View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Nottingham Trent Institutional Repository (IRep)

1 **Cytolethal distending toxin (CDT)-Negative *Campylobacter jejuni* strains and**
2 **anti-CDT neutralizing antibodies induced during human infection but not**
3 **chicken colonization**
4
5 Running title: Lack of CDT and host differences in neutralization.
6
7 **Manal AbuOun[1]*, Georgina Manning[1], Shaun A. Cawthraw[1], Anne Ridley[1], If**
8 **H. Ahmed[1], Trudy M. Wassenaar[2] and Diane G. Newell[1]**
9
10
11 [1]Veterinary Laboratories Agency (Weybridge), Surrey, United Kingdom. [2] Molecular
12 Microbiology and Genomics Consultants, Zotzenheim, Germany.
13
14 **Corresponding author:**
15 M. AbuOun
16 Department of Food and Environmental Safety,
17 Veterinary Laboratories Agency,
18 New Haw, Addlestone
19 Surrey KT15 3NB
20 United Kingdom
21 Tel + 44 1932357738
22 Fax + 44 1932357595
23 Email m.abuoun@vla.defra.gsi.gov.uk
24
25

25 **Summary**

26 The cytolethal-distending toxin (CDT), of *Campylobacter jejuni* was detectable, using
27 an *in vitro* assay in most, but not all, 24 strains tested. The reason for the absence of
28 toxin activity in these naturally-occurring CDT-negative *C. jejuni* strains was then
29 investigated at the genetic level. CDT is encoded by three highly conserved genes,
30 *cdtA, B* and *C*. In the CDT-negative strains, 2 types of mutation were identified. The
31 CDT activities of *C. jejuni* strains possessing both types of mutation were
32 successfully complemented with the functional genes of *C. jejuni* 11168. The first
33 type of mutation comprised a 667-bp deletion across *cdtA* and *cdtB* and considerable
34 degeneration in the remainder of the *cdt* locus. Using a PCR technique to screen for
35 this deletion, this mutation occurred in less than 3% of 147 human, veterinary and
36 environmental strains tested. The second type of mutation involved at least 4 non-
37 synonymous nucleotide changes but only the substitution of proline with serine at
38 CdtB-95 was considered important to CDT activity. This was confirmed by site-
39 directed mutagenesis. This type of mutation also occurred in less than 3% of strains as
40 determined using a LightCycler biprobe assay.
41 The detection of two CDT-negative clinical isolates questioned the role of CDT in
42 some cases of human campylobacteriosis. To determine if anti-CDT antibodies are
43 produced using human infection, a toxin neutralization assay was developed and
44 validated using rabbit antisera. Pooled human sera from infected patients neutralized
45 the toxin indicating expression and immunogenicity during infection. However, no
46 neutralizing antibodies were detected in colonized chickens despite the expression of
47 CDT in the avian gut as indicated by RT-PCR.

48

48 **Introduction**

49 *Campylobacter jejuni* and *C. coli* are major causes of acute human bacterial enteritis
50 in industrialised countries (35). These *Campylobacter* species asymptomatically
51 colonize the intestinal tract of most mammals and birds (24) and one major route of
52 human campylobacteriosis is assumed to be the consumption of contaminated poultry
53 meat products (10). The pathogenic mechanisms by which campylobacters cause
54 diarrhoea are as yet unknown, although motility, adhesion and invasion have been
55 implicated (38). Several toxic activities have been reported but their roles in disease
56 remain debatable (37).

57 The best-characterised Campylobacter toxin is the cytolethal-distending toxin (CDT).
58 CDT production has been described in several Gram-negative bacteria, including
59 *Escherichia coli* (30, 33), *Haemophilus ducreyi* (7), *Actinobacillus*
60 *actinomycetemcomitans* (21), *Shigella dysenteriae* (25, 26) and *Helicobacter spp.*(42).
61 However, not all these species are implicated in enteric disease. In *C. jejuni*, CDT
62 causes progressive cellular distension with eventual cell death (16). These
63 morphological changes appear to be a consequence of alterations in the progression of
64 the cell cycle, in particular cell cycle arrest in the G2/M phase (6, 8, 28, 41).

65 CDT production is dependent on the expression of three tandem genes, *cdtA*, *cdtB* and
66 *cdtC* (31). The CdtA, CdtB and CdtC proteins form a tripartite holotoxin complex
67 required for CDT activity (18). Current evidence indicates that *cdtB* encodes the
68 active/toxic component of the toxin while *cdtA* and *cdtC* are involved with binding to
69 and internalisation into the host cell (18, 19).

70 The role of CDT in human campylobacteriosis is unclear. Although, all *C. jejuni*
71 strains tested to date appear to possess the *cdt* genes (11, 12, 31), the levels of toxin
72 activities expressed are strain dependent, with 2 strains (~1.2 %) reported to produce

73 no detectable levels of CDT *in vitro* (2, 12). The explanation for such CDT-negative
74 strains is currently unknown. In this study we have investigated the molecular basis of
75 this using 8 *C. jejuni* CDT-negative strains isolated from human diarrhoeic stools
76 (n=2), bacteremia (blood)(n=2), sheep (n=1), poultry processing plant (n=1) and a
77 broiler (n=2). The results indicated that lack of the CDT phenotype was a
78 consequence of either major deletions (51bp and 667bp) in or around *cdtB* or one or
79 more point mutations within the *cdtABC* gene. Site directed mutagenesis and
80 complementation were used to confirm these observations. A PCR assay and a
81 LightCycler BiProbe assay were developed to screen 123 randomly selected
82 veterinary and human *Campylobacter* isolates for either the deletion or the
83 predominant point mutation. The isolation of CDT-negative strains from cases of
84 human campylobacteriosis questioned the role in disease of this potential virulence
85 factor. Therefore, we developed an assay to detect specific anti-CDT neutralizing
86 antibodies in the serum from infected individuals. The results of these studies
87 indicated that circulating antibody responses, which neutralize CDT activity, are
88 elicited during human infection but not during chicken colonization with *C. jejuni*.
89

90 **Materials and Methods**

91

92 **Bacterial Strains and Growth Conditions:** A panel of 24, human (n=10), veterinary
93 (n=6) and environmental (n=8), *C. jejuni* strains were initially tested for CDT activity.
94 In addition, 3 strains (81116, 11168, 81-176) of previously reported CDT activity and
95 a *cdtABC* mutant of 81-176 (81-176*cdt*), kindly provided by Carol Pickett (41), were
96 used as controls. The DNA from an additional 123 strains, isolated from humans,
97 poultry, cattle and sheep, were tested in the molecular screening assays developed.

98 Chemically competent *E.coli* TOPO10F' (Invitrogen, Paisley, UK) and DH5αMCR
99 (Invitrogen) strains were used for cloning and site directed mutagenesis studies,
100 respectively.
101 *C. jejuni* strains were grown for 24 h at 42 ^{o}C under microaerobic conditions (7.5 %
102 O_2, 7.5 % CO_2, 85 % N_2) on either Mueller-Hinton (M-H) or blood agar plates
103 supplemented with 10% sheep blood, 50 µg/ml Actidione and selective antibiotics
104 (Oxoid, Basingstoke, UK) (34). *C. jejuni* 81-176*cdt* was grown on media
105 supplemented with 50 µg/ml kanamycin. *E. coli* was grown in Luria-Bertani medium
106 (LB) under atmospheric conditions at 37 ^{o}C. Where necessary, LB medium was
107 supplemented with 50 µg/ml ampicillin or 20 µg/ml chloramphenicol. The *C. jejuni*
108 and *E. coli* strains were stored frozen at – 80 ^{o}C in 1 % (w/v) proteose peptone water
109 containing 10 % (v/v) glycerol or in LB broth containing 50 % (v/v) glycerol
110 respectively.
111
112 ***In Vitro* HeLa cell cytolethal distending toxin (CDT) assay:** The assay used in this
113 study was adapted from previously published CDT assays (14, 31). Cultured HeLa
114 cells (ECACC, Porton Down, UK) were maintained in complete Eagle's Minimal
115 Essential Medium (EMEM) with 10 % (v/v) foetal bovine serum, 1 % (v/v) L-
116 glutamine, 1 % (v/v) non-essential amino acids and 0.5 % (v/v) gentamicin) at 37 ^{o}C
117 in 5% CO_2. For the assay, HeLa cell concentrations were adjusted to 2 x 10^4 cells ml^{-1}
118 and 150 µl of this suspension was added to each well of a flat-bottomed, tissue culture
119 grade, 96-well plate. The cells were then cultured for 2-3 hours at 37 ^{o}C in 5% CO_2,
120 prior to the addition of bacterial lysates.
121 Lysates were prepared from bacteria grown on M-H agar plates for 24 hours at 42 ^{o}C
122 under microaerobic conditions and harvested into complete EMEM. The bacterial

123 suspensions were adjusted to OD_{550} 1.6, sonicated on ice (1.5 min at 30 amp with 6-

124 sec pulses) (Vibra Cell, Sonics and Materials Inc, USA) and cell debris removed by

125 centrifugation (6000 g for 10 min at 4 ^{o}C). The lysates were sterilised by filtration

126 using 0.22 μm filters (Minisart, Sartorius, Germany). Undiluted and 1 in 10 dilutions

127 of the bacterial lysates were added to HeLa cells and two-fold dilutions performed

128 across each plate, which were then incubated for 5 days at 37 ^{o}C in 5 % CO_2. The

129 HeLa cell monolayers were then fixed in 10 % (v/v) formalin, stained with 2.3 %

130 (w/v) crystal violet and examined by light microscopy. The toxin titres were

131 expressed as the reciprocal of the highest dilution that caused 50 % of cells to become

132 distended, and adjusted by dividing the OD_{550} of bacterial sonic lysates. Toxin titre for

133 each strain was tested in at least three independent assays blind.

134

135 **CDT antibody neutralization assay:** Hyperimmune rabbit antisera were produced

136 against the whole organisms of *C. jejuni* strains 81116 (R12), EF (R43) and C37596

137 (R42) as previously described (23). Serum from 10 chickens experimentally colonized

138 with *C. jejuni* 81116, at 9 weeks post-challenge, were collected as previously

139 described (3). Sera pooled from 6 human patients, taken 2-4 weeks after

140 campylobacter isolation (pooled positive sera) and 6 human donors, with no recorded

141 enteric infection (pooled negative sera), were used in the neutralization assay. The

142 collection, storage and characterisation of these sera has been described previously (4,

143 5).

144 All sera used were initially treated at 56 ^{o}C for 2 hours to inactivate complement,

145 before use in the i*n vitro* HeLa cell CDT assays. Prior to the addition to the HeLa

146 cells, 1/10 dilution of bacterial lysates were pre-incubated with R12 (1 in 50 dilution),

147 R42 (1 in 30 dilution), R43 (1 in 30 dilution), pooled chicken sera (1 in 30 dilution) or

148 pooled human sera (1in 30 dilution), at 37 °C for 1 hour. Treated lysates were
149 subsequently applied to the HeLa cells as described previously.
150
151 **Statistical Analyses:** Experimental results from at least 3 independent assays were
152 transformed to log (x+0.5) for analysis. Mean titres of each treatment were compared
153 to controls by two-way analysis of variance and wherever treatment differences were
154 $p<0.05$, indicated statistical significance.
155
156 **Western blotting:** Whole-cell protein profiles of *C. jejuni* 11168 and 81-176 were
157 prepared by separating on 12 % sodium dodecyl sulfate-polyacrylamide gel
158 electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membrane for
159 western blotting as described previously (3). Membrane strips of each strain were
160 incubated with the human and chicken sera (1/30 dilution) used for the CDT
161 neutralization studies, and bound antibodies detected using species-specific anti-
162 immunoglobulin antisera conjugated to horse-radish peroxidase according to the
163 manufacturers instructions (DakoCytomation Ltd, Cambridgeshire, UK and
164 Amersham Biosciences UK Limited, Buckinghamshire, UK respectively).
165
166 **Polymerase chain reaction (PCR) assay for *cdt* genes:** Chromosomal DNA was
167 isolated from strains by the CTAB/NaCl method (1). Recombinant Taq DNA
168 polymerase (Invitrogen) was used for all PCR reactions according to the
169 manufacturer's recommendations. Oligonucleotide primers used for PCR and
170 sequencing reactions are given in Table 1 (Sigma Genosys, Haverhill, UK). PCR
171 reactions were performed on a PE Applied Biosystems GeneAmp PCR 9700, with an

172 initial denaturation step of 95 ^{o}C for 5 min, followed by 25 cycles of 95 ^{o}C for 2 min,

173 60 ^{o}C for 2 min and 72 ^{o}C for 1.5 min and a final extension step at 72 ^{o}C for 10 min.

174

175 **Cloning and DNA sequence analysis of *cdt* genes:** PCR reactions were repeated

176 using Advantage cDNA Polymerase Mix , containing 3'- 5' proof reading activity

177 (BD Biosciences Clontech, Oxford, UK) according to the manufacturer's instructions.

178 PCR products were then cloned into pCR2.1-TOPO® (TOPO-TA cloning kit,

179 Invitrogen) and transformed into chemically competent *E. coli* TOPO10F' one shot®

180 cells (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was

181 isolated for analysis using a mini plasmid preparation kit (Qiagen Ltd, Crawley, UK)

182 and the DNA inserts confirmed by restriction analysis. Inserts were sequenced using

183 BigDye terminator cycle sequencing chemistry (Applied Biosystems, Warrington,

184 UK) according to the manufacturer's instructions. Sequenced products were separated

185 on an ABI Prism 377 automated DNA sequencer (Applied Biosystems). DNA

186 sequences were assembled and edited using SeqMan (DNAstar, Lasergene, Madison,

187 USA) and ClustalV alignments to the published *C. jejuni* 81-176 *cdt*ABC genes

188 (GenBank accession number U51121) were done in MegAlign 5.00 (DNAstar,

189 Lasergene, Madison, USA).

190

191 **Southern blot analysis:** Genomic DNA (10 μg) was digested with the restriction

192 endonuclease *Hind*III (Promega, Southampton, UK) overnight at 37 ^{o}C and used for

193 Southern blot analysis using the CDP-star chemiluminescent detection reagent

194 (Amersham Biosciences, Bucks UK) according to manufacturer's instructions.

195

196 **RNA extraction and reverse transcriptase PCR (RT-PCR):** Total RNA was

197 isolated from *C. jejuni* strains and chicken caecal contents using Tri reagent (Sigma

198 Aldrich, Poole, UK) according to the manufacturer's instructions. RNA was

199 suspended in an appropriate amount of RNase free water and RNA concentrations

200 estimated by spectrophotometry. RT-PCR was carried out using the high fidelity

201 ProSTAR HF single-tube RT-PCR system (Stratagene, Texas, USA), according to the

202 manufacturer's instructions. Since the *cdt* genes of *C. jejuni* strains are known to be

203 expressed in a single mRNA transcript (14), RT-PCR primers DS15 and DS18 (Table

204 1) were used to amplify the region between *cdtA* and *cdtB*. RT-PCR was performed

205 with an initial incubation step of 42 ^{o}C for 30 min, during which time cDNA was

206 synthesised from the RNA template. The reverse transcriptase was inactivated and the

207 cDNA denatured by incubation at 95 ^{o}C for 1 min, followed by an amplification

208 reaction, comprising 40 cycles of 95 ^{o}C for 30 sec, 60 ^{o}C for 30 sec and 68 ^{o}C for 2

209 min. RT-PCR products were visualised on 1.2 % (w/v) agarose gels.

210

211 **LightCycler BiProbe assay of *cdtB* polymorphism:** For the LightCycler, PCR

212 primers LC-T-F and LC-T-R (Table 1), were designed by alignment of the published

213 sequence of the *C. jejuni* 11168 *cdtB* gene with the *cdtB* gene from *C. jejuni* strain EF,

214 to amplify a 163 bp region encompassing codon 95 of *cdtB*. The polymorphism was

215 detected by melting curve analysis of the probe LC-T-probe (Table 1) after PCR

216 amplification. The probe was identical to the sequence of *C. jejuni* EF, containing a

217 serine at codon 95 (tct). Amplification and probe hybridisation were performed in 20

218 μl reactions using the LightCycler DNA Master SYBR Green I kit (Roche

219 Diagnositics Ltd, Lewes, UK) in a LightCycler instrument (Roche Diagnostics Ltd).

220 Typically reaction mixtures comprised 10 – 15 pmol DNA, 2 pmol of LC-T-F, 5 pmol

221 LC-T-R, 5 pmol LC-T-Probe and 3 mM $MgCl_2$ in 1x LightCycler DNA master mix

222 SYBR green mix. LightCycler PCR was performed for 50 cycles, with 15 s at 95 ^{o}C,

223 5s at 55 ^{o}C and 10s at 74 ^{o}C with a transition rate of 20 ^{o}C/s. Fluorescence was

224 measured at a wavelength of 540 nm (F1 channel) at the end of each amplification

225 step to monitor the accumulation of PCR product. Melting curve analysis was

226 performed immediately after amplification by heating the product to 94 ^{o}C (20°C/s),

227 cooling to 40 ^{o}C for 10 s, and then heating to 85 ^{o}C (0.1^{o}C/s). The final heating step

228 was performed under continuous fluorescence measurement. DNA from *C. jejuni* EF

229 and 11168 were included as positive controls and a no template control was included

230 in each run.

231

232 **Complementation studies of CDT negative strains:** Complementation of CDT-

233 negative strains was achieved by the introduction of the *Campylobacter* shuttle vector

234 vector, pUOA18 (36), expressing a 2.4-kb region from *C. jejuni* 11168 containing the

235 *cdt* genes (41). The region, included 206 bp of upstream and 106 bp of downstream

236 sequence, was amplified with ClCdtABC-F and ClCdtABC-R (Table 1) and initially

237 cloned into pCR2.1 TOPO. These primers included *Bam*HI restriction enzyme sites at

238 the 5' ends of both primers. Following *Bam*HI digestion and gel purification, the

239 insert was ligated into dephosphorylated, *Bam*HI digested pUOA18, to make the

240 construct pCDT, and transformed to DH5αMCR (Invitrogen). The construct was

241 isolated from *E. coli* and electroporated into *C. jejuni* EF and 99/68, using a method

242 adapted from Wassenaar *et al*. (39). Colonies that appeared on 20 mg/ml

243 chloramphenicol medium were tested for CDT activity in the *in vitro* CDT assay.

244

245 **Site directed mutagenesis of *cdt*B-95.** Pro-95 of CdtB was changed to Ser-95 using
246 the QuikChange® XL Site-Directed Mutagenesis Kit (Stratagene), according to the
247 manufacturer's instructions. Mutagenic primers (Table 1) were used to generate
248 $pCdtB^{P95S}$ and mutants identified by *Hae*III restriction digestion of the *cdtABC* genes
249 and sequencing of the entire insert.

250

251 **Chick colonization model:** A quantitative chick colonization model was used as
252 previously described (40). Briefly, groups of 10 specific pathogen-free, 1-day old
253 chickens (Charles River SPAFAS Inc., Hanover, Germany), housed in isolators, were
254 dosed by oral gavage with *C. jejuni* 81-176, 81-176 *cdt*ABC^{-}, C289/6. Doses,
255 administered in 100 µl 0.1 M PBS , ranged from 10^2 - 10^9 colony forming units
256 (c.f.u). Doses were prepared by harvesting bacteria, grown overnight on 10 % blood
257 agar plates at 42 ^{o}C, into sterile 0.1 M PBS. At five days post challenge, colonization
258 levels were determined by plating of dilutions of caecal contents. Colonization levels
259 were determined as c.f.u. per gram of caecal contents for individual birds. All animal
260 experiments were performed in accordance with the local ethics committee and UK
261 home office licence guidelines.

262

263 **Nucleotide sequence accession numbers:** The *cdt* gene sequences have been
264 submitted to the GenBank database under the following accession numbers: *C. jejuni*
265 C37596 - AY442300, *C. jejuni* C35926 - AY442302, *C. jejuni* C37533 - AY442301
266 and *C. jejuni* EF – AY445094.

267

268 **Results**

269

270 **Identification and characterisation of CDT-negative *C. jejuni* strains:** A panel of
271 24 selected *C. jejuni* strains were tested for CDT activity. A range of CDT-activities,
272 from titres of <50 (n=13), 50-150 (n=4), 150-300 (n=2) and >300 (n=1), were
273 observed but there was no obvious correlation with strain source (data not shown). Of
274 the 24 strains tested 4 (C37596, C35926, C37533 and EF) had no detectable CDT
275 activity. Two of these CDT-negative strains, C37596 and C35926, were isolated from
276 the blood of independent campylobacteriosis patients (within the UK in 2000) with
277 underlying medical problems, as discussed later. *C. jejuni* strain C37533 was isolated
278 from the faeces of the same patient as isolate C37596. None of these strains were
279 serotypable. The remaining CDT-negative strain (EF) was isolated from a chicken
280 carcass in the chilling area of a poultry-processing plant.
281 The presence of *cdt*ABC genes in all 24 strains was determined by PCR. In 21 of the
282 24 strains, the expected PCR amplicon size of 2143 bp, generated with primers CdtF
283 and CdtR, was observed (Fig. 1). However, in strains C37596, C35926 and C37533,
284 which were CDT-negative, a smaller product of 1400 bp was detected. The PCR
285 products of all 4 CDT-negative strains were cloned and sequenced. ClustalV
286 alignment, to the published *cdtABC* sequence of *C. jejuni* 81-176 (Accession number
287 U51121), revealed a 667-bp deletion between *cdtA* and *cdtB* and a separate 51-bp
288 deletion within the *cdtB* genes of strains C37596, C35926 and C37533, consistent
289 with the reduced PCR amplicon size observed by PCR (Fig. 2). The remainder of the
290 sequences from these three strains had only 51% identity, which was due to additional
291 indels (insertions-deletions) and substitutions throughout the remaining *cdtABC* gene
292 sequence.

293 We next determined, by Southern blot hybridisation, whether the 667 bp deleted
294 region between *cdtA* and *cdtB* was the result of recombination with another part of the
295 genome or whether it was completely lost from the genome. Using primers specific
296 for this region (C7del), a PCR product was generated from strain 11168, and used to
297 probe *Hind*III-digested genomic DNA of strains C37596, C35926 and C37533. The
298 results confirmed the absence of the region between *cdtA* and *cdtB* in the genomic
299 DNA of these strains (data not shown).
300 To determine the prevalence of the 667 bp deletion in *C. jejuni* isolates, the DNA of
301 an additional 123 randomly selected clinical and veterinary strains were screened by
302 PCR using the primers Cdt-F and Cdt-R. All but one strain, 99/68, produced a PCR
303 product of 2.14 kb. Strain 99/68, which was isolated from a broiler, produced an
304 amplicon of only 1400 bp indicative of a deletion. Cloning and sequencing of this
305 amplicon revealed 96% sequence identity with the *cdt* genes of C37596, C35926 and
306 C37533 previously identified as having the same deletions (data not shown). This
307 result indicates that significant deletions in the *cdt* genes occur in 2.7 % (4 of 147
308 isolates tested) of *C. jejuni* strains, but when such deletions in the *cdt* gene locus are
309 present, they were found between *cdtA* and *cdtB*.
310 Interestingly the sequence of the *cdt* genes from the CDT-negative strain, EF,
311 identified 18-nucleotide substitutions compared to previously published sequences of
312 *C. jejuni cdtABC* (11168 & 81-176). Sequence analysis indicated that all three reading
313 frames were open in *C. jejuni* EF. Of the 18 nucleotide substitutions, only four were
314 identified as non-synonymous: involving an alanine to a valine at codon 88 in CdtA; a
315 proline to serine and methionine to threonine at codons 95 and 120 of CdtB
316 respectively (Fig. 3); and an isoleucine to asparagine at codon 167 of CdtC. In order
317 to determine if the lack of CDT activity in strain EF was due to a loss of gene

318 expression rather than the substitutions, RT-PCR analysis was undertaken. Total RNA
319 extracted from *C. jejuni* EF was used as a template and RT-PCR was carried out,
320 using the DS15 and DS18 primers (Table 1). A mRNA transcript was detected from
321 both *C. jejuni* EF and 11168 (Fig. 4A), indicating that gene expression occurred and
322 that the lack of toxicity was most likely caused by the production of inactive toxin,
323 probably as a consequence of one or more of the amino acid substitutions.
324 Alignment of the CdtB amino acid sequence from other CDT-producing bacteria,
325 revealed Pro-95 to be a highly conserved residue (Fig. 3), suggesting that this residue
326 is important for the function of the protein. In order to determine the frequency of the
327 mutation at this residue, melting curve analysis using a LightCycler assay with a
328 probe designed to match the EF Ser-95 mutation was developed and performed on the
329 original 24 strains and the additional 123 strains (Fig. 5). The assay identified two
330 strains (99/373 and S58 from a human and a sheep respectively) in addition to EF,
331 which gave a melting temperature of 60 ^{o}C indicating the presence of the Ser-95
332 mutation. A third strain (99/12), also from a broiler, was identified, which had a lower
333 melting temperature of 48 ^{o}C, suggesting that more than one mutation was present
334 within the region probed. These observations were further confirmed by sequencing
335 of the *cdtABC* genes. Strain 99/373 and S58 were identical at the amino acid level to
336 *C. jejuni* EF, except that S58 did not have the substitution in CdtC at codon 167 of an
337 isoleucine to asparagine. In contrast, the sequence of 99/12 revealed several additional
338 amino acid substitutions to those found in *C. jejuni* EF; 8 of these occurred in CdtB
339 and 6 in CdtC. In addition to these substitutions, 4 nucleotide insertions and 1 deletion
340 were also identified. Overall the frequency of the Pro-95-Ser mutation as detected by
341 the LightCycler BiProbe assay, was 2.29 % (3 of 147 strains tested). The absence of
342 CDT activity in strains 99/373, S58 and 99/12 was confirmed using the *in vitro* HeLa

343 cell CDT assay (data not shown). By RT-PCR the *cdt* genes were transcribed in
344 strains 99/373, S58 and 99/12 (Fig. 4B), providing supporting evidence that once
345 again suggesting that these mutations were associated with the lack of CDT activity.
346
347 **Complementation and site directed mutagenesis**: To verify whether CDT negative
348 strains could produce active CDT, the complementation vector (pCDT), containing
349 functional *cdt* genes of *C. jejuni* 11168 was introduced into *C. jejuni* EF and 99/68.
350 All transformants tested produced active CDT in the *in vitro* CDT assays, with levels
351 comparable to *C. jejuni* 11168 (Fig. 6).
352 To ascertain whether the proline residue at CdtB-95 was essential for CDT activity,
353 site directed mutagenesis was used to mutate proline to serine at this residue in the
354 complementation vector pCdt to generate $pCdtB^{P95S}$. *C. jejuni* EF and 99/68 were
355 complemented with $pCdtB^{P95S}$ and transformants tested for CDT activity. The single
356 mutation at CdtB-95 to serine resulted in a 98.4 % ($p = 0.0004$) and 97.3 % ($p < 0.0001$)
357 reduction in CDT titre, for EF and 99/68 respectively (Fig. 6). These results indicated
358 the residue at CdtB-95 is critical for the toxicity of CDT in *C. jejuni*.
359
360 **The expression and immunogenicity of CDT during colonization:** As yet there are
361 no suitable in vivo models of campylobacteriosis (22), however the one day-old
362 chicken is an excellent model of colonization. Challenge of one-day-old chicks with
363 the CDT negative mutant of 81-176 gave the same level of colonization, up to 10^9 cfu
364 per gram of cecal contents, as the parent strain (data not shown) suggesting that the
365 absence of CDT expression does not affect colonization potential.
366 Despite this high level of colonization no clinical signs of disease were discernable in
367 chicks challenged with either the mutant or the wild type strain. These differences in

368 outcome of colonization in the chicken and human are well recognised but
369 unexplained (22). To determine whether the cdt genes were expressed during chicken
370 colonization, the caecal contents of chicks challenged for up to 10 days with CDT-
371 positive *C. jejuni* strain C289/6 was investigated by RT-PCR. The results indicated
372 that CDT is expressed during colonization of the avian gut (Fig. 7).
373 As determining the CDT expression in humans by a similar approach was not
374 feasible, an alternative approach was sought. For other bacteria, the development of
375 host circulating anti-toxin antibody responses has provided indicators of both *in vivo*
376 expression and the virulence potential of putative toxins (9, 13). Therefore an assay to
377 detect specific anti-CDT antibodies was developed. In this assay sera, from
378 experimentally colonized chickens, hyperimmunised rabbits and patients with
379 campylobacteriosis, were tested for their capacities to neutralize the *in vitro* CDT
380 activity from 3 CDT-positive *C. jejuni* strains 81116, 81-176 and 11168 which had
381 CDT titres of 23.6 (± 0.8), 16.2 (± 3.7), 28.6 (± 4.8) respectively. When lysates from
382 these strains were pre-treated with rabbit anti-*C. jejuni* 81116 antisera (R12) the CDT
383 activity was completely or largely neutralized (100%, 100% and 79% respectively)
384 (Fig. 8), where as no neutralisation was observed with sera from the preimmunised
385 rabbits. Thus indicating that the CDT expressed during *in vitro* culture is antigenic in
386 immunized rabbits. To confirm the specificity of this neutralizing activity, rabbit
387 antisera directed against two of the CDT-negative strains, C37596 (R42) and EF
388 (R43), were also tested in the neutralization assay. As expected R42 did not neutralize
389 the CDT activity of any of the strains tested, which is consistent with the presence of
390 the large deletions and substantial degeneration of the *cdt* locus in this strain. In
391 contrast R43 only partly neutralized (71%, 54% and 46%) the CDT activities of
392 strains 81116, 81-176 and 11168 respectively (Fig. 8), probably reflecting changes in

393 antigenic structure of CDT in strain EF as a consequence of the amino acid sequence
394 variation.
395 The neutralization assay was further used to determine the presence of anti-CDT
396 neutralizing antibodies in pooled sera from patients convalescing from
397 campylobacteriosis. Previous studies, using ELISA and western blotting (4),
398 demonstrated the presence and specificity of anti-campylobacter antibodies in the sera
399 from each individual patient. Complete (100 %) neutralization of the CDT activity
400 was observed in all strains (Fig. 8). This level of neutralization was significantly
401 higher ($p = 0.001$) than the level of neutralization obtained with pooled human sera
402 from blood donors with no history of enteric disease and no demonstrable anti-
403 campylobacter antibodies as detected by ELISA and western blotting (4), indicating
404 that CDT is both expressed during human infection and induces antibody responses.
405 Finally we investigated the presence of neutralizing anti-CDT antibodies in the pooled
406 sera of chickens experimentally colonized with *C. jejuni* 81116. The development of
407 humoral responses directed against the antigens of this strain during colonization has
408 been reported previously (3) and in two other strains by western blot (Fig. 9).
409 Interestingly, in contrast to antibodies from colonized humans and immunised rabbits,
410 these chicken antibodies demonstrated no detectable CDT neutralizing activity (Fig.
411 8.) against either the homologous strain or the two heterologous strains.
412
413 **Discussion**
414 The lack of a suitable small animal model of campylobacteriosis continues to be a
415 major limitation to our understanding of the bacterial pathogenic mechanisms
416 involved in campylobacteriosis. Thus potential *C. jejuni* virulence factors have been
417 largely defined on the basis of *in vitro* properties. Although, many toxin activities

418 have been observed in *C. jejuni* strains *in vitro,* to date only CDT has been sufficiently
419 characterised to suggest that this is a true virulence factor with a role in enteric
420 campylobacteriosis. Our observations confirmed previous reports (12, 31) that the
421 majority of *C. jejuni* strains express detectable CDT activity. However, clearly the
422 level of CDT activity expressed varied between strains and, moreover, some strains
423 expressed no detectable activity.
424 Although CDT-negative *C. jejuni* strains have been described previously (11, 12, 31),
425 the frequency, molecular basis and potential consequences of this negative phenotype
426 have not been fully investigated. From our studies two distinct types of naturally
427 occurring mutation have been identified in the *cdt* locus of CDT-negative strains. The
428 deletion mutation, detected in isolates from 2 unrelated patients and a broiler,
429 involved a 667-bp deleted region between *cdtA* and *cdtB,* and a separate 51 bp
430 deletion further downstream in *cdtB*, as well as several point mutations throughout the
431 remaining *cdt* genes. Southern blot analysis indicated that these observations were the
432 consequence of a genomic deletion rather than a transition. It, therefore, seems likely
433 that the major deletion occurred first followed over time by the subsequent
434 degeneration of the residual surrounding, now redundant, genetic material.
435 The recovery of such mutants from albeit immunocompromised patients with clinical
436 symptoms calls into question the role of active CDT in the pathogenesis of
437 campylobacteriosis. That CDT is not required for effective colonization of the avian
438 intestine was supported by results from this study using the experimental challenge of
439 one-day-old chicks with a defined *cdt* mutant and of the mammalian intestine by
440 previously published work using a *cdtB* mutant (32). However, without a suitable
441 animal model, the role of CDT in disease is more difficult to determine. Two out of
442 the three clinical infections with CDT-negative strains, were in patients with

443 underlying problems, of cirrhosis and/or neutropenia, which could potentially induce
444 an immune compromised status and thus influence the outcome of that infection.
445 Interestingly, both patients developed campylobacter bacteraemia. However, in one
446 of these patients, from whom strain C37596 was isolated, an earlier faecal isolate
447 (C37533) was also recovered during a period of acute enteritis and had the same
448 mutation as the blood isolate. The isolation of CDT negative faecal strains (C37533,
449 99/373) from patients with enteric disease, indicates that CDT expression is not
450 essential for the production of enteric disease in humans. Mechanisms other than CDT
451 expression may be involved in the outcome of campylobacteriosis. Similar large
452 deletions have been described in the toxin genes of other bacteria without obvious
453 consequences for disease outcome. For example large deletions (1.18 kb and 5.08 kb)
454 have been reported in the vacuolating cytotoxin (*vacA*) of *Helicobacter pylori* (15)
455 and yet these isolates were still associated with gastritis and peptic ulceration.
456 The second type of naturally occurring mutation identified in CDT-negative strains (4
457 out of 147 strains tested) from various sources (poultry processing plant, broiler,
458 sheep and a human), were point mutations resulting in at least 3 amino acid
459 substitutions. As RT-PCR demonstrated that the *cdt* genes of these strains were
460 transcribed, we hypothesised that these mutations resulted in the loss of CDT activity.
461 This hypothesis was further supported by complementation and site directed
462 mutagenesis studies in which the CDT activity was restored by *cdt* genes from a
463 CDT-positive strain.
464 Because CDT is a tripartite protein, in which the CdtA and C subunits are required for
465 the delivery of the CdtB subunit containing the enzymatically active site associated
466 with toxin activity (18), non-synomonous point mutations in these genes may have
467 different effects on phenotype. The comparisons of the sequences within this group of

468 strains suggest that the minimal set of mutations required to induce naturally
469 occurring CDT-negativity were represented in strain EF. In this strain a mutation
470 resulting in the replacement of alanine with valine, at codon 88 was detected in CdtA.
471 This change was also conserved in the other two CDT-negative strains within this
472 group. As both amino acids are aliphatic, hydrophobic and have non-polar side chains,
473 this transition is unlikely to substantially affect the structure or folding of the
474 polypeptide chain. Moreover, alignment of CdtA amino acid sequences from
475 *Haemophilus* and *Actinobacillus* species with that from strain EF, also showed a
476 valine at this position (data not shown) and as both these bacterial species produce
477 active CDT, it appears that this polymorphism within the CdtA subunit has little
478 consequence for the activity of this toxin. The mutation at codon 167 of CdtC
479 resulted in the replacement of isoleucine with asparagine and was found in an
480 additional *C. jejuni* strain 99/373, a human isolate. This results in a change from a
481 non-polar hydrophobic residue to a hydrophilic polar residue. Alignment of the CdtC
482 amino acid sequence from *Helicobacter hepaticus* with that from *C. jejuni* 11168
483 shows that the two sequences are identical at this position. This substitution may
484 therefore have possible a role in the lack of CDT activity in *C. jejuni* EF.
485 Moreover, there were two potentially important non-synonomous changes in *cdtB* in
486 this group of CDT-negative strains. All demonstrated a substitution of methionine by
487 threonine at CdtB-120. Although this could potentially influence the 3-dimensional
488 structure of CdtB, alignment of the amino acid sequences from other bacterial species
489 shows considerable variability at this position (Fig. 3.), suggesting that this mutation
490 in CdtB would have little, if any, consequence for CDT activity. In contrast, the
491 substitution of proline with serine at CdtB-95, appeared more important. Proline at
492 Cdt-95 appears a highly conserved residue (Fig. 3.) and its role in CDT activity has

493 now been confirmed using site directed mutagenesis. Interestingly, CDT is known to
494 have similarities with diphosphodiesterases, the active sites of which have been
495 identified (17, 20). However, neither CdtB-95 nor any other of the mutations
496 identified in *C. jejuni* EF fell into the conserved or functionally important residues
497 predicted for such enzymes. Although, CdtB-95 is clearly essential for CDT activity,
498 whether, any of the observed or, or as yet unidentified, mutations within the *cdt* locus
499 could explain the observed variability in levels of CDT activity detectable *in vitro* is
500 unknown.
501 For toxins expressed by related organisms, such as the VacA of *Helicobacter pylori*
502 (9, 29), the immunogenicity of the toxin during infection, as determined by western
503 blotting, is considered both evidence of *in vivo* expression and importance as a marker
504 of pathogenicity. Unfortunately, western blotting of *C. jejuni* cell preparations using
505 anti-CDT antibodies from human campylobacteriosis patient sera proved
506 unsuccessful. Therefore, we developed a CDT neutralization assay to detect serum
507 anti-CDT antibodies. This assay was validated using serum from a rabbit
508 hyperimmunised with the whole cells of CDT-expressing *C. jejuni* strain 81116. This
509 antiserum completely neutralized the CDT activity of this strain. The specificity of
510 these neutralizing antibodies was then demonstrated using rabbit antiserum directed
511 against strain C37596 that contained the large deletions in the *cdt* locus. This
512 antiserum failed to neutralize the toxin activity of *C. jejuni* 81116, 11168 and 81-176.
513 In contrast antiserum directed against strain EF, containing the point mutations partly
514 neutralized this activity. Comparison of the neutralizing capacity of the anti *C. jejuni*
515 81116 antisera against several strains suggests that the observed variation may be due
516 to a quantitative rather than a qualitative variation in toxin titre (due to coding region
517 differences), which may be under some regulatory control.

518 This CDT neutralization assay was then used to investigate the presence of
519 neutralizing antibodies in the sera of patients recovering from campylobacteriosis.
520 Because of the limited availability of such human sera, and the large volumes of sera
521 required for such studies, a pool of 6 patients' sera was used and compared with a
522 pool of 6 sera from donors with no laboratory confirmed history of enteric disease.
523 Previous studies using ELISA and western blotting had already demonstrated the
524 presence of specific anti-campylobacter antibodies in the sera from the individual
525 patients and the absence of these antibodies in the sera from the individual control
526 donors (4, 5). The results clearly demonstrated the complete neutralization of CDT
527 activity by the pooled sera from the campylobacteriosis patients. In contrast, the level
528 of neutralization by the pooled sera from the control donors was significantly lower
529 ($p=0.001$). These results suggest that CDT is expressed by *C. jejuni* during human
530 enteric infection and is antigenic, and that antibodies directed against this antigen can
531 neutralize the toxin activity.
532 Finally, the neutralization assay was also used to investigate CDT immunogenicity
533 during chicken colonization. This absence of detectable neutralizing antibodies,
534 despite a substantial antibody response in colonized chickens (Fig. 9) (3) and the
535 demonstrable expression of CDT in the avian gut, was surprising but suggests that
536 CDT is not antigenic in chickens. The reason for this is currently under investigation
537 but may reflect host-specific differences in immune responsiveness particularly as the
538 colonization model uses young chicks. It is possible that non-neutralizing antibodies
539 are induced. However, it is well recognised that colonization in chickens, unlike
540 humans, is asymptomatic. Thus the relationship between the lack of detectable
541 immunogenicity of CDT in the chicken and the absence of a disease outcome of

542 colonization may be a valuable future approach to the investigation of host-specific
543 differences in campylobacter infection.
544 In summary CDT remains the only clearly identified toxin in the genome sequence of
545 *C. jejuni* (27). Nevertheless, the recovery, albeit rarely, of naturally occurring CDT-
546 negative *C. jejuni* strains raises questions about the role of this toxin in
547 Campylobacter biology. However, infected patients, with disease symptoms, elicited
548 circulating and neutralizing antibodies directed against this toxin during infection.
549 Moreover, the absence of similar antibodies in colonized, asymptomatic chickens
550 suggests that the role of this toxin in disease requires considerable further
551 investigation.
552
553
554
555 **Acknowledgements:**
556 Many thanks to Jenny Frost (Health Protection Agency, Colindale), Exeter Public
557 Health Laboratories and CAMPYNET for providing some of the isolates used in this
558 study.
559 The work described in this paper was funded by the Department for Environment,
560 Food and Rural Affairs (Defra), Great Britain.

561 Table 1 – Primers and probes used in study

562

Primer or Probe	5'- 3' oligonucleotide sequence	Amplicon size	Comments
Cdt-F Cdt-R	gcggaaaattataatgaaattta gcaaggggctattccaaagc	2143bp	Amplification of *cdtABC* genes *
C7-F-Del C7-R-Del	cggatgagccttgcaaattc gatcaatcctcgctttaaaga	700 bp	Amplification of region between *cdtA* and *cdtB*
DS18‡ DS15‡	ccttgtgatggcaagcaatc acactccatttgctttctg	300 bp	Amplification of region between *cdtA* and *cdtB*‡
LC-T-F † LC-T-R †	gatatcttaatgatacaagaagc ctacatcaacgcgagaataa	162 bp	Amplification of region encompassing CdtB codon 95
LC-T-Probe †	cy5 - taaaaaccctatcagaccttga - biotin		Hybridises to CdtB codon 95
ClCdtABC-F ClCdtABC-R	ccaaagggtctttccaagag aaattattatagagcaaggtaaattac	2493 bp	Amplification of *cdtA*, *cdtB* and *cdtC* including promoter region[c]
SDMT1291-F SDMT1291-R	ggaatttaggaactctttcaaggtctga tagggtttttatttattattctcg cgagaataataaataaaaaccctatca gaccttgaaagagttcctaaattcc		Site directed mutagenesis primers

* *cdt* gene primers were designed to amplify a product from 41 nucleotides upstream of *cdtA*, including all of *cdtB* and the first 486 nucleotides of *cdtC*.

† LightCycler Primers and Probe were synthesised by MWG-Biotech UK Ltd (UK).

‡ RT-PCR primers used were taken from Hickey *at al*. (14).

563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580 581 582 583 584 585

586 **Figure legends:**

587

588 FIG. 1. Amplified products of *cdtA, B, and C* of selected strains, with known *in vitro*

589 CDT activity, using primers cdt-F and cdt-R.

590

591 FIG. 2. Schematic diagram of the *cdtABC* of *C. jejuni* 81-176 illustrating the locations

592 of the 667 and 50 bp deletions in strains C37596, C37533 and C35926. Arrows

593 indicate direction of transcription

594

595 FIG. 3. Alignment of the CdtB amino acid sequences of *C. jejuni* strains EF, 11168

596 and other bacterial species. Shaded areas highlight amino acids identical to *C. jejuni*

597 11168. Accession numbers are as follows: *C. jejuni* EF - AY445094, *C. jejuni* 11168

598 CdtB - CAB72564, *C. upsaliensis* CdtB - AAF98364, *A. actinmyetemcomitians* CdtB

599 - AAC70898. *H. ducreyi* CdtB - AAB57726, *H. hepaticus* CdtB - AAF19158 and *E.*

600 *coli* CdtB -2010282B. Asterisks indicate point mutations found in *C. jejuni* EF. The

601 amino acids are numbered continuously on the left.

602

603 FIG. 4. RT-PCR to detect the expression of CDT in *C. jejuni* EF and 11168, using

604 primers DS15 and DS18 to amplify a 450 bp region overlapping *cdtA* and *cdtB* (Panel

605 A) and strains, 99/373, S58 and 99/12 (Panel B). Lanes A – reverse transcribed RNA

606 sample, lanes B- RT negative controls; lanes C, DNA controls. Lanes containing a 1

607 kb ladder were included (MW).

608

609 FIG. 5. Melting curve analyses by LightCycler in 20 selected *C. jejuni* strains,

610 including EF, for the detection of polymorphism at CdtB-95. A negative control is

611 included. Vertical lines represent the presence of proline (codon - cct) (blue) (as

612 represented by strain 81116), serine (codon - tct) (green) (as represented by strains

613 EF, S58 and 99/373) or more than one bp mismatch (orange) (as represented by strain

614 99/12) at CdtB-95. A no template control (NTC) was used in each run.

615

616 FIG. 6. CDT activities of *C.jejuni* strains 99/68 and EF, complemented with a 2.4 kb

617 region from *C.jejuni* 11168 containing the *cdt* genes to give 99/68 pCDT and EF

618 pCDT, and the same strains in which site directed mutagenesis was performed to

619 replace proline at CdtB-95 with serine generating 99/68pCDT^{P95S} and EF pCDT^{P95S}.

620 Assays were performed in triplicate and *C. jejuni* 11168 was used as a positive

621 control.

622

623 FIG. 7. RT-PCR of total RNA extracted from the caecal contents of chickens

624 colonized with *C. jejuni* strain C289/6 (CDT-positive) and from campylobacter-free

625 birds, using primers DS15 and DS18 to detect the expression of *cdtA* and *cdtB*. *C.*

626 *jejuni* 11168 grown *in vitro* was used as the positive control. Lanes A: reverse

627 transcribed RNA sample; lanes B: RT negative controls; lanes C: DNA controls.

628 Lanes containing a 1 kb ladder were included (MW).

629

630 FIG. 8. Lysates of *C. jejuni* strains 81116, 81-176 and 11168 were treated with rabbit

631 antisera directed against strain 81116 whole cells (R12) ▩ , strain EF (R43) ■ ,

632 pooled campylobacteriosis patient sera ▦ , pooled experimentally colonized

633 chicken sera (||) prior to CDT activity assays. Sera from pooled normal human blood

634 donors ▧ as well as sera from pre-immunized rabbits (§) and uncolonized chickens

635 (data not shown) were used as controls. The lysates were tested for CDT activity and
636 the % neutralization determined by comparison with untreated lysates.
637
638 FIG. 9. Western blots showing reactivity of sera from human blood donors (A),
639 human campylobacterosis patients (B), uncolonized chickens (C) and experimentally
640 *C. jejuni* 81116 colonised chickens (D) to total protein profiles of *C. jejuni* 11168 and
641 81-176. Molecular mass markers are shown on right of figure (MW).
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659

660

661

662

663

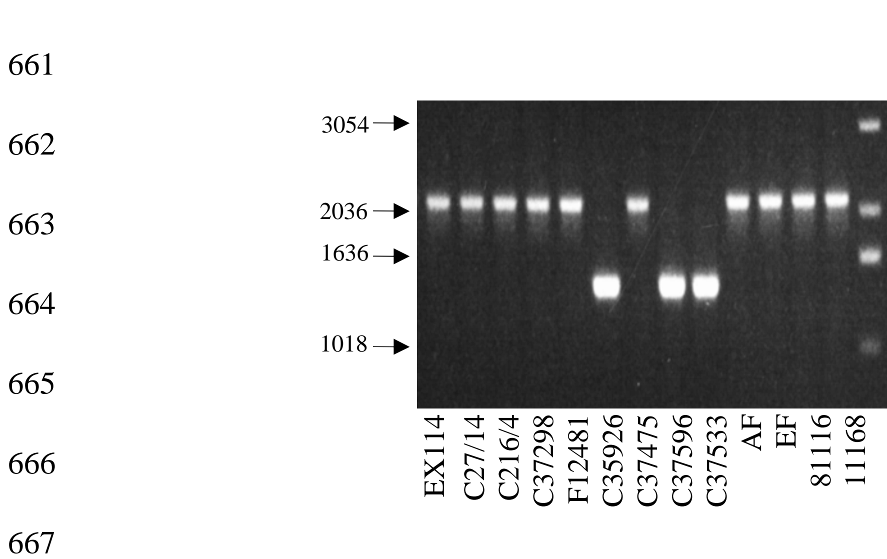
664

665

666

667

668



669 FIG. 1. Amplified products of *cdtA, B, and C* of selected strains, with known *in vitro*

670 CDT activity, using primers cdt-F and cdt-R.

671

672

673

674

675

676

677

678

679

680

681

682

683

684

685

686 cdtA cdtB cdtC 81176

687 667 bp 50 bp

688

689 FIG. 2. Schematic diagram of the *cdtABC* of *C. jejuni* 81-176 illustrating the locations

690 of the 667 and 50 bp deletions in strains C37596, C37533 and C35926. Arrows

691 indicate direction of transcription

692

693

694

695

696

697

698

699

700

701

702

703

704

705

706

707

708

709

710

711

712

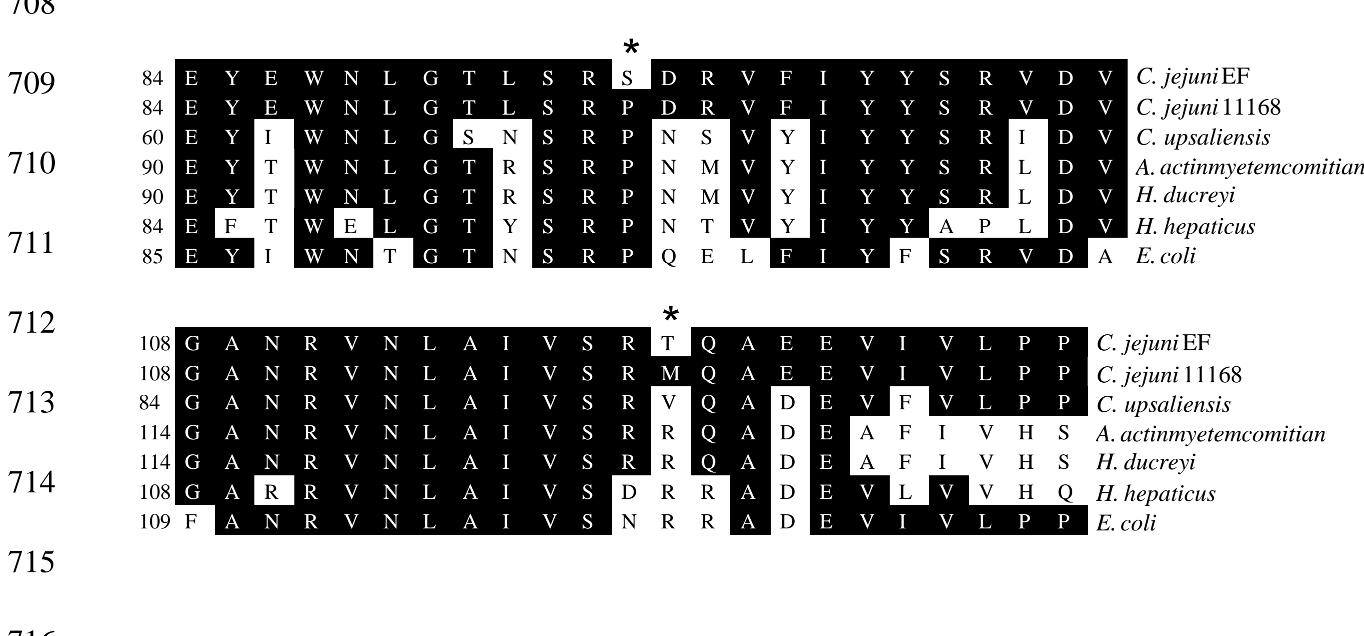
713

714

715

716

717



718 FIG. 3. Alignment of the CdtB amino acid sequences of *C. jejuni* strains EF, 11168
719 and other bacterial species. Shaded areas highlight amino acids identical to *C. jejuni*
720 11168. Accession numbers are as follows: *C. jejuni* EF - AY445094, *C. jejuni* 11168
721 CdtB - CAB72564, *C. upsaliensis* CdtB - AAF98364, *A. actinmyetemcomitians* CdtB
722 - AAC70898. *H. ducreyi* CdtB - AAB57726, *H. hepaticus* CdtB - AAF19158 and *E.*
723 *coli* CdtB -2010282B. Asterisks indicate point mutations found in *C. jejuni* EF. The
724 amino acids are numbered continuously on the left.

725

726

727

728

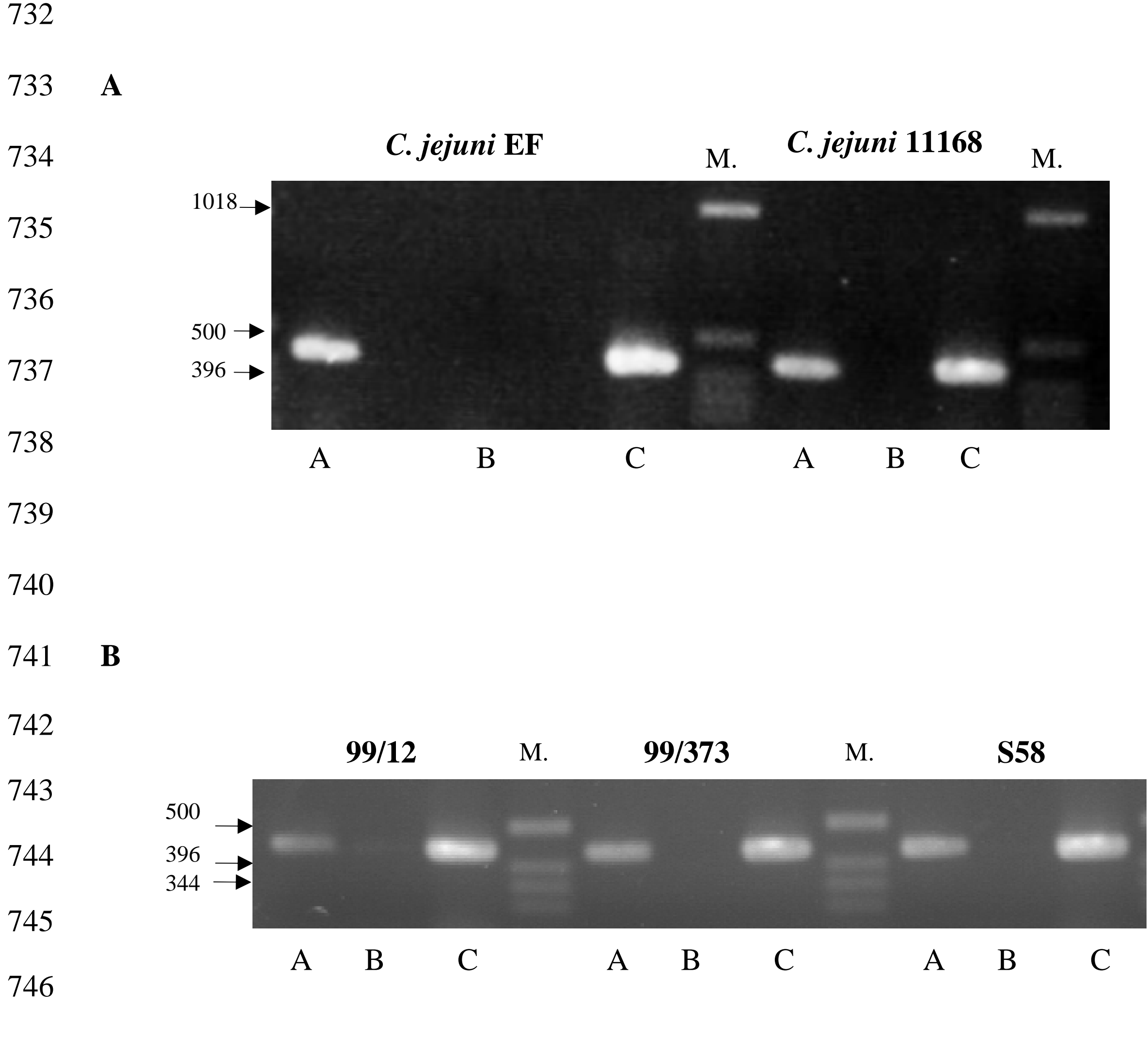
729

730

731

732

733 **A**



748 FIG. 4. RT-PCR to detect the expression of CDT in *C. jejuni* EF and 11168, using
749 primers DS15 and DS18 to amplify a 450 bp region overlapping *cdtA* and *cdtB* (Panel
750 A) and strains, 99/373, S58 and 99/12 (Panel B). Lanes A – reverse transcribed RNA
751 sample, lanes B- RT negative controls; lanes C, DNA controls. Lanes containing a 1
752 kb ladder were included (M).

753

754

755

756

757

758

759

760

761

762

763

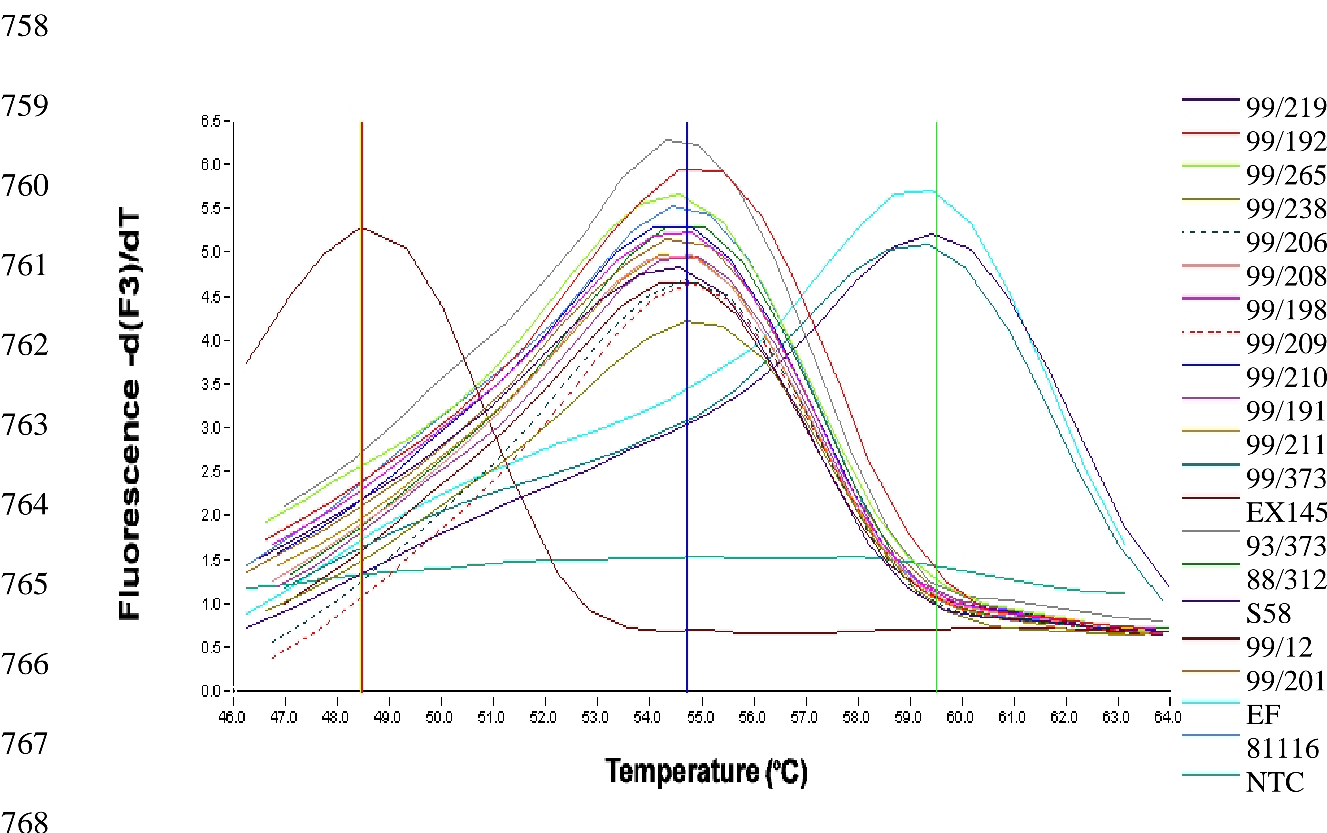
764

765

766

767

768



769

770 FIG. 5. Melting curve analyses by LightCycler in 20 selected *C. jejuni* strains,

771 including EF, for the detection of polymorphism at CdtB-95. A negative control is

772 included. Vertical lines represent the presence of proline (codon - cct) (blue) (as

773 represented by strain 81116), serine (codon - tct) (green) (as represented by strains

774 EF, S58 and 99/373) or more than one bp mismatch (orange) (as represented by strain

775 99/12) at CdtB-95. A no template control (NTC) was used in each run.

776

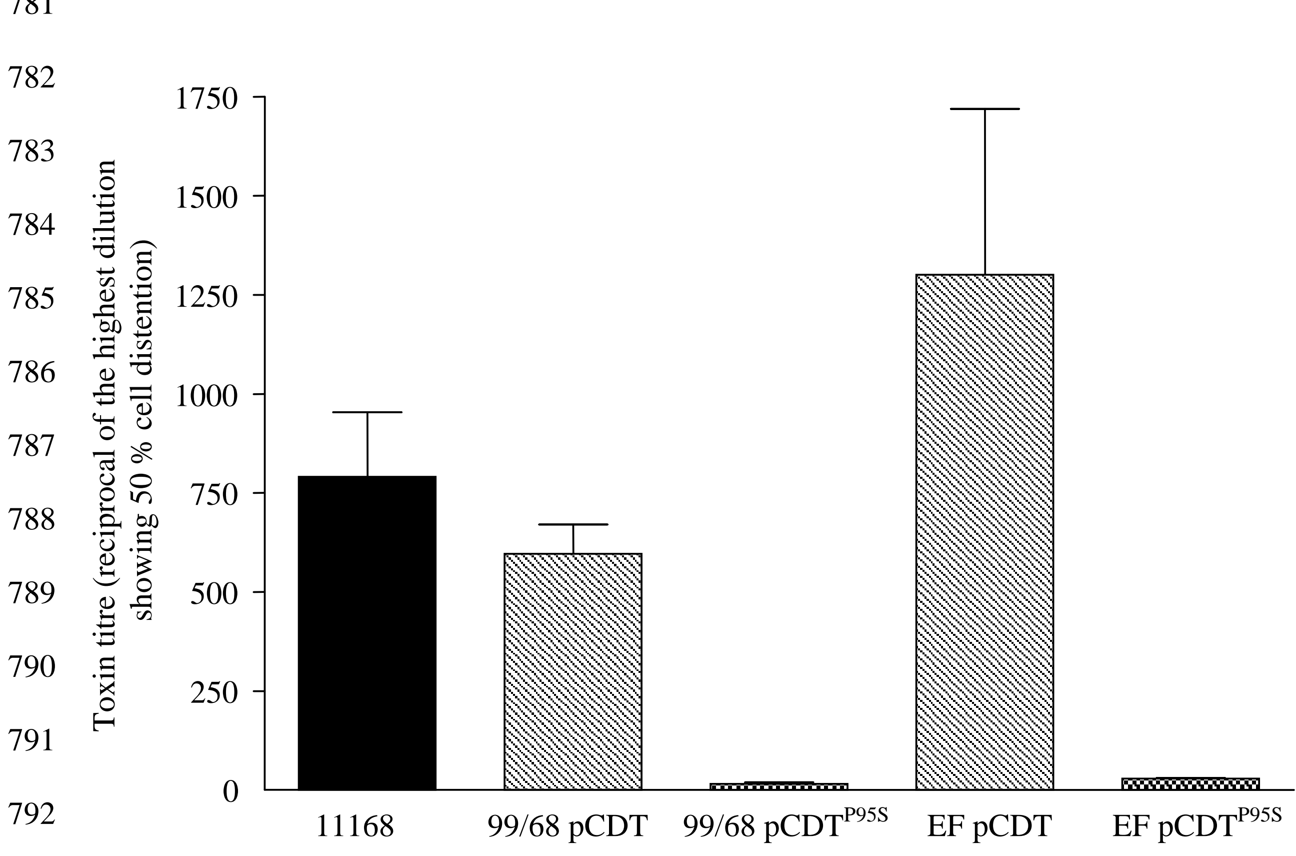
777

778

779

780

781
782
783
784
785
786
787
788
789
790
791
792

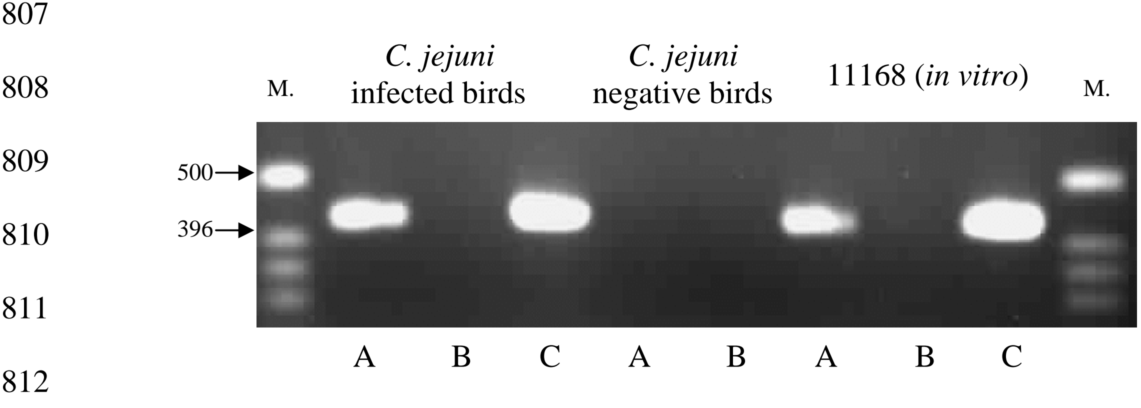


793
794

795 FIG. 6. CDT activities of *C.jejuni* strains 99/68 and EF, complemented with a 2.4 kb
796 region from *C.jejuni* 11168 containing the *cdt* genes to give 99/68 pCDT and EF
797 pCDT, and the same strains in which site directed mutagenesis was performed to
798 replace proline at CdtB-95 with serine generating 99/68pCDT^{P95S} and EF pCDT^{P95S}.
799 Assays were performed in triplicate and *C. jejuni* 11168 was used as a positive
800 control.

801
802
803
804
805

806
807
808
809
810
811
812
813



814 FIG. 7. RT-PCR of total RNA extracted from the caecal contents of chickens
815 colonized with *C. jejuni* strain C289/6 (CDT-positive) and from campylobacter-free
816 birds, using primers DS15 and DS18 to detect the expression of *cdtA* and *cdtB*. *C.*
817 *jejuni* 11168 grown *in vitro* was used as the positive control. Lanes A: reverse
818 transcribed RNA sample; lanes B: RT negative controls; lanes C: DNA controls.
819 Lanes containing a 1 kb ladder were included (M).
820
821
822
823
824
825
826
827
828
829
830

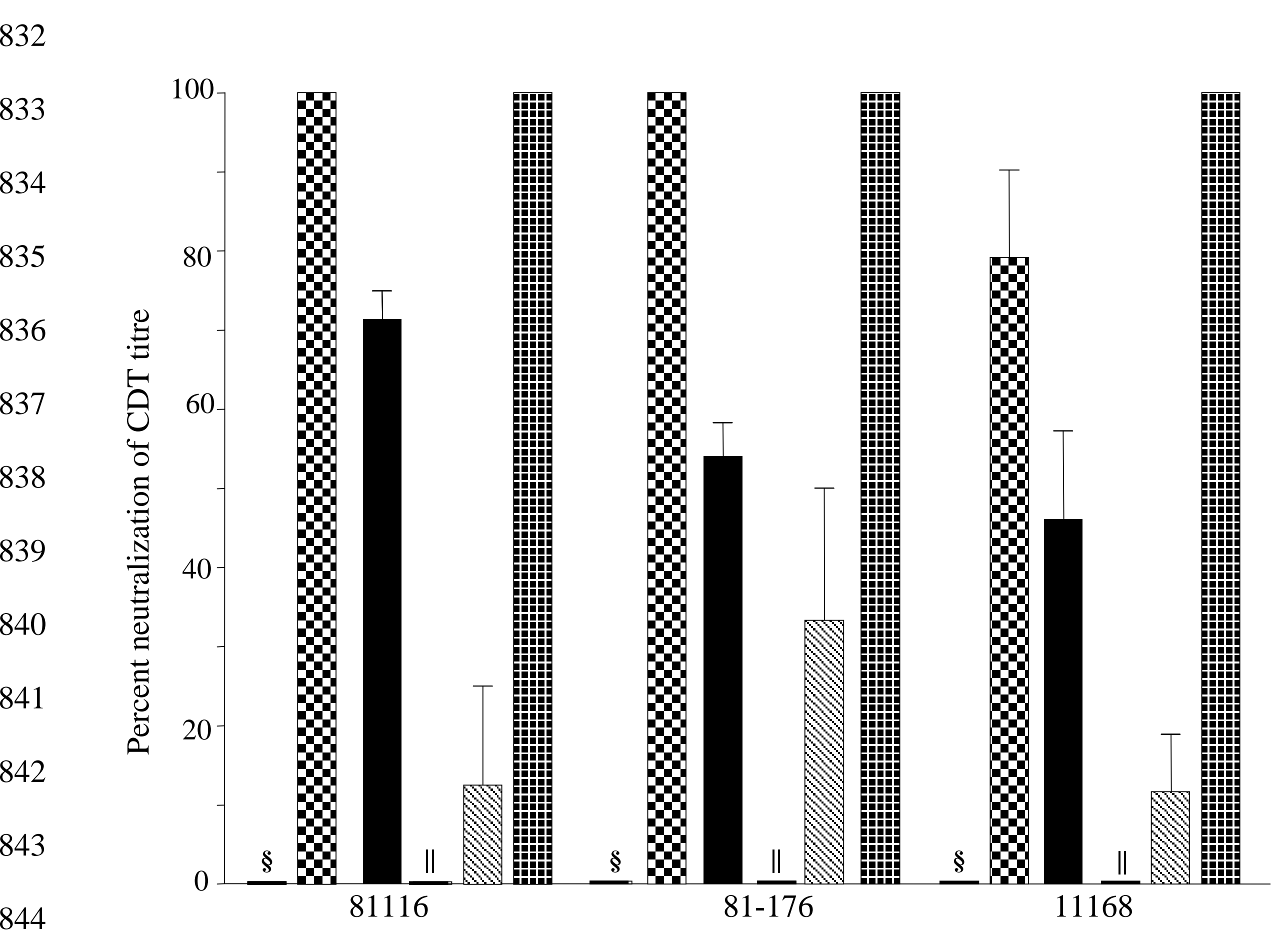
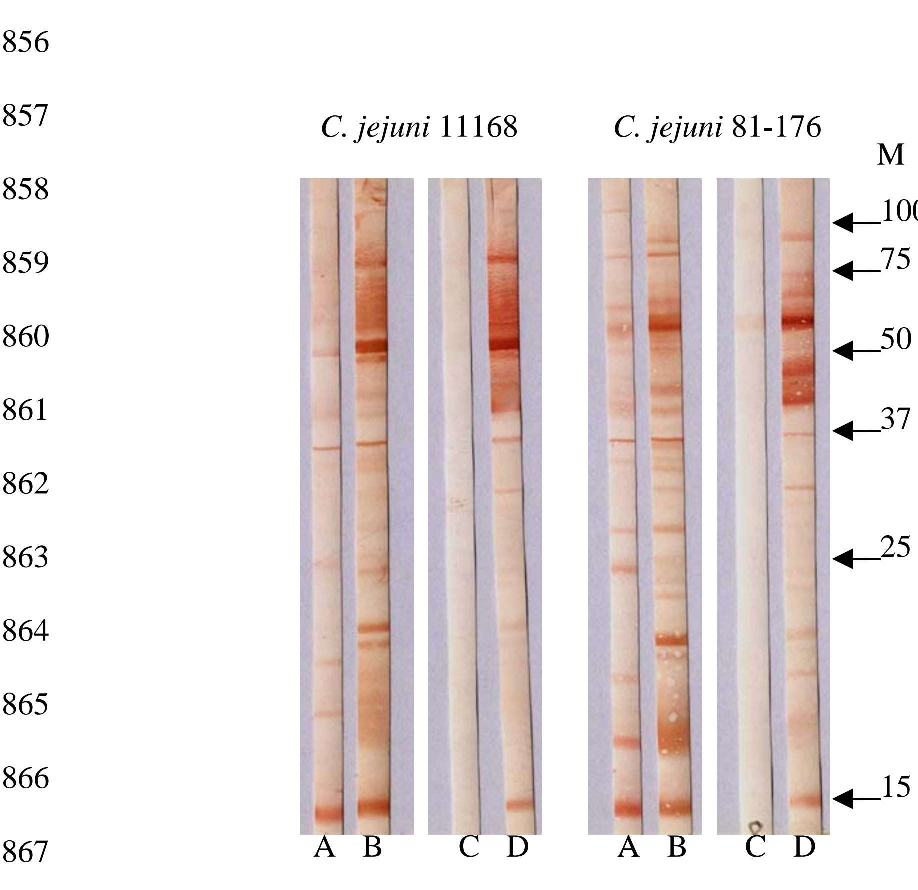


FIG. 8. Lysates of *C. jejuni* strains 81116, 81-176 and 11168 were treated with rabbit antisera directed against strain 81116 whole cells (R12), strain EF (R43), pooled campylobacteriosis patient sera, pooled experimentally colonized chicken sera (||) prior to CDT activity assays. Sera from pooled normal human blood donors as well as sera from pre-immunized rabbits (§) and uncolonized chickens (data not shown) were used as controls. The lysates were tested for CDT activity and the % neutralization determined by comparison with untreated lysates.

855

856

857



858

859

860

861

862

863

864

865

866

867

868

869

870 FIG.9. Western blots showing reactivity of sera from human blood donors (A),

871 human campylobacterosis patients (B), uncolonized chickens (C) and experimentally

872 *C. jejuni* 81116 colonised chickens (D) to total protein profiles of *C. jejuni* 11168 and

873 81-176. Molecular mass markers are shown on right of figure (M).

874

875

876

877

878

879

880 **References:**

881 1. **Ausubel, S. F., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A.**
882 **Smith, and K. Struhl.** 1994. Current Protocols In Molecular Biology. John
883 Wiley & Sons, New York.

884 2. **Bang, D. D., F. Scheutz, P. Ahrens, K. Pedersen, J. Blom, and M. Madsen.**
885 2001. Prevalence of cytolethal distending toxin (*cdt*) genes and CDT
886 production in *Campylobacter spp*. isolated from Danish broilers. J Med
887 Microbiol **50:**1087-94.

888 3. **Cawthraw, S., R. Ayling, P. Nuijten, T. Wassenaar, and D. G. Newell.**
889 1994. Isotype, specificity, and kinetics of systemic and mucosal antibodies to
890 *Campylobacter jejuni* antigens, including flagellin, during experimental oral
891 infections of chickens. Avian Dis **38:**341-9.

892 4. **Cawthraw, S. A., R. A. Feldman, A. R. Sayers, and D. G. Newell.** 2002.
893 Long-term antibody responses following human infection with *Campylobacter*
894 *jejuni*. Clin Exp Immunol **130:**101-6.

895 5. **Cawthraw, S. A., L. Lind, B. Kaijser, and D. G. Newell.** 2000. Antibodies,
896 directed towards *Campylobacter jejuni* antigens, in sera from poultry abattoir
897 workers. Clin Exp Immunol **122:**55-60.

898 6. **Comayras, C., C. Tasca, S. Y. Peres, B. Ducommun, E. Oswald, and J. De**
899 **Rycke.** 1997. *Escherichia coli* cytolethal distending toxin blocks the HeLa cell
900 cycle at the G2/M transition by preventing cdc2 protein kinase
901 dephosphorylation and activation. Infect Immun **65:**5088-95.

902 7. **Cope, L. D., S. Lumbley, J. L. Latimer, J. Klesney-Tait, M. K. Stevens, L.**
903 **S. Johnson, M. Purven, R. S. Munson, Jr., T. Lagergard, J. D. Radolf, and**

904 **E. J. Hansen.** 1997. A diffusible cytotoxin of *Haemophilus ducreyi*. Proc Natl
905 Acad Sci U S A **94:**4056-61.
906 8. **Cortes-Bratti, X., E. Chaves-Olarte, T. Lagergard, and M. Thelestam.**
907 1999. The cytolethal distending toxin from the chancroid bacterium
908 *Haemophilus ducreyi* induces cell-cycle arrest in the G2 phase. J Clin Invest
909 **103:**107-15.
910 9. **Cover, T. L., P. Cao, U. K. Murthy, M. S. Sipple, and M. J. Blaser.** 1992.
911 Serum neutralizing antibody response to the vacuolating cytotoxin of
912 *Helicobacter pylori*. J Clin Invest **90:**913-8.
913 10. **Deming, M. S., R. V. Tauxe, P. A. Blake, S. E. Dixon, B. S. Fowler, T. S.**
914 **Jones, E. A. Lockamy, C. M. Patton, and R. O. Sikes.** 1987. *Campylobacter*
915 enteritis at a university: transmission from eating chicken and from cats. Am J
916 Epidemiol **126:**526-34.
917 11. **Eyigor, A., K. A. Dawson, B. E. Langlois, and C. L. Pickett.** 1999.
918 Cytolethal distending toxin genes in *Campylobacter jejuni* and *Campylobacter*
919 *coli* isolates: detection and analysis by PCR. J Clin Microbiol **37:**1646-50.
920 12. **Eyigor, A., K. A. Dawson, B. E. Langlois, and C. L. Pickett.** 1999.
921 Detection of cytolethal distending toxin activity and *cdt* genes in
922 *Campylobacter* spp. isolated from chicken carcasses. Appl Environ Microbiol
923 **65:**1501-5.
924 13. **Gosciniak, G., A. Przondo-Mordarska, B. Iwanczak, and E. Poniewierka.**
925 2000. Neutralization of vacuolating activity of cytotoxin by serum antibodies
926 of *Helicobacter pylori* infected patients. Acta Microbiol Pol **49:**113-20.
927 14. **Hickey, T. E., A. L. McVeigh, D. A. Scott, R. E. Michielutti, A. Bixby, S.**
928 **A. Carroll, A. L. Bourgeois, and P. Guerry.** 2000. *Campylobacter jejuni*

929 cytolethal distending toxin mediates release of interleukin-8 from intestinal
930 epithelial cells. Infect Immun **68:**6535-41.

931 15. **Ito, Y., T. Azuma, S. Ito, H. Suto, H. Miyaji, Y. Yamazaki, Y. Kohli, and**
932 **M. Kuriyama.** 1998. Full-length sequence analysis of the *vacA* gene from
933 cytotoxic and noncytotoxic *Helicobacter pylori*. J Infect Dis **178:**1391-8.

934 16. **Johnson, W. M., and H. Lior.** 1988. A new heat-labile cytolethal distending
935 toxin (CLDT) produced by *Campylobacter* spp. Microb Pathog **4:**115-26.

936 17. **Lara-Tejero, M., and J. E. Galan.** 2000. A bacterial toxin that controls cell
937 cycle progression as a deoxyribonuclease I-like protein. Science **290:**354-7.

938 18. **Lara-Tejero, M., and J. E. Galan.** 2001. CdtA, CdtB, and CdtC form a
939 tripartite complex that is required for cytolethal distending toxin activity.
940 Infect Immun **69:**4358-65.

941 19. **Mao, X., and J. M. DiRienzo.** 2002. Functional studies of the recombinant
942 subunits of a cytolethal distending holotoxin. Cell Microbiol. **4:**245-55.

943 20. **Matsuo, Y., A. Yamada, K. Tsukamoto, H. Tamura, H. Ikezawa, H.**
944 **Nakamura, and K. Nishikawa.** 1996. A distant evolutionary relationship
945 between bacterial sphingomyelinase and mammalian DNase I. Protein sci
946 **5:**2459-67.

947 21. **Mayer, M. P., L. C. Bueno, E. J. Hansen, and J. M. DiRienzo.** 1999.
948 Identification of a cytolethal distending toxin gene locus and features of a
949 virulence-associated region in *Actinobacillus actinomycetemcomitans*. Infect
950 Immun **67:**1227-37.

951 22. **Newell, D. G.** 2001. Animal models of *Campylobacter jejuni* colonization and
952 disease and the lessons to be learned from similar *Helicobacter pylori* models.
953 Symp Ser Soc Appl Microbiol**:**57S-67S.

954 23. **Newell, D. G., H. McBride, and A. D. Pearson.** 1984. The identification of
955 outer membrane proteins and flagella of *Campylobacter jejuni*. J Gen
956 Microbiol **130:**1201-8.
957 24. **Newell, D. G., J. E. Shreeve, M. Toszeghy, G. Domingue, S. Bull, T.**
958 **Humphrey, and G. Mead.** 2001. Changes in the carriage of Campylobacter
959 strains by poultry carcasses during processing in abattoirs. Appl Environ
960 Microbiol. **67:**2636-40.
961 25. **Okuda, J., M. Fukumoto, Y. Takeda, and M. Nishibuchi.** 1997.
962 Examination of diarrheagenicity of cytolethal distending toxin: suckling
963 mouse response to the products of the *cdtABC* genes of *Shigella dysenteriae*.
964 Infect Immun **65:**428-33.
965 26. **Okuda, J., H. Kurazono, and Y. Takeda.** 1995. Distribution of the
966 cytolethal distending toxin A gene (*cdtA*) among species of *Shigella* and
967 *Vibrio*, and cloning and sequencing of the *cdt* gene from *Shigella dysenteriae*.
968 Microb Pathog **18:**167-72.
969 27. **Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D.**
970 **Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K.**
971 **Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail,**
972 **M. A. Rajandream, K. M. Rutherford, A. H. van Vliet, S. Whitehead, and**
973 **B. G. Barrell.** 2000. The genome sequence of the food-borne pathogen
974 *Campylobacter jejuni* reveals hypervariable sequences. Nature **403:**665-8.
975 28. **Peres, S. Y., O. Marches, F. Daigle, J. P. Nougayrede, F. Herault, C.**
976 **Tasca, J. De Rycke, and E. Oswald.** 1997. A new cytolethal distending toxin
977 (CDT) from *Escherichia coli* producing CNF2 blocks HeLa cell division in
978 G2/M phase. Mol Microbiol **24:**1095-107.

979 29. **Perez-Perez, G. I., W. R. Brown, T. L. Cover, B. E. Dunn, P. Cao, and M.
980 J. Blaser.** 1994. Correlation between serological and mucosal inflammatory
981 responses to *Helicobacter pylori*. Clin Diagn Lab Immunol **1:**325-9.
982 30. **Pickett, C. L., D. L. Cottle, E. C. Pesci, and G. Bikah.** 1994. Cloning,
983 sequencing, and expression of the *Escherichia coli* cytolethal distending toxin
984 genes. Infect Immun **62:**1046-51.
985 31. **Pickett, C. L., E. C. Pesci, D. L. Cottle, G. Russell, A. N. Erdem, and H.
986 Zeytin.** 1996. Prevalence of cytolethal distending toxin production in
987 *Campylobacter jejuni* and relatedness of *Campylobacter* sp. *cdtB* gene. Infect
988 Immun **64:**2070-8.
989 32. **Purdy, D., C. M. Buswell, A. E. Hodgson, K. McAlpine, I. Henderson, and
990 S. A. Leach.** 2000. Characterisation of cytolethal distending toxin (CDT)
991 mutants of *Campylobacter jejuni*. J Med Microbiol **49:**473-9.
992 33. **Scott, D. A., and J. B. Kaper.** 1994. Cloning and sequencing of the genes
993 encoding *Escherichia coli* cytolethal distending toxin. Infect Immun **62:**244-
994 51.
995 34. **Skirrow, M. B.** 1977. *Campylobacter* enteritis: a "new" disease. Br Med J
996 **2:**9-11.
997 35. **Tauxe, R. E.** 1992. Epidemiology of *Campylobacter jejuni* infections in th
998 united states and other industrialized nations., p. 9-19. *In* I. Nachamkin, M. J.
999 Blaser & L. S. Tompkins (ed.), *Campylobacter jejuni*: Current Status and
1000 Future Trends. American Society for Microbiology, Washington, DC.
1001 36. **Wang, Y., and D. E. Taylor.** 1990. Chloramphenicol resistance in
1002 *Campylobacter coli*: nucleotide sequence, expression, and cloning vector
1003 construction. Gene **94:**23-8.

1004 37. **Wassenaar, T. M.** 1997. Toxin production by *Campylobacter* spp. Clin
1005 Microbiol Rev **10:**466-76.
1006 38. **Wassenaar, T. M., and M. J. Blaser.** 1999. Pathophysiology of
1007 *Campylobacter jejuni* infections of humans. Microbes Infect **1:**1023-33.
1008 39. **Wassenaar, T. M., B. N. Fry, and B. A. van der Zeijst.** 1993. Genetic
1009 manipulation of Campylobacter: evaluation of natural transformation and
1010 electro-transformation. Gene **132:**131-5.
1011 40. **Wassenaar, T. M., B. A. van der Zeijst, R. Ayling, and D. G. Newell.** 1993.
1012 Colonization of chicks by motility mutants of *Campylobacter jejuni*
1013 demonstrates the importance of flagellin A expression. J Gen Microbiol
1014 **139:**1171-5.
1015 41. **Whitehouse, C. A., P. B. Balbo, E. C. Pesci, D. L. Cottle, P. M. Mirabito,**
1016 **and C. L. Pickett.** 1998. *Campylobacter jejuni* cytolethal distending toxin
1017 causes a G2-phase cell cycle block. Infect Immun **66:**1934-40.
1018 42. **Young, V. B., K. A. Knox, and D. B. Schauer.** 2000. Cytolethal distending
1019 toxin sequence and activity in the enterohepatic pathogen *Helicobacter*
1020 *hepaticus*. Infect Immun **68:**184-91.