Brazilian Journal of Microbiology 44, 3, 693-699 (2013)
ISSN 1678-4405

Research Paper

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

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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.*,

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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 (Wassenaar, 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 (Abuoun *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 *hip* 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 714 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 $MgCl_2$, 100 mM TRIS-HCl, pH 9.0); 1.5 mM $MgCl_2$; 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).

Detection of *cdt* genes in *C. coli*: Multiplex-PCR was carried out as previously described, with different primers (*CcspAU1, CcspAR1, CcSPBU5, CcSPBR5*) and (*CcSPBU5, CcSPBR5, CcspCU1, CcspCR1*), 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 1 - Classification of *Campylobacter* spp. strains according to food source, point of sale, and detection of the *cdt* gene complex.

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

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 Unites States, Eyigor *et al.* (1999) isolated 70/105 (67%) *C. jejuni* and 35/105 (33%) *C. coli* in poultry carcasses. Rozynek *et al.* (2005) found

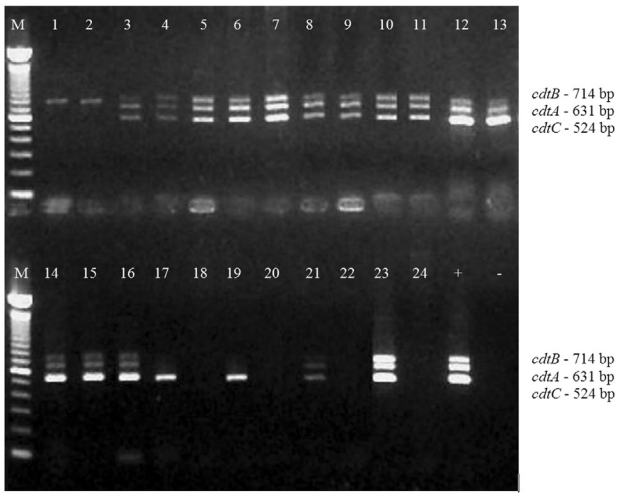

Figure 1 - Multiplex-PCR for *cdt* genes (*cdtA, cdtB* and *cdtC*) in *C. jejuni* strains. M: molecular weight marker (100 bp DNA Ladder - Invitrogen); 1 to 24: *C. jejuni* strains; + Positive control (*C. jejuni* ATCC 33291); - Negative control.

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

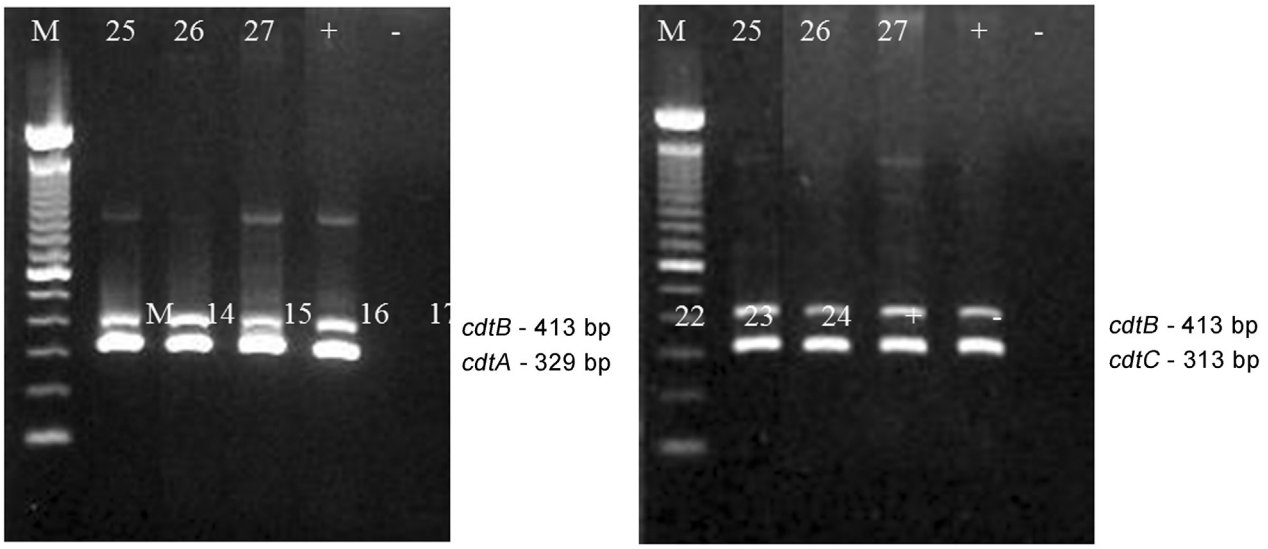

Figure 2 - Multiplex-PCR for *cdt* genes (*cdtA, cdtB* and *cdtC*) in *C. coli.* strains M: molecular weight marker (100 bp DNA Ladder - Invitrogen); 25 to 27: *C. coli* strains; (+): Positive control (*C. coli* CDC A3315); (-): Negative control.

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).

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