Detection of CDT toxin genes in *Campylobacter* spp. strains isolated from broiler carcasses and vegetables in São Paulo, Brazil

Aline Feola de Carvalho¹, Daniela Martins da Silva¹, Sergio Santos Azevedo², Rosa Maria Piatti¹, Margareth Elide Genovez¹, Eliana Scarcelli¹

¹Instituto Biológico, Centro de Pesquisa e Desenvolvimento de Sanidade Animal, São Paulo, SP, Brazil. ²Universidade Federal de Campina Grande, Campina Grande, PB, Brazil.

Submitted: June 08, 2011; Approved: April 01, 2013.

**Abstract**

Campylobacteriosis is a worldwide distributed zoonosis. One of the main virulence factors related to *Campylobacter* spp. in animals and humans is the cytolethal distending toxin (CDT), encoded by three adjacent genes (*cdtA*, *cdtB*, *cdtC*). The occurrence of *Campylobacter* spp. in samples of vegetables has not been reported in Brazil yet, and has seldom been described in the international literature. The detection of CDT in these strains has not been reported, either. The objectives of the present study were to determine the occurrence of *Campylobacter* spp. strains carrying virulence factors in samples of poultry and vegetables (lettuce and spinach) from different points of sale, thus verifying if vegetables are as an important vehicle for potentially virulent *Campylobacter* spp. strains as poultry. Twenty four strains were identified as *Campylobacter jejuni* by phenotypic and genotypic methods: 22 from broiler carcasses and two from lettuce samples. Three strains were identified as *Campylobacter coli*: two from broiler carcasses and one from lettuce. The presence of the *cdt* genes were detected in 20/24 (83.3%) *C. jejuni* strains, and 3/3 (100%) *C. coli* strains. The isolation of *Campylobacter* spp. strains with the *cdt* gene cluster in lettuce samples points to a new possible source of contamination, which could have an impact in the vegetable production chain and risk to public health. Results show that potentially virulent *C. jejuni* and *C. coli* strains remain viable in samples of broiler carcasses and vegetables at the points of sale.

**Key words**: *Campylobacter* spp., cytolethal distending toxin (CDT), broiler carcasses, vegetables, Multiplex-PCR.

---

**Introduction**

Campylobacteriosis is a worldwide distributed zoonosis. *Campylobacter jejuni* and *Campylobacter coli* are ubiquitous microorganisms, which are found in the environment, as well as in the gastrointestinal tract of animals, where they live as pathogenic or commensal agents (Altekruse, 1998; Scarcelli *et al.*, 2005). These species are the most common causes of diarrhea in children in developing countries, and of gastro-enteritis in industrialized countries (Carvalho *et al.*, 2001).

Foodborne transmission is the main route in human contamination by *Campylobacter* spp. The most significant risk factors include the contamination of poultry carcasses in slaughterhouses and consequent consumption and/or handling of raw or undercooked meat. The ingestion of unpasteurized milk or untreated water, cross-contamination during food preparation or direct contact with infected animals are other risk factors (Rozynek *et al.*, 2005; Hussain *et al.*, 2007).

Environmental routes of transmission of *Campylobacter* spp. should also be emphasized, including plants, insects, and non-chlorinated water contaminated by animal feces, which few studies have considered as a vehicle for the dissemination of the microorganism, particularly to foods of non-animal origin (Kumar *et al.*, 2001). Fresh vegetables, especially when raw, may also be a source of transmission for several human pathogens (Abadias *et al.*, 2007).
2008). In this case, contamination by Campylobacter spp. may occur before or after their purchase (Evans et al., 2003).

The cytolethal distending toxin (CDT) is one of the main virulence factors related to Campylobacter spp. pathogenesis in humans and animals. It causes diarrhea by interfering with the division and differentiation of cells in intestinal crypts (Wassenberg, 1997; Park, 2002). The toxin activity is encoded by the cdt gene cluster, made up of three adjacent genes: cdtA, cdtB and cdtC (Martinez et al., 2006). All the three subunits are required for full toxin activity; cdtB encodes the active/toxic component of the toxin, while cdtA and cdtC are involved with binding to and internalization into the host cell (Abouon et al., 2005; Jeon et al., 2005).

The protein produced by gene cdtB (CdtB) potentiates a cascade leading to cell cycle block, whereas the proteins of genes cdtA and cdtC function as dimeric subunits, with bind cdtB and delivers in to the cell interior. Once in the cell, CdtB enters the nucleus and exhibits a DNase-I like that results in DNA double-strand breaks. CDT blocks the G2/M phase of eukaryotic cells prior to cell division, induces a cytoplasmic distention and ultimately causes cell death (Jeon et al., 2005; Smith and Bayles, 2006).

The result of CDT activity can differ depending on the type of eukaryotic cell affected. CDT contributes to campylobacteriosis pathogenesis by inhibiting both cellular and humoral immunity via apoptosis of immune cells, and generating the necrosis of epithelial cells and fibroblasts involved in the repair of lesions produced by pathogens, which results in slow healing and production of disease symptoms (Smith and Bayles, 2006).

The occurrence of Campylobacter spp. in vegetables has rarely been described in the literature, and CDT detection in these strains has never been reported (Park and Sanders, 1992; Kumar et al., 2001; Chai et al., 2007). However, it is essential to assess if vegetables are as an important vehicle for Campylobacter spp. as poultry meat, which is known as the main source of foodborne transmission of campylobacteriosis. Lettuce and spinach are described in the international literature as the main vegetable sources of human infection by Campylobacter spp. (Park and Sanders, 1992; Kumar et al., 2001; Brandl et al., 2004; Hussain et al., 2007). Nevertheless, this is the first study in Brazil on CDT found in vegetables.

The objectives of the present study were to determine the occurrence of Campylobacter spp. strains carrying cdt genes in samples of poultry and vegetables (lettuce and spinach), and to verify if vegetables are as an important vehicle for potentially virulent Campylobacter spp. strains as poultry at different points of sale.

Materials and Methods

A total of 194 broiler carcasses refrigerated were analyzed. The samples were purchased in two supermarkets (n = 40), two street markets (n = 40), and two slaughterhouses (n = 114) in São Paulo. As for vegetables, 80 samples were purchased in two supermarkets (n = 20 samples of spinach, and n = 20 of lettuce) and two street markets (n = 20 samples of spinach, and n = 20 of lettuce) in the same state. Poultry carcasses and vegetables were collected from two supermarkets and two street markets, 20 specimens by collection, in a total of eight samplings, between April and October, 2008. Two samplings were performed in two slaughterhouses, with the collection of 60 carcasses in the first samplings and 54 in the second, carried out between March and June, 2009.

Samples were submitted for bacteriological examination. The isolation and biochemical identification of C. jejuni and C. coli was performed according to the Bacteriological Analytical Manual (Hunt et al., 2001; Carvalho et al., 2010).

In order to confirm the biochemical identification, the isolated strains were submitted to two-step PCR reactions with primers described by Linton et al. (1997). The first was targeted to the hiep gene, which encodes the enzyme hippuricase, responsible for the amplification of a 735-base pair (bp) fragment only found in C. jejuni; and the second was targeted to the gene encoding the enzyme aspartokinase, which amplifies a 500-bp fragment found in C. coli.

Isolated Campylobacter strains had their DNA extracted with the commercial kit Ilustra Bacterial Genomic PREP Mini Spin (GE Healthcare), used according to the manufacturer’s instructions.

Analysis of the amplified products was carried out by electrophoresis in 2.0% agarose gel stained with ethidium bromide (0.5 µg/mL). Gels were photographed under ultraviolet light (300-320 nm) with a Kodak DC/120 Zoom digital camera. Images were analyzed using the 1D Image Analysis software (Kodak Digital Science). Campylobacter jejuni ATCC 33291 and Campylobacter coli CDC A3315 were used as positives controls.

Multiplex-PCR with primers described by Asakura et al. (2008) was used to detect the simultaneous presence of cdtA, cdtB and cdtC in the C. jejuni and C. coli strains that were isolated in the study. In C. jejuni, the cdtA gene corresponds to a fragment of 631 bp; cdtB, to a fragment of 413 bp; and cdtC, to a fragment of 524 bp.

In C. coli, the cdtA gene corresponds to a fragment of 329 bp; cdtB, to a fragment of 413 bp; and cdtC, to a fragment of 313 bp. Due to the similarity between the number of base pairs in the fragments amplified corresponding to genes cdtA (329pb) and cdtC (313pb), two Multiplex-PCR reactions were carried out: one with cdtA and cdtB primers, and the other with cdtB and cdtC primers.

Detection of cdt genes in C. jejuni: PCR buffer 10 X (500 mM KCl, 15 mM MgCl2, 100 mM TRIS-HCl, pH 9.0); 1.5 mM MgCl2; 200 mM dNTPs (200 mM of each nucleotide dCTP, dATP, dGTP, dTTP); 20 pmol of each primer (CjspAU2, CjspAR2, CjSPBU5, CjSPBR6,
CjspCU1 and CjspCR2); 1.0 U Taq DNA polymerase (Invitrogen), and 10 μL of extracted DNA were mixed to a final volume of 50 μL. Thermocycler PT 200 (MJ Research) was used in an amplification cycle preceded by initial denaturation at 94 °C for 5 min, and followed by 30 cycles of denaturation at 94 °C for 30 s, hybridization at 55 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 10 min, in a procedure adapted from Asakura et al. (2008). The analysis of the amplified products was carried out according to the procedures used for the hippuricase and aspartokinase genes.

Statistical analysis was based on the chi-square test (X^2) and Fisher’s exact test, with significance level set at p < 0.05, according to Callegari-Jacques (2003). Statistical analyses were carried out in EpiInfo 6.04 (Dean, 1994).

Results

Campylobacter spp. strains were isolated in 21/194 (10.8%) broiler carcasses and in 3/40 (7.5%) samples of lettuce. As for spinach, none of the samples were positive for Campylobacter spp. Twenty four strains were identified as C. jejuni: 22 from broiler carcasses, and two from lettuce samples. Three strains were identified as C. coli: two from broiler carcasses, and one from lettuce samples. C. jejuni and C. coli strains isolated in samples of animal and vegetable origin were found in supermarkets, street markets and slaughterhouses (Table 1).

All C. jejuni and C. coli strains were identified by phenotypic methods and confirmed by genotyping using PCR for detection of the genes encoding the enzymes hippuricase and aspartokinase.

The cdt gene cluster was detected in 20/24 (83.3%) C. jejuni strains: 18 from broiler carcasses (seven purchased in slaughterhouses, seven in supermarkets, and four in street markets), and two from lettuce samples purchased in street markets. The cdt gene complex was not found in 4/24 (16.7%) C. jejuni strains (Table 1; Figure 1) isolated in broiler carcasses from slaughterhouses (strains 18, 20, 22 and 24).

Genes cdtA, cdtB and cdtC were simultaneously detected in 16/20 (80%) C. jejuni strains positive in Multiplex-PCR. In 2/20 (10%) strains, only gene cdtB was found in broiler carcasses from street markets (strains 1 and 2). In 2/20 (10%) strains, only gene cdtC was found in broiler carcasses from slaughterhouses (strains 17 and 19) (Table 1; Figure 1).

The presence of the cdt gene cluster was detected in 3/3 (100%) C. coli strains, two from broiler carcasses and one from a lettuce sample, both purchased in street markets.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Food source</th>
<th>Point of sale</th>
<th>cdtA</th>
<th>cdtB</th>
<th>cdtC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SM</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SM</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SM</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>C. jejuni</td>
<td>L</td>
<td>SM</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SM</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>C. jejuni</td>
<td>L</td>
<td>SM</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SL</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SL</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SL</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SL</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SL</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SL</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SL</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SL</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>22</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SL</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SL</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>C. jejuni</td>
<td>BC</td>
<td>SL</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>C. coli</td>
<td>BC</td>
<td>SM</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>26</td>
<td>C. coli</td>
<td>BC</td>
<td>SM</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>27</td>
<td>C. coli</td>
<td>L</td>
<td>SM</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

BC: broiler carcasses; L: lettuce; SM: street market; SP: supermarket; SL: slaughterhouse.

All C. coli strains showed cdtA, cdtB and cdtC, simultaneously (Table 1, Figure 2).

No statistically significant differences were observed in relation to the number of Campylobacter spp. positive samples isolated from broiler carcasses and vegetables (p = 0.060); or in animal (p = 0.820) and vegetable samples (p = 0.241) purchased in supermarkets, street markets and slaughterhouses. No statistically significant differences were observed in relation to the presence of cdt genes in broiler carcasses and vegetables (p = 1.000).

Discussion

C. jejuni and C. coli strains were isolated from 21/194 (10.8%) broiler carcasses, a lower frequency to that reported in the literature. In the United States, Eyigor et al. (1999) isolated 70/105 (67%) C. jejuni and 35/105 (33%) C. coli in poultry carcasses. Rozynk et al. (2005) found
53/92 (57.6%) strains of C. jejuni, and 39/92 (42.4%) of C. coli in poultry carcasses in Poland. Mena et al. (2008) identified 99/165 (60.3%) strains of Campylobacter spp. in poultry samples in Portugal. Prencipe et al. (2007) isolated Campylobacter spp. in 178/392 (45.4%) poultry carcasses from supermarkets and butcheries in Italy. Kang et al. (2006) isolated Campylobacter spp. in 570/923 (61.8%) poultry samples from traditional markets, retailers and warehouses in Korea.

The contamination of poultry carcasses often occurs during slaughter, more commonly during scalding and evisceration, when the surface of the carcass is in contact with intestinal microorganisms. Studies in Brazil and other countries have shown a prevalence of 60% to 100% positive results for C. jejuni and C. coli in poultry carcasses and organs analyzed immediately after evisceration. However, such frequency decreased to 15% to 20% in samples refrigerated or frozen for different periods of time (Sakuma et al., 1992; Castro et al., 1997; Cortez et al., 2006; Prencipe et al., 2007).

Higher levels of chlorine in processing water, coupled with improved hygienic conditions in slaughterhouses may significantly reduce carcass contamination (Mead et al., 1995). However, some Campylobacter spp. strains may survive cleaning and disinfection procedures in poultry slaughterhouses, and may contaminate the carcasses during processing (Peyrat et al., 2008). This may be due to the resistance of Campylobacter spp. strains to disinfectants, mainly chlorine, or to the inefficiency of the cleaning and disinfection procedures used in slaughterhouses.

C. jejuni and C. coli strains were isolated from 3/40 (7.5%) lettuce samples, and this is the first report about Campylobacter spp. recovered from these samples purchased in São Paulo, Brazil. This frequency was greater than that described by Park and Sanders (1992), who observed 3.1% lettuce samples positive for Campylobacter spp. The same authors identified C. jejuni strains in 3.3% of spinach samples, whereas no strain was isolated from this vegetable in the present study. Abadias et al. (2008) did not isolate Campylobacter spp. in lettuce and spinach samples from supermarkets in Spain.

Brandl et al. (2004) isolated C. jejuni from spinach and radish samples. According to these authors, this bacterium is found in the roots, remaining viable in the soil and rhizosphere, and thus leading to sporadic cases of campylobacteriosis associated with the ingestion of these foods. In Pakistan, Hussain et al. (2007) studied the prevalence of Campylobacter spp. in foodstuffs available in retailers, and
nine (40.9%) of the vegetable and fruit salad samples were positive for Campylobacter spp. Kumar et al. (2001) analyzed different vegetable samples collected in markets in India, and isolated 2/56 (3.57%) C. jejuni, one in spinach and the other in fenugreek.

The presence of C. jejuni and C. coli in vegetables collected in Brazil may indicate fecal contamination. Campylobacter spp. inhabits the intestinal tract of animals, mainly birds, and their excreta may contaminate the environment (Kumar et al., 2001). Natural fertilizers or water used for irrigation may also be contamination sources (Butzler and Oosterman, 1991). Cross-contamination of vegetables with kitchen utensils used for cutting other foods, such as poultry, may occur during handling and packaging (De-Boer and Hahne, 1990). The use of contaminated water in washing or handling at the point of sale should also be considered as a risk factor for cross-contamination (Chai et al., 2007).

Campylobacter spp. strains were isolated from lettuce samples purchased in street markets (3/20; 15%), where handling of these vegetables is more frequent. In order to keep their appearance fresh, vegetables are constantly sprinkled with water that is stored in containers and kept behind the stalls, which is an unhygienic procedure. Although vegetables sold in street markets appear to be freshly harvested because they are regularly sprayed, this procedure preserves the viability of Campylobacter spp.

Cdt genes were detected in 83.3% (20/24) of the C. jejuni strains isolated from broiler carcasses and lettuce heads. In the present study, the percentage of C. jejuni strains that carried the cdt complex was similar to that reported in the international literature. In Poland, Rozynek et al. (2005) detected the three cdt genes in 100% of the C. jejuni strains found in poultry carcasses from supermarkets and slaughterhouses. Samosornsuk et al. (2007), in Thailand, detected the cdt genes in 95% of the C. jejuni strains isolated from poultry. Talukder et al. (2008) identified C. jejuni in patients with diarrhea in Bangladesh, and identified the genes cdtA, cdtB and cdtC in 97.5% of the strains. According to these authors, CDT production is associated with the strains that cause enteritis in humans.

The presence and expression of the three genes are necessary for functional activity of the CDT toxin (Jeon et al., 2005). Therefore, 80% (16/20) of the 20 strains that carried the cdt genes were potentially virulent, once they had cdtA, cdtB and cdtC, simultaneously. In 20% (4/20) of the strains, only one cdt gene was detected (cdtB or cdtC).

According to Martinez et al. (2006), all C. jejuni strains have cdt genes, and most of them show toxin activity. However, there are exceptions of rare isolates that mutate and do not show activity of these genes. These authors also sequenced and characterized CDT-negative genes, and observed the presence of cdtA, cdtB and cdtC pseudo-genes with deletions in their sequences. Asakura et al. (2007) also observed that some cdt genes may not be identified because of mutations, such as nucleotide deletion, insertion or substitution, and suggest that these mutations may affect toxin activity.

As for C. coli strains isolated from broiler carcasses and lettuce, 100% (3/3) were positive for the three cdt genes simultaneously. In the present study, the percentage of C. coli strains that carried the cdt complex was similar to that reported by other authors in different countries. In Portugal, Fernandes et al. (2010) detected cdtA and cdtC in 99.4% and cdtB in 98.8% of the strains isolated from food samples. In Italy, Ripabelli et al. (2010) detected the cdt gene complex in 94.4% of the C. coli strains isolated from foods, animals and humans.

There are no reports in Brazil and other countries on the study of cdt genes in Campylobacter spp. strains isolated from vegetables, making this group of foods a new source of contamination with C. jejuni and C. coli strains potentially virulent for humans and animals, with possible public health risk.

This is the first time that the cdt gene cluster was detected in C. coli strains from broiler carcasses, and C. jejuni and C. coli strains in samples of lettuce, in the state of São Paulo, Brazil. These results may provide yet unpublished data on the occurrence of Campylobacter spp. strains that carry the genes for CDT from foods of animal, and mainly vegetable origin. They also show that potentially virulent strains remain viable in broiler carcasses and in lettuce samples in the points of sale. Therefore, the contamination of foodstuffs by these microorganisms should not be ignored. Preparation should be carried out in optimal conditions of hygiene, especially for ready-to-eat products, or whenever there is a possibility of cross-contamination during handling.

Acknowledgments

Grant#2007/52441-0, São Paulo Research Foundation (FAPESP).

References


Sakuma H, Franco DGM, Fernadez H (1992) Occurrence of 
Campylobacter jejuni an Campylobacter coli in retail raw 

Samosornsuk W, Asakura M, Yoshida E, Taguchi T, Nishimura 
K, Eampokalap B, Phongsisay V, Chaicumpa W, Yamasaki 
gene-based species-specific Multiplex-PCR assay for the 


Talukder K A, Aslam M, Islam Z, Azmi IJ, Dutta DK, Hossain S, 
Nur-E-Kamal A, Nair GB, Cravioto A, Sack DA, Endtz HP 

Clin Microbiol Rev 10:466-476.

All the content of the journal, except where otherwise noted, is licensed under a 
Creative Commons License CC BY-NC.