*, 2 *C. lanienae*, and 2 *C. lari*) were used in this study. The frequency of virulence genes in all the isolates were detected in different proportions ranging from 34-93% using Polymerase Chain Reaction (PCR) assay. Cytolethal Distending Toxin A (CDTA), Cytolethal Distending Toxin B (CDTB) and Cytolethal Distending Toxin C (CDTC) genes were found in 66.1%, 65.3% and 66.9% of the *Campylobacter* isolates tested, respectively ($P > 0.05$). Of the 19 isolates, only two (one *C. jejuni*, one *C. coli*) showed morphological changes such as cell swelling, expansion, growth, and cell shape change in HeLa cell cultures. CDT and virulence genes were detected at low frequencies in *C. jejuni*, *C. coli* and *C. lari* isolates that were obtained from clinically healthy broilers. Although valuable information was attained about the pathogenicity of *C. lanienae*, additional studies using animal models are necessary for clarification.

1. Introduction

Campylobacter spp., particularly *Campylobacter jejuni* and *Campylobacter coli*, are considered to be the most common bacterial causes of gastrointestinal diseases in humans worldwide. *Campylobacter* lives commensally and asymptotically in the gastrointestinal tract of various wild and domestic animals with poultry being the most important source of infection in humans (Deming, 1987; Newell, 2001; Acik, 2013b). It has been estimated that approximately 50 to 80 % of *Campylobacter* infections in humans originated from the chicken reservoir (EFSA, 2010). Handling,

preparation and consumption of poultry meat are widely referred as the risk factors associated with *Campylobacter* infections in humans (EFSA, 2011). Although *C. jejuni* is considered to be responsible for 90% of *Campylobacter* infections, *C. coli* and *C. lari* pose risk for human health as well (EFSA and ECDC, 2009). It is still questionable whether *C. lanienae* strains isolated from humans and domestic animals are pathogenic. Specific pathogenicity mechanisms in these pathogens have not yet been clearly revealed probably due to the lack of an effective small animal model for

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Campylobacter spp. Many studies have previously been conducted to elucidate the mechanism by which campylobacters express certain virulence genes (Acik, 2013a; Ingala, 2014; Koolman, 2016). It has been reported that virulence factors include bacterial adherence to intestinal mucosa, flagella-mediated motility, invasive capability and the ability to produce toxins (Negretti, 2017). Cytolethal Distending Toxin (*CDT*) is a class of heterotrimeric genotoxins (formed by *CDTA*, *CDTB*, *CDTC* subunits) that is widely produced by several pathogenic bacteria, including *Escherichia coli*, *Haemophilus ducreyi*, *Shigella dysenteriae*, and *Helicobacter* spp (Okuda, 1997; Ge, 2005; Gargi, 2013) In addition, *CDT* which has been detected in *Campylobacter* isolates of human and animal origin is one of the most important virulence factors related to the pathogenesis of these agents. It has been shown that *CDT* lead to elongation followed by progressive cellular distention and eventually death in a variety cell lines including Vero, HEp-2, HeLa and Caco-2 cell cultures (Peres, 1997; Malagon, 2010).

CDTB is the biologically active subunit whereas the roles of *CDTA* and *CDTC* are still rather unclear due possibly to their combination with the bacterial outer membrane which probably causes cross contamination. Apart from *CDT*, other virulence factors that play role in the pathogenesis of *Campylobacter* spp. include *flaA*, *cadF*, *racR* and *dnaJ* gene regions which are responsible for colonization and adherence and, *virB11*, *ciaB* and *pldA* gene regions which are responsible for invasion. The high prevalence of *flaA* and *CadF* genes in *Campylobacter* isolates have been reported, while the frequency of other virulence factors varies by the species of origin. For instance, *VirB* gene which is found on the *pVir* plasmid gene is the least frequently reported virulence factor with the proportions ranging from 0% to 20% (Acik, 2013a; Zhang, 2016). Research studies regarding the prevalence of virulence genes in *Campylobacter* isolates of broiler origin are rather limited. In addition, there is a paucity of information about the frequency of *CDT* genes in the strains isolated from gall bladders of broilers. Therefore, the present study was carried out to investigate the presence and frequency of virulence and *CDT* genes in *Campylobacter* isolates of broiler origin and, to

evaluate their cytotoxic effects on HeLa cell cultures.

2. Materials and Methods

2.1. Bacterial strains

A total of 121 *Campylobacter* strains (*C. jejuni*: 106, *C. coli*:11, *C. lanienae*: 2 and *C. lari*: 2) isolated from intestinal content and gall bladder samples of broiler chickens which were slaughtered at three different abattoirs located in eastern Turkey between May 2011 and February 2012. were used in this study. Of the isolates, 72 were originated from intestinal content samples and the remaining 49 were from gall bladder samples. The isolation and identification of *Campylobacter* spp. were performed using routine bacteriological examination and PCR assays (Acik, 2013c). All the isolates used in this study were cultured on Blood Agar Base No:2 at 42 °C in microaerobic conditions and were kept in nutrient broth containing 15% glycerol at -80 °C until analysis.

2.2. DNA extraction and PCR

The method used for DNA extraction has been described previously (Acik, 2005). Briefly, bacterial suspension was treated with TNES (Tris–NaCl–EDTA–SDS) and proteinase K, which was followed by a boiling step and phenol extraction. Then, precipitation with sodium acetate and ethanol was applied. PCR was performed using the primers and heat cycles given previously by Acik (2013c) to investigate the presence of virulence and *CDT* genes in *Campylobacter* isolates.

The PCR was performed in a TC512 Temperature Cycling System (Techne, Staffordshire, UK) in a total of 50 µL containing 5 µL of 10x PCR buffer (750mM Tris-HCl, pH 8.8, 200mM (NH₄)₂SO₄, 0.1% Tween 20), 5 µL of 2mM MgCl₂, 250mM of each deoxynucleotide triphosphate, 1.25 U of Taq DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany), 20 pmol of each primer, and 5 µL of template DNA.

2.3. Examination of *CDT* activity

CDT activity of 19 *Campylobacter* species; 2 *C. lanienae*, 2 *C. lari*, 5 *C. coli* (2 were carrying *CDT* gene and 3 were not) and 10 randomly

selected *C. jejuni* isolates (five were carrying *CDT* gene and five were not) was examined in HeLa cell culture. *Campylobacter* isolates were inoculated into Minimum Essential Medium (MEM) after *Campylobacter* Blood-Free Selective Agar (CCDA) cultivation. Bacterial concentration of medium was adjusted to 2×10^8 CFU/ml (0,125 at OD 600) spectrophotometrically. Also, bacterial concentration was confirmed with colony counting method. Bacteria in MEM medium were lysed with sonication. The suspension was centrifuged at 10,000 g for 20 minutes at 4°C to remove cell debris and bacteria that were not lysed and, was sterilized by filtration through 0.22 µl filters. The filtrate was diluted tenfold with polyethylene glycol and stored at -20 °C until use. HeLa cells were seeded into plates containing 0.5 ml medium and 2×10^4 bacterial cells. MEM medium was used for two-fold dilutions of filtrate. The final dilution was incubated at 37 °C in 5% CO₂ for three days. Actin filaments were stained with Alexa Fluor 488-conjugated phalloidin (Invitrogen) as described previously by Acik (2013c). Then, morphological changes occurring in HeLa cells were examined under fluorescence.

2.4. Statistical Analysis

Fisher's exact test was used to evaluate the differences between various parameters. $P < 0.05$ was considered as statistically significant.

3. Results

The overall percentages of the *CDTA*, *CDTB* and *CDTC* genes detected in *Campylobacter* isolates were 66.1%, 65.3% and 66.9%,

respectively. Prevalence of *CDT* genes in *C. jejuni* and *C. coli* isolates of gall bladder origin was higher than that of intestinal content origin. Both *C. lari* strains were positive for the *CDT* genes whereas *C. lanienae* strains were found to be negative. While 75% of *C. coli* isolates from intestinal contents were positive for *cdt* genes, nearly half of the *C. jejuni* isolates were not. In addition, approximately 80% of *C. jejuni* isolates and 100% of *C. coli* isolates from gall bladders were positive for *CDT* genes (Table 1). The isolates were also analysed by PCR for the presence of seven different virulence genes and the results are presented in Table 2. The most frequently detected virulence gene in the isolates was *flaA* gene (95%) followed by *CadF* gene (93.4%). The prevalence of other virulence genes was found to vary between 34% and 53%. With the exception of *flaA* (100%) and *CadF* (50%) genes, none of the virulence genes were detected in 2 *C. lanienae* isolates.

3.1. CDT activity

Capability of toxin production of isolates was evaluated by examining the HeLa cell cultures. Of the 19 isolates, only two (one *C. jejuni*, one *C. coli*) showed morphological changes such as cell swelling, expansion, growth, and cell shape change. No sign of *CDT* activity was detected in the other *CDT* gene positive *C. jejuni* and *C. coli* isolates, and *CDT* gene negative *Campylobacter* isolates. Similarly, no *CDT* toxin production was observed in *C. lanienae* and *C. lari* isolates.

Table 1. PCR results of *CDT* genes in *Campylobacter* species isolated from broilers.

Sample	Species	Number of isolates	<i>CDT</i> genes (%)		
			<i>CDTA</i> (%)	<i>CDTB</i> (%)	<i>CDTC</i> (%)
Intestinal content	<i>C.jejuni</i>	60	31(51,7)	31(51,7)	32(53,3)
	<i>C.coli</i>	8	6(75)	6(75)	6(75)
	<i>C.lanienae</i>	2	0(0)	0(0)	0(0)
	<i>C.lari</i>	2	2(100)	2(100)	2(100)
	Total	72	39(54,2)	39(54,2)	40(55,6)
Gall bladder	<i>C.jejuni</i>	46	38(82,6)	37(80,4)	38(82,6)
	<i>C.coli</i>	3	3(100)	3(100)	3(100)
	Total	49	41(83,7)	40(81,6)	41(83,7)
Overall Total	121	80(66,1)	79(65,3)	81(66,9)	

$P > 0,05$

Table 2. PCR results of virulence genes in *Campylobacter* species isolated from broilers

Number of virulence genes (%)									
Sample	Species	Number of isolates	<i>racR</i> (%)	<i>dnaJ</i> (%)	<i>ciaB</i> (%)	<i>pldA</i> (%)	<i>flaA</i> (%)	<i>cadF</i> (%)	<i>virB11</i> (%)
Intestinal content	<i>C.jejuni</i>	60	16(26,7)	24(40)	15(25)	17(28,3)	58(96,7)	58(96,7)	26(43,3)
	<i>C.coli</i>	8	6(75)	5(62,5)	5(62,5)	4(50)	6(75)	6(75)	6(75)
	<i>C.lanienae</i>	2	0(0)	0(0)	0(0)	0(0)	2(100)	1(50)	0(0)
	<i>C.lari</i>	2	2(100)	2(100)	1(50)	1(50)	2(100)	1(50)	0(0)
	Total	72	24(33,3)	31(43,1)	21(29,2)	22(30,6)	68(94,4)	66(91,7)	32(44,4)
Gall bladder	<i>C.jejuni</i>	46	33(71,7)	33(71,7)	19(41,3)	23(50)	44(95,7)	44(95,7)	10(21,7)
	<i>C.coli</i>	3	1(33,3)	0(0)	1(33,3)	1(33,3)	3(100)	3(100)	0(0)
	Total	49	34(69,4)	33(67,3)	20(40,8)	24(49)	47(95,9)	47(95,9)	10(20,4)
Overall Total		121	58(47,9)	64(52,9)	41(33,9)	46(38)	115(95)	113(93,4)	42(34,7)

P>0,05

4. Discussion

In the current study, broiler-originated isolates were examined to determine presence and prevalence of *CDT* and virulence genes which have important roles in pathogenesis of *Campylobacter*. *CDT* which causes inflammation in the intestines of humans and animals is the most studied member of virulence factors. Although quantitative data toward the prevalence of *CDT* genes among campylobacters from various sources have been published previously, little is known about these toxin genes in *Campylobacter* isolates of broiler origin. Different proportions ranging from 90 to 100% in developed countries and 30 to 93% in other countries have been reported for the prevalence of *CDT* genes in recent studies (Bang, 2001; Okoyama, 2006; El-Jakee, 2015; Kavan, 2015). The results of the present study indicated that prevalence of the toxin genes in broiler isolates was lower when compared with those reported in developed countries. Although deletions and mutations may be responsible for the dissimilarity in prevalences of *CDT* genes in *Campylobacter* species, the role of geographic variation should not be ruled out. In other words, *Campylobacter* infections are known as “developed countries disease” and the prevalence of infection in humans in these countries has been reported to be fairly high. Therefore, it is plausible to expect higher prevalence rates for the presence of toxin and virulence genes in developed countries than developing countries.

Incompatible results have been reported in previous studies about the prevalence of toxin genes by species. For instance; *CDT* genes were detected in all *C. coli* isolates in one study

(Carvalho, 2013), whereas they were reported in 93% of *C. jejuni* isolates and only in 56% of *C. coli* isolates in another study (Kavan, 2015). In the present study, the prevalence of *CDT* genes in *C. coli* isolates originated from both intestinal content and gall bladder samples of broilers was found to be higher than that of *C. jejuni*.

The toxin genes were not detected in two *C. lanienae* isolates in the present study. In our previous study conducted in intestinal samples of sheep collected in the same study area, *C. lanienae* isolates with the proportions ranging from 50 to 75% were determined to carry *CDT* genes (Acik, 2013c). On the other hand, these genes were found out in both isolates of *C. lari* in this study which is in contrast with the findings of Jain (2008) who reported lack of toxin genes in this species. In spite of these controversial results, it should be underlined that the number of *C. lari* and *C. lanienae* isolates investigated here is rather limited to make plausible comments about the presence and prevalence of toxin genes within these species.

Although the prevalence of *CDT* genes in broiler originated *Campylobacters* except *C. lanienae* was quite high, the toxin production capability of these agents in HeLa cell culture was low. Similar results have also been reported previously. Ripabelli (2010) declared the existence of *CDT* genes in *Campylobacter* isolates as 100%, but none of the isolates caused toxin production in Vero and Hep-2 cell cultures. This situation may be explained with the difference between *in vitro* and *in vivo* gene expression. As a matter of fact, high toxin production capacity of *Campylobacter* isolates was represented in *in vivo* studies. For this reason, animal experiments would be

meaningful to exhibit the toxin production capacity faithfully. The type of cell culture is also important for the detection of *CDT* production capacity (Bang, 2003). Different cell cultures have been used to detect toxin activity but, HeLa cell culture has been regarded as the most effective and suitable one. *CDT* production capacities differ from each other in *Campylobacter* species. Pickett (1996) detected lower production capacity in *C. coli* than *C. jejuni* in cell cultures. In the present study, *CDT* production capacity was found to be quite high in one *C. jejuni* and in one *C. coli* isolates but none of the other *CDT* positive isolates showed toxin activity.

Among the virulence genes, *flaA* was found at high rates (75-100%) in *Campylobacter* species in this study. When the results were considered at species level, the *flaA* gene was detected in 100% of *C. lanieane* and *C. lari*, and 95-96% of *C. jejuni* isolates. However, a lower *flaA* gene proportion was observed in *C. coli* isolates. The *flaA* gene is notified as highly protected in *Campylobacter* species and represented in almost all *Campylobacter* isolates. However, this gene was absent in 5% of the broiler originated isolates in this study. Interestingly, none of the *flaA* negative isolates were positive for the other six virulence genes or *CDT* genes. This may arise from mutation of isolates which lose their virulence genes. Nachamkin (1993) reported mutations in *flaA* gene but not in *flaB* gene which is necessary for colonization.

CadF gene is one of the outer membrane proteins of *Campylobacter* that play role in the adhesion of the bacteria to the gastrointestinal epithelium and colonization mechanisms. Ziprin (2001) showed that *cadF* mutated *C. jejuni* isolates could not colonize chicken guts. This gene area which is highly protected in *C. jejuni* and *C. coli* is essential for the appearance of campylobacteriosis. In the present study, the percentage of *cadF* gene varied from 50 to 100% in overall isolates. This gene was detected at high proportions in all the species except *C. coli* isolated in this study which is compatible with the reports of previous studies (Konkel, 1999; Talukder, 2008; Zhang, 2016). *CadF* gene could not be detected in more than half of the *C. coli* strains isolated from intestinal content samples of broilers. Besides, other virulence genes and *CDT* genes were detected at low proportions in

cadF negative *C. coli* isolates. In this study, *racR*, *dnaJ*, *ciaB*, and *pldA* genes which have different and important roles in the pathogenesis of *Campylobacter* were detected in 25–64% of the isolates. However, Talukder (2008) reported the presence of *racR*, *dnaJ*, *pldA*, and *ciaB* virulence genes at significantly high proportions (95–100%) in human isolates. The different sample types might be responsible for this. Also in the human study, patients with diarrhea were sampled, whereas samples were collected from apparently healthy broilers in the present study. Although *flaA* and *cadF* genes were detected in *C. lanienae* isolates other virulence genes were absent in this species. Even though, these results present a clue for low pathogenicity of *C. lanienae*, *in vivo* experiments with more isolates obtained from large field surveys need to be set up to enlighten this matter.

VirB11 genes detected by Bacon (2000) were reported to play a role in the pathogenesis of *Campylobacters*. Although the pVir gene was attributed to play important role in *C. jejuni* infections in humans, no relation between this gene and diarrhea was revealed in campylobacter infections in humans. Higher proportions were obtained for the prevalence of *virB11* gene in *C. jejuni* isolates (43.3% from intestinal content and 21.7% from gall bladder samples) when compared with previous studies. The existence of this gene in *Campylobacter* isolates originated from various animals was reported at the rates of 6-15%, but it was not detected at all in some studies (Biswas, 2011; Acik, 2013a; Zhang, 2016).

Conclusion

CDT and virulence genes which participate in the pathogenesis of *Campylobacter* infections were detected at lower frequencies in *C. jejuni*, *C. coli* and *C. lari* isolates that were obtained from clinically healthy broilers, when compared with the previous studies. Besides, HeLa cell culture showed that *CDT* production capacity of the isolates was low. Although valuable information was attained about the pathogenicity of *C. lanienae*, additional studies using animal models are necessary for clarification.

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