EPIDEMIOLOGY AND BACTERIAL TYPING

Prevalence of cytolethal distending toxin (*cdt*) genes and CDT production in *Campylobacter* spp. isolated from Danish broilers

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The pathogenesis of campylobacter infection in man is largely unknown, although cytolethal distending toxin (CDT) has been incriminated as a virulence factor. However, little is known about the *cdt* genes in *Campylobacter* spp. isolated from broiler chickens. A total of 350 cloacal swabs was collected and tested by conventional culture and PCR. Of the 114 *Campylobacter* isolates obtained, 101 (88.6%) were identified as *C. jejuni* and 13 (11.4%) as *C. coli* by conventional methods. *cdt* genes were detected by PCR in all the isolates except one *C. jejuni* isolate. Cytotoxic effects were produced in a Vero cell line, by 100 of the *C. jejuni* isolates. In contrast, 10 *C. coli* isolates produced much lower levels of toxin and 3 produced no detectable toxin. These results confirm the common occurrence of campylobacter infection in chickens and indicate that *cdt* genes are commonly present in both *C. jejuni* and *C. coli* isolates from broilers, but that there are distinct differences in CDT production in these two closely related species.

Introduction

Campylobacteriosis is one of the most common bacterial intestinal disorders of man in many industrialised countries. In the USA, 2–8 million cases, which cause an estimated 200–800 deaths, are reported annually [1, 2]. In Denmark, human campylobacteriosis has recently increased rapidly from 1129 reported cases in 1992 [3] to >4000 cases in 1999 [4]. Large outbreaks of human campylobacteriosis are rare, but sporadic cases are common and are often linked to the consumption of undercooked food products contaminated with campylobacters [5–7]. A high prevalence of campylobacter infection has been reported in poultry flocks in several countries, e.g., the USA [8] and Denmark [9]. Campylobacters can survive during poultry processing and the bacteria may spread to uncontaminated poultry products. The most common cause of *Campylobacter*-induced human enteritis is *C. jejuni*, which may account for as much as 90% of all

Campylobacter isolates from broilers in Denmark [4] and other industrial countries [7, 10, 11].

In poultry, *Campylobacter* spp. are considered to be commensals because the infection does not cause any clinical symptoms [12]. However, the specific virulence mechanisms of *Campylobacter* spp. have not yet been adequately elucidated in human disease, although several virulence determinants have been proposed for *Campylobacter* spp., including flagella-mediated motility, adherence to the intestinal mucosa, invasive capability and the ability to produce toxin [13–15].

The best characterised of the proposed campylobacter toxins is the cytolethal distending toxin (CDT), which has been detected in several *Campylobacter* spp. [16, 17]. CDT causes progressive cellular distention and ultimately death in Chinese hamster ovary (CHO), Vero, Hep-2, HeLa and Caco-2 cells [16–20].

The *C. jejuni cdt* genes have been cloned and sequenced [21]; they consist of three adjacent genes, *cdtA*, *cdtB* and *cdtC*, that encode proteins with predicted mol. wts of c. 30, 29 and 21 kDa, respectively. The functions of the CDT proteins are unknown.

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However, it has been shown recently by DNA content analysis of cells treated with campylobacter CDT that the CDT causes several cell types to become blocked in either G2 or early M phase of their cell cycle before cell division [20, 22]. This indicates that the action of CDT differs from that of other bacterial toxins [20, 23, 24].

Little is known about the prevalence of the *cdt* genes among *Campylobacter* spp. isolated from broilers. In the present study, *Campylobacter* spp. were isolated from individual chickens and identified by conventional microbiological culture and by PCR. The *cdt* genes were detected by PCR and the CDT activity of culture supernates was tested in tissue cultures.

Materials and methods

Broiler samples

Ten cloacal swabs from each of 35 chicken broiler flocks were collected randomly at the abattoir immediately before slaughter. The swabs were transferred from the abattoir to the laboratory by mail in screw-cap centrifugation tubes containing 12 ml of Brain Heart Infusion (BHI) transport medium (BHI broth; Difco) supplemented with sterile defibrinated calf blood 5% v/v, and agar 0.5% (Oxoid) pH 7.4, on the day of sample collection. On arrival at the laboratory, each swab was transferred immediately to 0.3 ml of sterile water and left at room temperature for 10–20 min to release the bacteria. The suspensions of faeces and bacteria were used directly for detection of campylobacters.

Isolation and identification of *Campylobacter* spp. by conventional culture methods

A loopful (10 μ l) of the suspensions was spread on the surface of a charcoal cefoperazone deoxycholate agar (CCDA) plate (Oxoid CM 739 with cefoperazone selective supplement SR 155E). The plate was incubated in micro-aerobic conditions (O₂ 6%, CO₂ 6%, H₂ 4% and N₂ 84%) at 42°C for 48 h. Colonies suspected as being *Campylobacter* spp. were examined for cell morphology by phase-contrast microscopy (Olympus B 201). The isolates were purified further on blood agar plates (BA; Blood Agar Base No. 2, Oxoid, supplemented with sterile defibrinated calf blood 5%). Single colonies were picked and streaked on wet BA plates, and further characterised to species level by their catalase reaction, ability to hydrolyse hippurate and indoxyl acetate and their susceptibility to nalidixic acid and cephalothin according to standard procedures [25, 26]. Isolates were stored with glycerol 15% at –80°C until required for further investigations.

Bacterial reference strains

Reference strains *C. jejuni* CCUG 11284, *C. coli* CCUG 11283, the verotoxin-producing *Escherichia*

coli strain H 19 (SSI no. D2165, serotype O26:H11) and the CDT-producing *E. coli* strain E6468/62 (SSI no. D2253, serotype O127:H6) were used as controls for the PCR studies and for the cell culture assays (see below). The strains were stored in serum-broth containing glycerol 15% at –80°C.

DNA techniques

Total bacterial cell DNA was isolated by QIA Amp kit (QIAGEN, Germany) from overnight cultures on BA. The DNA was eluted in 200 μ l of sterile water and stored at –20°C. Restriction endonucleases were used in accordance with the supplier's specifications (New England Biolabs).

Identification of *Campylobacter* spp. by PCR

For species identification, a PCR was performed initially with the universal *Campylobacter* 16S rRNA primer [27] for the detection of the *Campylobacter* genus. All PCR-positive samples were then subjected to a second PCR with different 16S rRNA species-specific primer sets [27, 28] for different *Campylobacter* spp. (Table 1). For differentiation of *C. jejuni* from *C. coli*, a third PCR was performed with hippuricase gene primers [29]. The hippuricase gene has been shown to be specific for *C. jejuni* [29, 30]. The PCR was performed in a Peltier Thermal Cycler PTC-200 (MJ Research, Massachusetts, USA) for 30 cycles. The PCR conditions for each primer set are presented in Table 1. All PCR mixtures (50 μ l) contained 0.2 mM (each) dATP, dCTP, dGTP and dTTP; 1.5 mM MgCl₂; 5 μ l (20 pmole) of each primer (DNA Technology, Aarhus, Denmark); 1 $\times$ Taq DNA polymerase buffer and Taq DNA polymerase (Boehringer-Mannheim, Germany) 0.5 μ l (2.5 U).

Detection of *cdt* genes from *Campylobacter* isolates by PCR and nucleotide sequencing of the *cdtB* gene

Chromosomal DNA from *C. jejuni* and *C. coli* isolates was used as templates in PCR reactions with the primers VAT2 and WM11 (Fig. 1) complementary to the *cdtB* gene as described by Pickett *et al.* [21]. The parameters of PCR for *cdt* gene detection reactions were 30 cycles of 94°C for 1 min, 42°C for 2 min and 72°C for 3 min.

The *cdtB* gene PCR-negative *Campylobacter* isolates were further screened for the *cdt* genes with the primers GNW and IVH, complementary to the sequences of the *cdtA* gene or with the primers VAT2 and LPF-X, complementary to the sequences of the *cdtB* and *cdtC* genes, respectively [21] (Fig. 1).

For DNA sequencing of the *cdtB* gene, PCR amplification products were purified with a QIA Quick Spin Column (QIAGEN). DNA sequencing was performed

Table 1. List of primers, size of amplicons and PCR conditions used in this study

PCR reaction no.	Primers	Sequences and amplicon size	PCR conditions [reference]
1	16S rRNA (for <i>Campylobacter</i> genus)	5'-GGATGACACTTTTCGGAGC-3' 5'-CATTGTAGCACGTGTGTC-3' Amplicon: 816 bp	94°C 1 min 55°C 1 min, 25 cycles 72°C 1 min [27]
2	16S rRNA (for <i>C. jejuni</i> , <i>C. coli</i>)	5'-AATCTAATGGCTTAACCATTA-3' 5'-GTAAGTAGTTAGTATTCCGG-3' Amplicon: 937 bp	94°C 1 min 58°C 1 min, 25 cycles 72°C 1 min [28]
3	16S rRNA (for <i>C. fetus</i>)	5'-GCAAGTCGAACGGAGTATTTA-3' 5'-GCAGCACCTGTCTCAACT-3' Amplicon: 997 bp	94°C 1 min 65°C 1 min, 25 cycles 72°C 1 min [27]
4	16S rRNA (for <i>C. hyointestinalis</i>)	5'-GCAAGTCGAACGGAGTATTTA-3' 5'-GCGATTCCGGTCTCATGCTC-3' Amplicon: 1287 bp	94°C 1 min 65°C 1 min, 25 cycles 72°C 1 min [27]
5	16S rRNA (for <i>C. upsaliensis</i>)	5'-GGGACAACACTTAGAAATGAG-3' 5'-CACTTCCGTATCTCTACAGA-3' Amplicon: 878 bp	94°C 1 min 60°C 1 min, 25 cycles 72°C 1 min [27]
6	16S rRNA (for <i>C. helveticus</i>)	5'-GGGACAACACTTAGAAATGAG-3' 5'-CCGTGACATGGCTGATTAC-3' Amplicon: 1225 bp or 1375 bp	94°C 1 min 60°C 1 min, 25 cycles 72°C 1 min [27]
7	16S rRNA (for <i>C. lari</i>)	5'-CAAGTCTCTTGTGAAATCCAAC-3' 5'-ATTAGAGTGCTCACCCGAAG-3' Amplicon: 561 bp	94°C 1 min 55°C 1 min, 25 cycles 72°C 1 min [27]
8	<i>HipO</i> (for <i>C. jejuni</i>)	5'-GTACTGAAAATTTAGTGGCG-3' 5'-GAGCTTTTAGCAAACCTTCC-3' Amplicon: 1130 bp	94°C 1 min 55°C 1 min, 25 cycles 72°C 1 min [29]
9	<i>CdtB</i> toxin gene	5'-GTAAAAATCCCCTGCTATCAACCA-3' 5'-GTTGGCACTTGGAATTTGCAAGGC-3' Amplicon: 495 bp	94°C 1 min 42°C 2 min, 30 cycles 72°C 3 min [21]

Fig. 1. Map of *C. jejuni* *cdt* genes and locations of the PCR primers. Arrows indicate the size and direction of transcription of the *cdt* genes. Large arrowheads indicate the location and orientations of the primers [21], and this study].

with an ABI automatic sequencer with the Prism BigDye terminator cycle sequencing kit (Applied Biosystem, Foster City, CA, USA) and the *cdtB* gene primers (Table 1).

Preparation of cell-free bacterial culture supernates for toxin assays

Cell-free bacterial culture supernates were prepared according to the method described by Florin and Antillon [18], with modifications. Bacteria from frozen stocks were inoculated on wet BA plates and incubated

under micro-aerobic conditions at 42°C for 48 h. A loopful of bacteria was transferred to a sterile 2-ml Eppendorf tube containing 1 ml of Veal Infusion Broth (VIB; Difco) 25g/L and the bacterial suspension was vortex mixed. The tubes were incubated at 42°C for 72 h under micro-aerobic conditions. To enable a comparison of the ability of different strains to produce CDT, the bacterial suspensions were adjusted to a defined optical density corresponding to 10⁸cfu/ml at OD₆₀₀ with an ELISA reader (Multiskan MS Labsystem, Life Science Inter. Comp. Finland). The cell-free bacterial culture supernates were collected by centrifugation at 13 000 rpm for 5 min at 5°C and filter sterilised (0.22 µm, Millipore, USA). Ten µl of the filtrates were inoculated onto BA plates and incubated at 37°C under both aerobic and micro-aerobic conditions to test for sterility.

Detection of CDT activity by cell culture assays

Nine-day-old chicken embryos were used to prepare cell lines according to the method described by Schat and Purchase [31]. The cells were used at a concentration of 0.5 × 10⁸ cells/ml.

Vero cells were prepared according to Konowalchuk *et al.* [32], with modifications. The cells were cultivated in Eagle's Minimal Essential Medium (MEM) with heat

inactivated (56°C, 30 min) fetal calf serum (FCS) 10%, 4 mM L-glutamine and gentamicin 60 mg/L. Overnight cultures in plastic flasks were trypsinised with 4 ml of trypsin 20% for 5–6 min. After removal of the trypsin, the cells were suspended in 15–20 ml of MEM to produce a final concentration 0.1×10^8 cells/ml. Samples of the cell suspension (150 µl) were transferred to each of 96 wells in a microtitration plate and incubated overnight at 37°C in a moist chamber. The following day, the medium was replaced with fresh MEM containing FCS 3%, 200 µl/well. Then 30 µl of cell-free bacterial culture supernate were added to each well. Each sample was added to duplicate wells at the desired dilutions. Duplicate assays of a given supernate were highly reproducible even after storage for 4–6 months at –20°C. Toxin production by each isolate was tested in at least three independent assays. Repeat assays of a given isolate with culture supernates prepared at different times did not vary by more than one dilution step. Supernates from a culture of the verotoxin-producing *E. coli* H19 (D2165, O26:H11) and the CDT-producing *E. coli* E6468/62 (D2253, O127:H6) strains were used as positive controls and VIB medium as a negative control. The control samples were always included in duplicate wells on each plate. The cytotoxic effects were monitored daily for 4–6 days by phase-contrast microscopy.

The CDT titres were determined by performing two-fold dilutions of the bacterial cell-free supernates in 96-well microtitration plates. The titre of a given sample was defined as the highest dilution that caused at least 30% of the cells in a well to be rounded or distended, as described previously [33].

Results

Isolation and identification of *Campylobacter* spp. isolates from faecal samples

Of the 350 samples tested, 111 (32%) samples were positive for *Campylobacter* spp. The method for

isolation of *Campylobacter* spp. was designed to isolate 1 cfu of a *Campylobacter* sp./chicken. However, if there appeared to be more than one type of colony morphology on the CCDA or BA plates then all the colony types were examined further. This resulted in the additional testing of one isolate from each of three chickens; two of these chickens carried two different *C. jejuni* isolates and one chicken carried *C. jejuni* and *C. coli*. Overall, 114 *Campylobacter* isolates were obtained from 15 broiler flocks.

A comparison of the methods for identification of *Campylobacter* isolates is presented in Table 2. By means of conventional culture and identification methods, 101 (88.6%) isolates were identified as *C. jejuni* and 13 (11.4%) were identified as *C. coli*. With the PCR methods, 114 samples were positive with the 16S rRNA universal primer that is specific for the *Campylobacter* genus. The second PCR was performed with different 16S rRNA species-specific primers. All of the 114 samples were PCR-positive with the 16S rRNA primers specific for *C. jejuni* and *C. coli*, whereas no specific PCR products were detected with the 16S rRNA primers specific for other *Campylobacter* species (Table 2). *C. jejuni* and *C. coli* were differentiated by a third PCR with hippuricase gene primers. Of 114 samples that were PCR-positive for the specific *C. jejuni* and *C. coli* 16S rRNA primers, 103 (90.4%) samples were positive with hippuricase primers and 11 (9.6%) were negative (Table 2).

Detection of *cdtB* gene sequences from *Campylobacter* isolates by PCR

The primers VAT2 and WMI1 were used to screen for the presence of *cdtB* sequences in all 114 isolates (Table 3). A single PCR product of c. 500 bp was observed in the PCR reaction with DNA from 98 *C. jejuni* and 13 *C. coli* isolates (Fig. 2). Three isolates, SC-6, SC-61 and SC-770, were *cdtB*-PCR-negative. The PCR amplicons observed were very close to the expected size of 495 bp for a product amplified from

Table 2. Comparison of methods for identification of *Campylobacter* isolated from broiler chickens

Species	Number (%) identified by				
	Culture	16S rRNA un*	PCR 16S rRNA ss†	Hipo‡	
				+	–
<i>Campylobacter</i> spp.	114	114	...		
<i>C. jejuni</i>	101 (88.6)	...	101	101	
<i>C. coli</i>	13 (11.4)	...	13	2	11
<i>C. lari</i>	0	...	0		
<i>C. upsaliensis</i>	0	...	0		
<i>C. hyointestinalis</i>	0	...	0		
<i>C. fetus</i>	0	...	0		
<i>C. helveticus</i>	0	...	0		
Total	114	114	114	103	11

*16S rRNA universal primers.

†16S rRNA primers for specific species.

‡Hippuricase gene primers.

Table 3. Effects of CDT from *C. jejuni* and *C. coli* isolates from Danish broilers in Vero and chicken embryo cell tissue culture assays

Species	Number of isolates	<i>cdt</i> genes detected (%)	Tissue culture assays for CDT*			
			Vero		Chicken embryo	
			+	–	+	–
<i>C. jejuni</i>	101	101 [†] (100)	100	1	89	12
<i>C. coli</i>	13	13 (100)	10	3	10	3
Total	114	114	110	4	99	15

*The effect of CDT on tissue culture assays was determined as described in *Materials and methods*.

[†]The *cdt* genes were detected by different primer sets (Fig. 1).

Fig. 2. *cdtB*-PCR products obtained with primers VAT2(a) and WMI1(b) with *Campylobacter* DNA as templates. Lane 1 (in a and b), 0.3 µg of DNA mass ladder mol. wt standard; 2 (in a), negative control; 20 (in b), positive control with DNA from reference strain *C. jejuni* CCUG-11284; 3–20 (a) and 2–8 (b), the *cdtB*-PCR products obtained with different DNA templates from *C. jejuni* and *C. coli* (9–19 in b) isolates from Danish broilers.

cdtB by these primers (Fig. 1). Two PCR amplicons from each species were selected randomly and purified for sequencing. The sequencing data of these PCR amplicons confirmed that the 500-bp PCR products represented an amplified product from the *cdtB* gene of both selected *C. jejuni* and *C. coli* isolates.

The three *C. jejuni* isolates SC-6, SC-61 and SC-770, that were *cdtB*-PCR-negative with VAT2–WMI1 primers, were screened for *cdt* genes with different primer combinations (GNW–IVH or VAT2–LPFX); PCR products of 165 bp and 1050 bp, respectively, were amplified with the DNA from these three strains.

Differentiation of C. jejuni from C. coli by restriction analysis of cdtB gene PCR product with EcoRI

It has been shown that the sequence of the 500-bp *cdtB* gene from selected *C. jejuni* strains contains an *EcoRI* restriction site, whereas this restriction site is not present in the *cdtB* sequences of some *C. coli* strains [34]. The *EcoRI* restriction endonuclease fragment patterns of 500-bp PCR products of all the isolates were examined. The 500-bp PCR products of all the *C.*

jejuni, including the hippuricase-positive *C. coli* isolates tested, were cut once with *EcoRI* generating 350-bp and 150-bp fragments, whereas none of the 500-bp PCR products from hippuricase-negative *C. coli* was cut (Fig. 3).

Vero and chicken embryo cell assay results

In the Vero cell assay, 100 (99%) of 101 *C. jejuni* isolates produced CDT; one *C. jejuni* isolate (SC-6) produced no toxin. However, with chicken embryo cells, 89 (88.1%) of the isolates produced CDT and 12 (11.8%) produced little or no CDT. Of 13 *C. coli* isolates, 10 isolates produced CDT in both assays, whereas three isolates produced no toxin in either Vero or chicken embryo cell assays (Table 3). The CDT titres produced by different isolates were determined by performing two-fold serial dilutions of the cell-free supernates on Vero cells and it was observed that the CDT activities differed among isolates. Of 101 *C. jejuni* isolates, 47 (46.5%) isolates produced a CDT titre of 1 in 64, 25 (24.7%) isolates produced a CDT titre of 1 in 32, 24 (23.7%) isolates produced a titre of 1 in 16, 4 (3.9%) isolates produced a CDT titre of 1 in 8 and one was negative. However, the *C. coli* isolates

Fig. 3. *EcoRI* restriction of *cdtB* gene PCR products from representative *C. coli* (a) and *C. jejuni* (b) chicken isolates. Lanes 1 and 20 (a and b), 0.3 μ g of 100-bp DNA ladder mol. wt standard. (a) *cdtB*-PCR products from *C. coli* strains. In even lanes, non-treated *cdtB*-PCR products from *C. coli* strains: CCUG-11283, SC-732, SC-733, SC-734, SC-736, SC-737, SC-738, SC-739, SC-741, and in odd lanes, *EcoRI*-treated PCR products from the same strains, respectively. (b) *cdtB*-PCR products from *C. jejuni* isolates. In even lanes, non-treated *cdtB*-PCR products from *C. jejuni* strains CCUG-11284, SC-328, SC-333, SC-335, SC-357, SC-360, SC-418, SC-452, SC-637 and in odd lanes *EcoRI*-treated PCR products from the respective strains.

produced much lower CDT titres. Of 13 *C. coli* isolates originating from four flocks, 3 produced no toxin, 9 produced a CDT titre of 1 in 8, and only one isolate produced a CDT titre of 1 in 16 (Fig. 4).

Discussion

In this study, the frequency of different *Campylobacter* species in individual chickens was investigated. *Campylobacter* isolates were identified to species level by two techniques – conventional phenotypic character-

isation and PCR. Identification by conventional methods is laborious and time-consuming (*c.* 2–3 days). However, *Campylobacter* spp. can be identified within 10–15 h by the PCR method described above, [35]. The results of the two methods were essentially in agreement except that two isolates identified as *C. coli* by conventional methods (hippurate hydrolysis negative) were positive by hippuricase-PCR, suggesting that they were hippurate hydrolysis-negative *C. jejuni*. Such strains have been described as variants of *C. jejuni* containing a mutation in the hippuricase gene [30].

The data from *EcoRI* restriction endonuclease analysis of the *cdtB* PCR products in this study confirmed that all 103 *C. jejuni* isolates tested – including the two hippuricase PCR-positive *C. coli* isolates – contained the *EcoRI* restriction site, whereas this restriction site was absent in 11 *C. coli* isolates. In view of these results PCR with hippuricase primers and *EcoRI* restriction analysis of *cdtB*-PCR amplicons may be useful for establishing species identity of these two closely related species. Taken together, the results of all the methods indicated that 90.4% of the isolates were *C. jejuni* and 9.6% were *C. coli*. No other *Campylobacter* sp. was isolated. The results confirmed the predominance of *C. jejuni* among strains isolated from broilers.

Fig. 4. CDT titre produced by *Campylobacter* spp. isolated from Danish broilers, ■, *C. coli*; ▨, *C. jejuni*.

Recently, several publications have reported on the frequency of *cdt* genes among *Campylobacter* isolates from different sources [17, 18, 34, 36–38]. Little is known about the frequency of these toxin genes among *Campylobacter* isolates from broilers. In this study, the prevalence and expression of the *cdt* genes in *Campylobacter* isolates from Danish broilers was determined. A PCR method was used to detect the *cdtB* gene in the *Campylobacter* isolates. With the combination of VAT2–WMI1 primers, the *cdtB* gene was detected in 111 of 114 isolates. The *cdtB* genes of the three *C. jejuni* isolates, SC-6, SC-61 and SC-770, were detected only when different primer sets complementary to the *cdtA* and *cdtC* genes were used. Testing for CDT production by the 114 isolates with cell culture assays revealed that one of the three *cdtB*-PCR-negative isolates (*C. jejuni* SC-6) was the only *C. jejuni* isolate that did not produce CDT, either in Vero or in chicken embryo cell assays. Strains SC-61 and SC-770 produced CDT, but with very low titres. The fact that the *cdt* genes of these three isolates were amplified only with different primer sets and that they produced little or no CDT toxin could be the result of a mutation in the coding region of *cdtB* gene in these isolates. This may have influenced the production of active CDT by these isolates as well as the annealing of primer WMI1 to the *cdtB* gene in the PCR tests.

The production of CDT by the isolates was also tested with Vero and chicken embryo cells. In all, 110 (96%) of 114 *Campylobacter* isolates produced CDT in the Vero cell test, whereas only 99 (86.8%) of 114 demonstrated CDT production in the chicken embryo cell assay.

The effects of CDT on Vero cells observed in this study were similar to the effects of CDT on Vero cells and Chinese hamster ovary cells observed by others [16, 39]. All but one of the *C. jejuni* isolates (100 of 101) produced CDT in the Vero cell assays, whereas *C. coli* isolates produced little or no CDT in either Vero or chicken embryo cell assays. The frequency of CDT-producing *Campylobacter* isolates in this study (96.5%) was similar to the reported 99% in one of the previous studies [38], but higher than the frequency of 17% [18], or 28.6% found by others [17]. There may be several explanations for this variation. Firstly, the *Campylobacter* isolates used in this study were fresh isolates. Also, the method of analysis of CDT production in this study was similar to that used by Johnson and Lior [16], who used the unconcentrated culture supernates, but was different from the cell lysate assays used in other studies [17, 21]. In this study, the cell-free supernates were tested immediately after preparation. Both the Vero and chicken embryo cells used in this study were < 2 days old after being trypsinised and seeded.

Previous studies have suggested that a substantial proportion of *C. jejuni* strains might not produce

CDT [16]. In this study, nearly all *C. jejuni* isolates produced CDT, although the CDT titre differed among the isolates. However, the Vero cell line used in this study has been tested and shown to be very sensitive to both *E. coli* Verotoxin [33] and CDT toxin (D. D. Bang and F. Scheutz, unpublished results).

Florin and Antillon [18] suggested that at least two different cell types should be used to detect CDT production by campylobacters. One of the cell lines should be Vero cells and the other could be either HeLa cells or a fibroblast cell line, as these cell lines were considered to be more sensitive. Vero and chicken embryo fibroblast cells were used in the present study. The frequency of CDT-producing *Campylobacter* isolates on chicken embryo fibroblast cells in the present study was lower than that obtained in the Vero cell assays. This could be due to the high cell densities (0.5×10^8 cells/ml) used in chicken fibroblast assays. We have observed a large cell density-dependent difference in the sensitivity of CDT detection (D. D. Bang and K. Handberg, unpublished results).

In conclusion, the results presented here confirm the very common occurrence of *C. jejuni* and *C. coli* in broiler chickens and indicate that the *cdt* genes may frequently be present in both *C. jejuni* and *C. coli* isolates from broilers. However, the role of CDT in campylobacteriosis is still in question. The study of regulation and expression of the *cdt* genes *in vivo* and *in vitro*, especially testing of strains with defined mutations of these genes, in animal models will be of great help to improve the understanding of the role of CDT in the pathogenesis of campylobacteriosis.

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