Cytolethal distending toxin (CDT)-Negative *Campylobacter jejuni* strains and anti-CDT neutralizing antibodies induced during human infection but not chicken colonization

Running title: Lack of CDT and host differences in neutralization.

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Summary

The cytolethal-distending toxin (CDT), of *Campylobacter jejuni* was detectable, using an *in vitro* assay in most, but not all, 24 strains tested. The reason for the absence of toxin activity in these naturally-occurring CDT-negative *C. jejuni* strains was then investigated at the genetic level. CDT is encoded by three highly conserved genes, *cdtA, B* and *C*. In the CDT-negative strains, 2 types of mutation were identified. The CDT activities of *C. jejuni* strains possessing both types of mutation were successfully complemented with the functional genes of *C. jejuni* 11168. The first type of mutation comprised a 667-bp deletion across *cdtA* and *cdtB* and considerable degeneration in the remainder of the *cdt* locus. Using a PCR technique to screen for this deletion, this mutation occurred in less than 3% of 147 human, veterinary and environmental strains tested. The second type of mutation involved at least 4 non-synonymous nucleotide changes but only the substitution of proline with serine at CdtB-95 was considered important to CDT activity. This was confirmed by site-directed mutagenesis. This type of mutation also occurred in less than 3% of strains as determined using a LightCycler biprobe assay.

The detection of two CDT-negative clinical isolates questioned the role of CDT in some cases of human campylobacteriosis. To determine if anti-CDT antibodies are produced using human infection, a toxin neutralization assay was developed and validated using rabbit antisera. Pooled human sera from infected patients neutralized the toxin indicating expression and immunogenicity during infection. However, no neutralizing antibodies were detected in colonized chickens despite the expression of CDT in the avian gut as indicated by RT-PCR.

Introduction

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

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

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

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

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

Materials and Methods

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

Chemically competent *E.coli* TOPO10F' (Invitrogen, Paisley, UK) and DH5αMCR (Invitrogen) strains were used for cloning and site directed mutagenesis studies, respectively.

C. jejuni strains were grown for 24 h at 42 ^{o}C under microaerobic conditions (7.5 % O_2, 7.5 % CO_2, 85 % N_2) on either Mueller-Hinton (M-H) or blood agar plates supplemented with 10% sheep blood, 50 μg/ml Actidione and selective antibiotics (Oxoid, Basingstoke, UK) (34). *C. jejuni* 81-176*cdt* was grown on media supplemented with 50 μg/ml kanamycin. *E. coli* was grown in Luria-Bertani medium (LB) under atmospheric conditions at 37 ^{o}C. Where necessary, LB medium was supplemented with 50 μg/ml ampicillin or 20 μg/ml chloramphenicol. The *C. jejuni* and *E. coli* strains were stored frozen at – 80 ^{o}C in 1 % (w/v) proteose peptone water containing 10 % (v/v) glycerol or in LB broth containing 50 % (v/v) glycerol respectively.

***In Vitro* HeLa cell cytolethal distending toxin (CDT) assay:** The assay used in this study was adapted from previously published CDT assays (14, 31). Cultured HeLa cells (ECACC, Porton Down, UK) were maintained in complete Eagle's Minimal Essential Medium (EMEM) with 10 % (v/v) foetal bovine serum, 1 % (v/v) L-glutamine, 1 % (v/v) non-essential amino acids and 0.5 % (v/v) gentamicin) at 37 ^{o}C in 5% CO_2. For the assay, HeLa cell concentrations were adjusted to 2 x 10^4 cells ml^{-1} and 150 μl of this suspension was added to each well of a flat-bottomed, tissue culture grade, 96-well plate. The cells were then cultured for 2-3 hours at 37 ^{o}C in 5% CO_2, prior to the addition of bacterial lysates.

Lysates were prepared from bacteria grown on M-H agar plates for 24 hours at 42 ^{o}C under microaerobic conditions and harvested into complete EMEM. The bacterial

suspensions were adjusted to OD_{550} 1.6, sonicated on ice (1.5 min at 30 amp with 6-sec pulses) (Vibra Cell, Sonics and Materials Inc, USA) and cell debris removed by centrifugation (6000 g for 10 min at 4 ^{o}C). The lysates were sterilised by filtration using 0.22 μm filters (Minisart, Sartorius, Germany). Undiluted and 1 in 10 dilutions of the bacterial lysates were added to HeLa cells and two-fold dilutions performed across each plate, which were then incubated for 5 days at 37 ^{o}C in 5 % CO_2. The HeLa cell monolayers were then fixed in 10 % (v/v) formalin, stained with 2.3 % (w/v) crystal violet and examined by light microscopy. The toxin titres were expressed as the reciprocal of the highest dilution that caused 50 % of cells to become distended, and adjusted by dividing the OD_{550} of bacterial sonic lysates. Toxin titre for each strain was tested in at least three independent assays blind.

CDT antibody neutralization assay: Hyperimmune rabbit antisera were produced against the whole organisms of *C. jejuni* strains 81116 (R12), EF (R43) and C37596 (R42) as previously described (23). Serum from 10 chickens experimentally colonized with *C. jejuni* 81116, at 9 weeks post-challenge, were collected as previously described (3). Sera pooled from 6 human patients, taken 2-4 weeks after campylobacter isolation (pooled positive sera) and 6 human donors, with no recorded enteric infection (pooled negative sera), were used in the neutralization assay. The collection, storage and characterisation of these sera has been described previously (4, 5).

All sera used were initially treated at 56 ^{o}C for 2 hours to inactivate complement, before use in the i*n vitro* HeLa cell CDT assays. Prior to the addition to the HeLa cells, 1/10 dilution of bacterial lysates were pre-incubated with R12 (1 in 50 dilution), R42 (1 in 30 dilution), R43 (1 in 30 dilution), pooled chicken sera (1 in 30 dilution) or

pooled human sera (1in 30 dilution), at 37 ^{o}C for 1 hour. Treated lysates were subsequently applied to the HeLa cells as described previously.

Statistical Analyses: Experimental results from at least 3 independent assays were transformed to log (x+0.5) for analysis. Mean titres of each treatment were compared to controls by two-way analysis of variance and wherever treatment differences were $p<0.05$, indicated statistical significance.

Western blotting: Whole-cell protein profiles of *C. jejuni* 11168 and 81-176 were prepared by separating on 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membrane for western blotting as described previously (3). Membrane strips of each strain were incubated with the human and chicken sera (1/30 dilution) used for the CDT neutralization studies, and bound antibodies detected using species-specific anti-immunoglobulin antisera conjugated to horse-radish peroxidase according to the manufacturers instructions (DakoCytomation Ltd, Cambridgeshire, UK and Amersham Biosciences UK Limited, Buckinghamshire, UK respectively).

Polymerase chain reaction (PCR) assay for *cdt* genes: Chromosomal DNA was isolated from strains by the CTAB/NaCl method (1). Recombinant Taq DNA polymerase (Invitrogen) was used for all PCR reactions according to the manufacturer's recommendations. Oligonucleotide primers used for PCR and sequencing reactions are given in Table 1 (Sigma Genosys, Haverhill, UK). PCR reactions were performed on a PE Applied Biosystems GeneAmp PCR 9700, with an

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

Cloning and DNA sequence analysis of *cdt* genes: PCR reactions were repeated using Advantage cDNA Polymerase Mix , containing 3'- 5' proof reading activity (BD Biosciences Clontech, Oxford, UK) according to the manufacturer's instructions. PCR products were then cloned into pCR2.1-TOPO® (TOPO-TA cloning kit, Invitrogen) and transformed into chemically competent *E. coli* TOPO10F' one shot® cells (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was isolated for analysis using a mini plasmid preparation kit (Qiagen Ltd, Crawley, UK) and the DNA inserts confirmed by restriction analysis. Inserts were sequenced using BigDye terminator cycle sequencing chemistry (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Sequenced products were separated on an ABI Prism 377 automated DNA sequencer (Applied Biosystems). DNA sequences were assembled and edited using SeqMan (DNAstar, Lasergene, Madison, USA) and ClustalV alignments to the published *C. jejuni* 81-176 *cdt*ABC genes (GenBank accession number U51121) were done in MegAlign 5.00 (DNAstar, Lasergene, Madison, USA).

Southern blot analysis: Genomic DNA (10 μg) was digested with the restriction endonuclease *Hind*III (Promega, Southampton, UK) overnight at 37 ^{o}C and used for Southern blot analysis using the CDP-star chemiluminescent detection reagent (Amersham Biosciences, Bucks UK) according to manufacturer's instructions.

RNA extraction and reverse transcriptase PCR (RT-PCR): Total RNA was isolated from *C. jejuni* strains and chicken caecal contents using Tri reagent (Sigma Aldrich, Poole, UK) according to the manufacturer's instructions. RNA was suspended in an appropriate amount of RNase free water and RNA concentrations estimated by spectrophotometry. RT-PCR was carried out using the high fidelity ProSTAR HF single-tube RT-PCR system (Stratagene, Texas, USA), according to the manufacturer's instructions. Since the *cdt* genes of *C. jejuni* strains are known to be expressed in a single mRNA transcript (14), RT-PCR primers DS15 and DS18 (Table 1) were used to amplify the region between *cdtA* and *cdtB*. RT-PCR was performed with an initial incubation step of 42 ^{o}C for 30 min, during which time cDNA was synthesised from the RNA template. The reverse transcriptase was inactivated and the cDNA denatured by incubation at 95 ^{o}C for 1 min, followed by an amplification reaction, comprising 40 cycles of 95 ^{o}C for 30 sec, 60 ^{o}C for 30 sec and 68 ^{o}C for 2 min. RT-PCR products were visualised on 1.2 % (w/v) agarose gels.

LightCycler BiProbe assay of *cdtB* polymorphism: For the LightCycler, PCR primers LC-T-F and LC-T-R (Table 1), were designed by alignment of the published sequence of the *C. jejuni* 11168 *cdtB* gene with the *cdtB* gene from *C. jejuni* strain EF, to amplify a 163 bp region encompassing codon 95 of *cdtB*. The polymorphism was detected by melting curve analysis of the probe LC-T-probe (Table 1) after PCR amplification. The probe was identical to the sequence of *C. jejuni* EF, containing a serine at codon 95 (tct). Amplification and probe hybridisation were performed in 20 µl reactions using the LightCycler DNA Master SYBR Green I kit (Roche Diagnositics Ltd, Lewes, UK) in a LightCycler instrument (Roche Diagnostics Ltd).

Typically reaction mixtures comprised 10 – 15 pmol DNA, 2 pmol of LC-T-F, 5 pmol LC-T-R, 5 pmol LC-T-Probe and 3 mM $MgCl_2$ in 1x LightCycler DNA master mix SYBR green mix. LightCycler PCR was performed for 50 cycles, with 15 s at 95 ^{o}C, 5s at 55 ^{o}C and 10s at 74 ^{o}C with a transition rate of 20 ^{o}C/s. Fluorescence was measured at a wavelength of 540 nm (F1 channel) at the end of each amplification step to monitor the accumulation of PCR product. Melting curve analysis was performed immediately after amplification by heating the product to 94 ^{o}C (20^{o}C/s), cooling to 40 ^{o}C for 10 s, and then heating to 85 ^{o}C (0.1^{o}C/s). The final heating step was performed under continuous fluorescence measurement. DNA from *C. jejuni* EF and 11168 were included as positive controls and a no template control was included in each run.

Complementation studies of CDT negative strains: Complementation of CDT-negative strains was achieved by the introduction of the *Campylobacter* shuttle vector vector, pUOA18 (36), expressing a 2.4-kb region from *C. jejuni* 11168 containing the *cdt* genes (41). The region, included 206 bp of upstream and 106 bp of downstream sequence, was amplified with ClCdtABC-F and ClCdtABC-R (Table 1) and initially cloned into pCR2.1 TOPO. These primers included *Bam*HI restriction enzyme sites at the 5' ends of both primers. Following *Bam*HI digestion and gel purification, the insert was ligated into dephosphorylated, *Bam*HI digested pUOA18, to make the construct pCDT, and transformed to DH5αMCR (Invitrogen). The construct was isolated from *E. coli* and electroporated into *C. jejuni* EF and 99/68, using a method adapted from Wassenaar *et al*. (39). Colonies that appeared on 20 mg/ml chloramphenicol medium were tested for CDT activity in the *in vitro* CDT assay.

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

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

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

Results

Identification and characterisation of CDT-negative *C. jejuni* strains: A panel of 24 selected *C. jejuni* strains were tested for CDT activity. A range of CDT-activities, from titres of <50 (n=13), 50-150 (n=4), 150-300 (n=2) and >300 (n=1), were observed but there was no obvious correlation with strain source (data not shown). Of the 24 strains tested 4 (C37596, C35926, C37533 and EF) had no detectable CDT activity. Two of these CDT-negative strains, C37596 and C35926, were isolated from the blood of independent campylobacteriosis patients (within the UK in 2000) with underlying medical problems, as discussed later. *C. jejuni* strain C37533 was isolated from the faeces of the same patient as isolate C37596. None of these strains were serotypable. The remaining CDT-negative strain (EF) was isolated from a chicken carcass in the chilling area of a poultry-processing plant.

The presence of *cdt*ABC genes in all 24 strains was determined by PCR. In 21 of the 24 strains, the expected PCR amplicon size of 2143 bp, generated with primers CdtF and CdtR, was observed (Fig. 1). However, in strains C37596, C35926 and C37533, which were CDT-negative, a smaller product of 1400 bp was detected. The PCR products of all 4 CDT-negative strains were cloned and sequenced. ClustalV alignment, to the published *cdtABC* sequence of *C. jejuni* 81-176 (Accession number U51121), revealed a 667-bp deletion between *cdtA* and *cdtB* and a separate 51-bp deletion within the *cdtB* genes of strains C37596, C35926 and C37533, consistent with the reduced PCR amplicon size observed by PCR (Fig. 2). The remainder of the sequences from these three strains had only 51% identity, which was due to additional indels (insertions-deletions) and substitutions throughout the remaining *cdtABC* gene sequence.

We next determined, by Southern blot hybridisation, whether the 667 bp deleted region between *cdtA* and *cdtB* was the result of recombination with another part of the genome or whether it was completely lost from the genome. Using primers specific for this region (C7del), a PCR product was generated from strain 11168, and used to probe *Hind*III-digested genomic DNA of strains C37596, C35926 and C37533. The results confirmed the absence of the region between *cdtA* and *cdtB* in the genomic DNA of these strains (data not shown).

To determine the prevalence of the 667 bp deletion in *C. jejuni* isolates, the DNA of an additional 123 randomly selected clinical and veterinary strains were screened by PCR using the primers Cdt-F and Cdt-R. All but one strain, 99/68, produced a PCR product of 2.14 kb. Strain 99/68, which was isolated from a broiler, produced an amplicon of only 1400 bp indicative of a deletion. Cloning and sequencing of this amplicon revealed 96% sequence identity with the *cdt* genes of C37596, C35926 and C37533 previously identified as having the same deletions (data not shown). This result indicates that significant deletions in the *cdt* genes occur in 2.7 % (4 of 147 isolates tested) of *C. jejuni* strains, but when such deletions in the *cdt* gene locus are present, they were found between *cdtA* and *cdtB.*

Interestingly the sequence of the *cdt* genes from the CDT-negative strain, EF, identified 18-nucleotide substitutions compared to previously published sequences of *C. jejuni cdtABC* (11168 & 81-176). Sequence analysis indicated that all three reading frames were open in *C. jejuni* EF. Of the 18 nucleotide substitutions, only four were identified as non-synonymous: involving an alanine to a valine at codon 88 in CdtA; a proline to serine and methionine to threonine at codons 95 and 120 of CdtB respectively (Fig. 3); and an isoleucine to asparagine at codon 167 of CdtC. In order 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

cell CDT assay (data not shown). By RT-PCR the *cdt* genes were transcribed in strains 99/373, S58 and 99/12 (Fig. 4B), providing supporting evidence that once again suggesting that these mutations were associated with the lack of CDT activity.

Complementation and site directed mutagenesis: To verify whether CDT negative strains could produce active CDT, the complementation vector (pCDT), containing functional *cdt* genes of *C. jejuni* 11168 was introduced into *C. jejuni* EF and 99/68. All transformants tested produced active CDT in the *in vitro* CDT assays, with levels comparable to *C. jejuni* 11168 (Fig. 6).

To ascertain whether the proline residue at CdtB-95 was essential for CDT activity, site directed mutagenesis was used to mutate proline to serine at this residue in the complementation vector pCdt to generate pCdtB^{P95S}. *C. jejuni* EF and 99/68 were complemented with pCdtB^{P95S} and transformants tested for CDT activity. The single mutation at CdtB-95 to serine resulted in a 98.4 % ($p = 0.0004$) and 97.3 % ($p < 0.0001$) reduction in CDT titre, for EF and 99/68 respectively (Fig. 6). These results indicated the residue at CdtB-95 is critical for the toxicity of CDT in *C. jejuni*.

The expression and immunogenicity of CDT during colonization: As yet there are no suitable in vivo models of campylobacteriosis (22), however the one day-old chicken is an excellent model of colonization. Challenge of one-day-old chicks with the CDT negative mutant of 81-176 gave the same level of colonization, up to 10^9 cfu per gram of cecal contents, as the parent strain (data not shown) suggesting that the absence of CDT expression does not affect colonization potential.

Despite this high level of colonization no clinical signs of disease were discernable in 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

antigenic structure of CDT in strain EF as a consequence of the amino acid sequence variation.

The neutralization assay was further used to determine the presence of anti-CDT neutralizing antibodies in pooled sera from patients convalescing from campylobacteriosis. Previous studies, using ELISA and western blotting (4), demonstrated the presence and specificity of anti-campylobacter antibodies in the sera from each individual patient. Complete (100 %) neutralization of the CDT activity was observed in all strains (Fig. 8). This level of neutralization was significantly higher ($p= 0.001$) than the level of neutralization obtained with pooled human sera from blood donors with no history of enteric disease and no demonstrable anti-campylobacter antibodies as detected by ELISA and western blotting (4), indicating that CDT is both expressed during human infection and induces antibody responses. Finally we investigated the presence of neutralizing anti-CDT antibodies in the pooled sera of chickens experimentally colonized with *C. jejuni* 81116. The development of humoral responses directed against the antigens of this strain during colonization has been reported previously (3) and in two other strains by western blot (Fig. 9). Interestingly, in contrast to antibodies from colonized humans and immunised rabbits, these chicken antibodies demonstrated no detectable CDT neutralizing activity (Fig. 8.) against either the homologous strain or the two heterologous strains.

Discussion

The lack of a suitable small animal model of campylobacteriosis continues to be a major limitation to our understanding of the bacterial pathogenic mechanisms involved in campylobacteriosis. Thus potential *C. jejuni* virulence factors have been 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

underlying problems, of cirrhosis and/or neutropenia, which could potentially induce an immune compromised status and thus influence the outcome of that infection. Interestingly, both patients developed campylobacter bacteraemia. However, in one of these patients, from whom strain C37596 was isolated, an earlier faecal isolate (C37533) was also recovered during a period of acute enteritis and had the same mutation as the blood isolate. The isolation of CDT negative faecal strains (C37533, 99/373) from patients with enteric disease, indicates that CDT expression is not essential for the production of enteric disease in humans. Mechanisms other than CDT expression may be involved in the outcome of campylobacteriosis. Similar large deletions have been described in the toxin genes of other bacteria without obvious consequences for disease outcome. For example large deletions (1.18 kb and 5.08 kb) have been reported in the vacuolating cytotoxin (*vacA*) of *Helicobacter pylori* (15) and yet these isolates were still associated with gastritis and peptic ulceration.

The second type of naturally occurring mutation identified in CDT-negative strains (4 out of 147 strains tested) from various sources (poultry processing plant, broiler, sheep and a human), were point mutations resulting in at least 3 amino acid substitutions. As RT-PCR demonstrated that the *cdt* genes of these strains were transcribed, we hypothesised that these mutations resulted in the loss of CDT activity. This hypothesis was further supported by complementation and site directed mutagenesis studies in which the CDT activity was restored by *cdt* genes from a CDT-positive strain.

Because CDT is a tripartite protein, in which the CdtA and C subunits are required for the delivery of the CdtB subunit containing the enzymatically active site associated with toxin activity (18), non-synomonous point mutations in these genes may have different effects on phenotype. The comparisons of the sequences within this group of

strains suggest that the minimal set of mutations required to induce naturally occurring CDT-negativity were represented in strain EF. In this strain a mutation resulting in the replacement of alanine with valine, at codon 88 was detected in CdtA. This change was also conserved in the other two CDT-negative strains within this group. As both amino acids are aliphatic, hydrophobic and have non-polar side chains, this transition is unlikely to substantially affect the structure or folding of the polypeptide chain. Moreover, alignment of CdtA amino acid sequences from *Haemophilus* and *Actinobacillus* species with that from strain EF, also showed a valine at this position (data not shown) and as both these bacterial species produce active CDT, it appears that this polymorphism within the CdtA subunit has little consequence for the activity of this toxin. The mutation at codon 167 of CdtC resulted in the replacement of isoleucine with asparagine and was found in an additional *C. jejuni* strain 99/373, a human isolate. This results in a change from a non-polar hydrophobic residue to a hydrophilic polar residue. Alignment of the CdtC amino acid sequence from *Helicobacter hepaticus* with that from *C. jejuni* 11168 shows that the two sequences are identical at this position. This substitution may therefore have possible a role in the lack of CDT activity in *C. jejuni* EF.

Moreover, there were two potentially important non-synonomous changes in *cdtB* in this group of CDT-negative strains. All demonstrated a substitution of methionine by threonine at CdtB-120. Although this could potentially influence the 3-dimensional structure of CdtB, alignment of the amino acid sequences from other bacterial species shows considerable variability at this position (Fig. 3.), suggesting that this mutation in CdtB would have little, if any, consequence for CDT activity. In contrast, the substitution of proline with serine at CdtB-95, appeared more important. Proline at 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.
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Table 1 – Primers and probes used in study

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 † LC-T-Probe †	gatatcttaatgatacaagaagc ctacatcaacgcgagaataa cy5 - taaaaaccctatcagaccttga - biotin	162 bp	Amplification of region encompassing CdtB codon 95 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).

Figure legends:

FIG. 1. Amplified products of *cdtA, B, and C* of selected strains, with known *in vitro* CDT activity, using primers cdt-F and cdt-R.

FIG. 2. Schematic diagram of the *cdtABC* of *C. jejuni* 81-176 illustrating the locations of the 667 and 50 bp deletions in strains C37596, C37533 and C35926. Arrows indicate direction of transcription

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

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

FIG. 5. Melting curve analyses by LightCycler in 20 selected *C. jejuni* strains, including EF, for the detection of polymorphism at CdtB-95. A negative control is

included. Vertical lines represent the presence of proline (codon - cct) (blue) (as represented by strain 81116), serine (codon - tct) (green) (as represented by strains EF, S58 and 99/373) or more than one bp mismatch (orange) (as represented by strain 99/12) at CdtB-95. A no template control (NTC) was used in each run.

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

FIG. 7. RT-PCR of total RNA extracted from the caecal contents of chickens colonized with *C. jejuni* strain C289/6 (CDT-positive) and from campylobacter-free birds, using primers DS15 and DS18 to detect the expression of *cdtA* and *cdtB. C. jejuni* 11168 grown *in vitro* was used as the positive control. Lanes A: reverse transcribed RNA sample; lanes B: RT negative controls; lanes C: DNA controls. Lanes containing a 1 kb ladder were included (MW).

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

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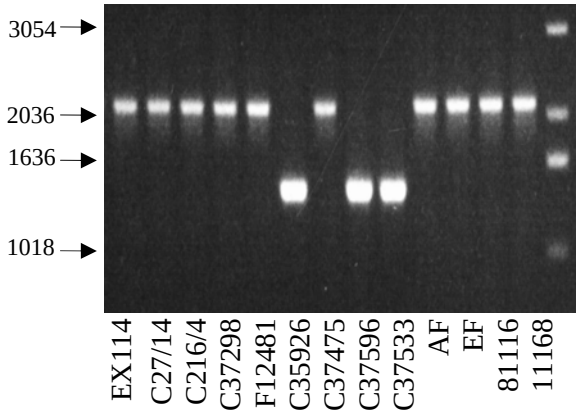

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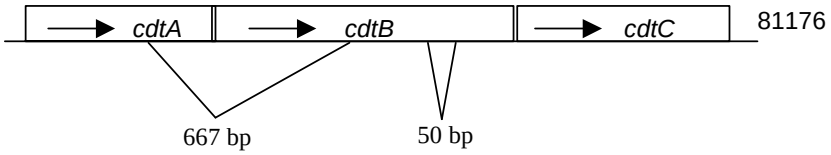

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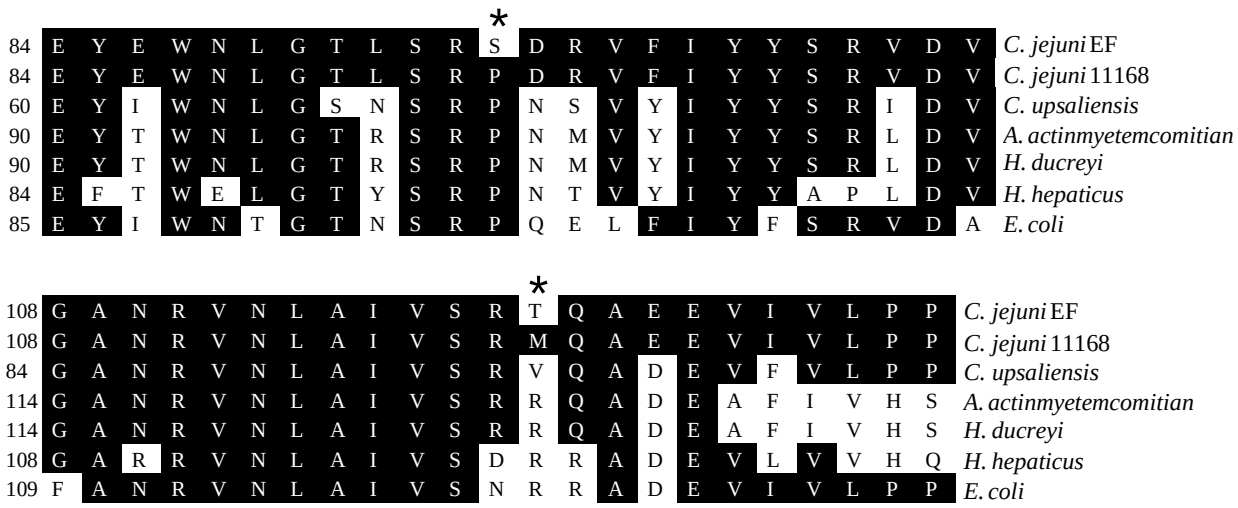

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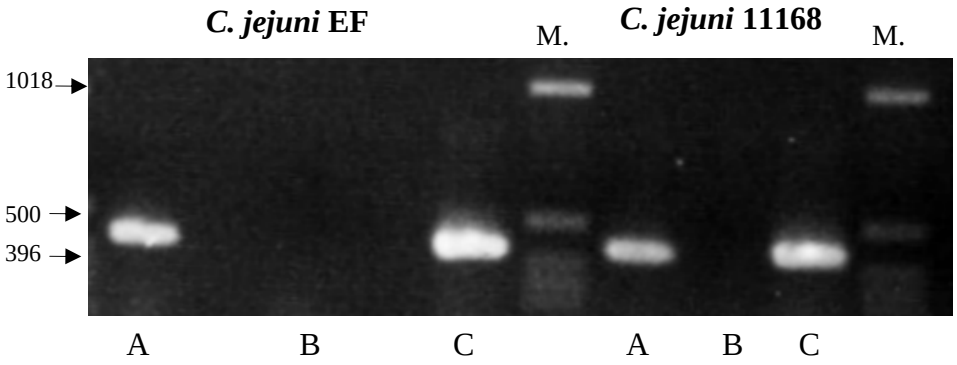
A



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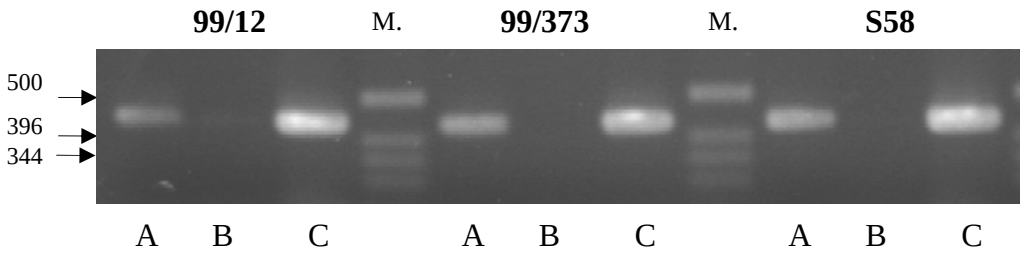

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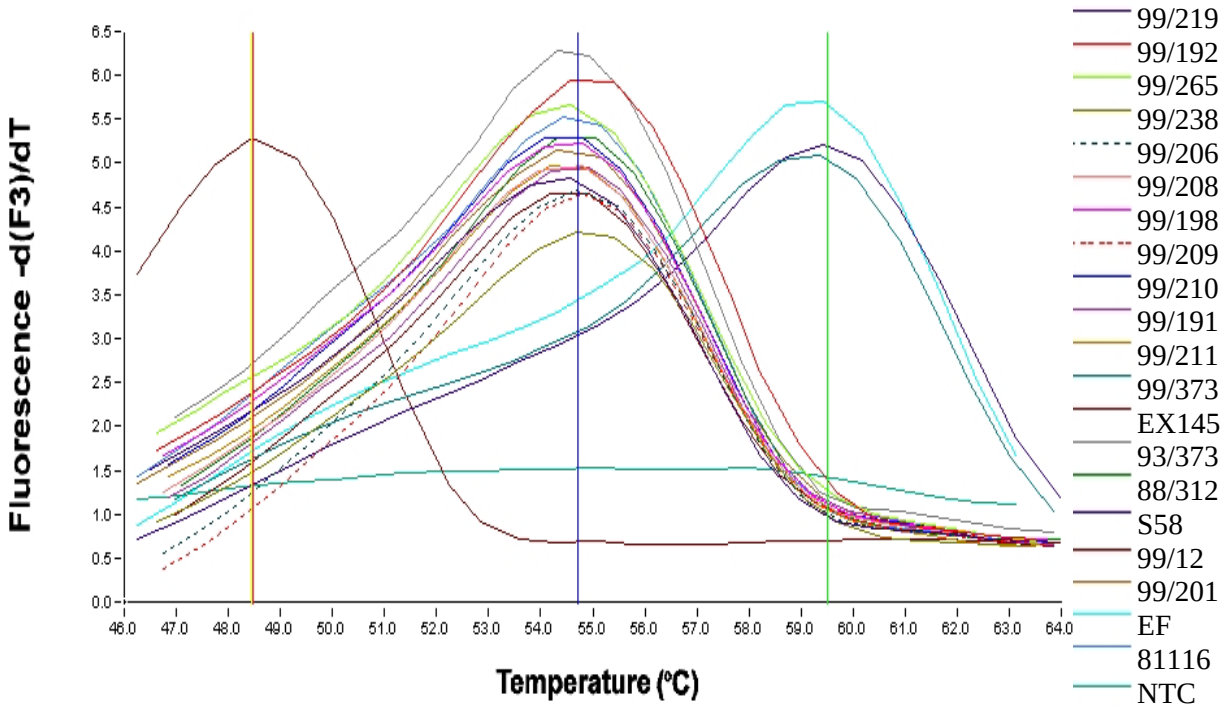

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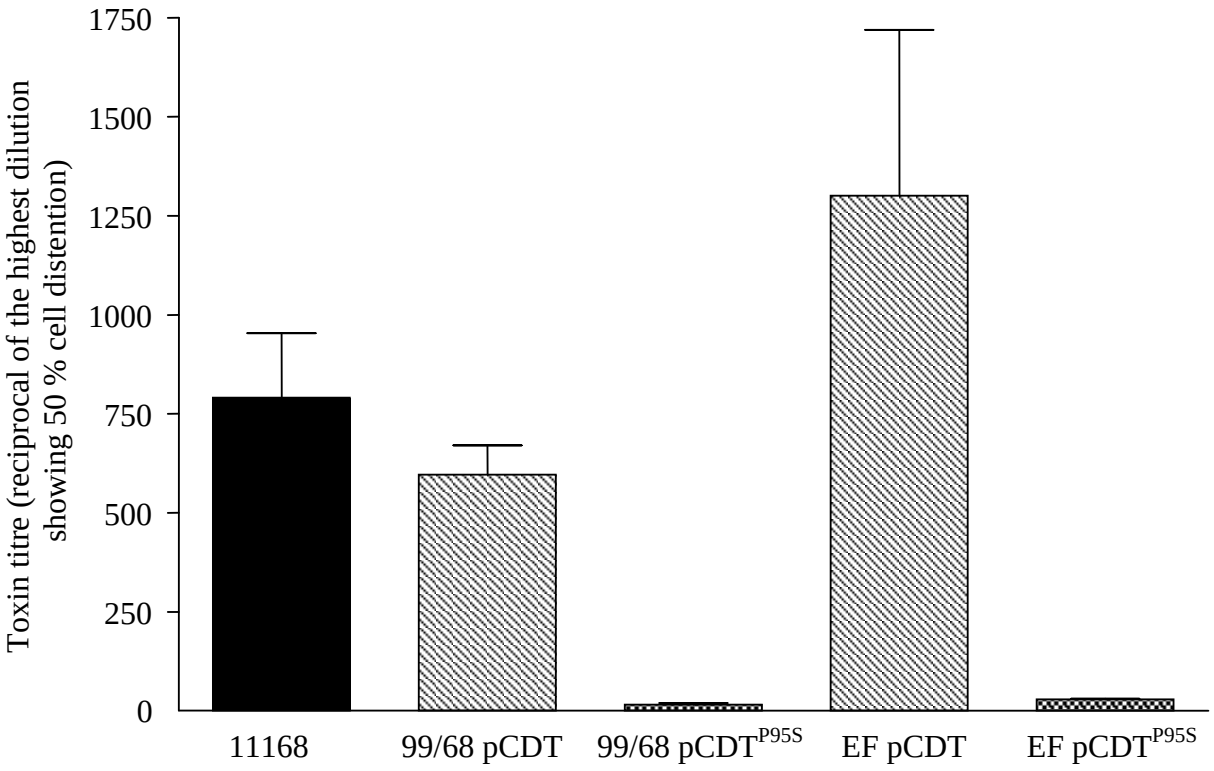

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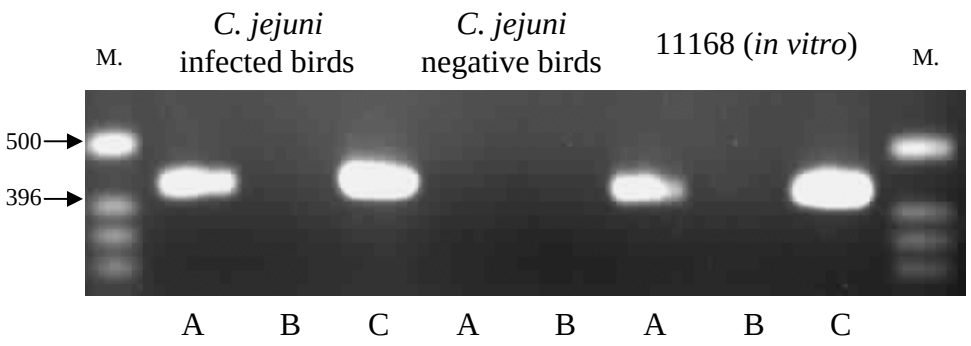

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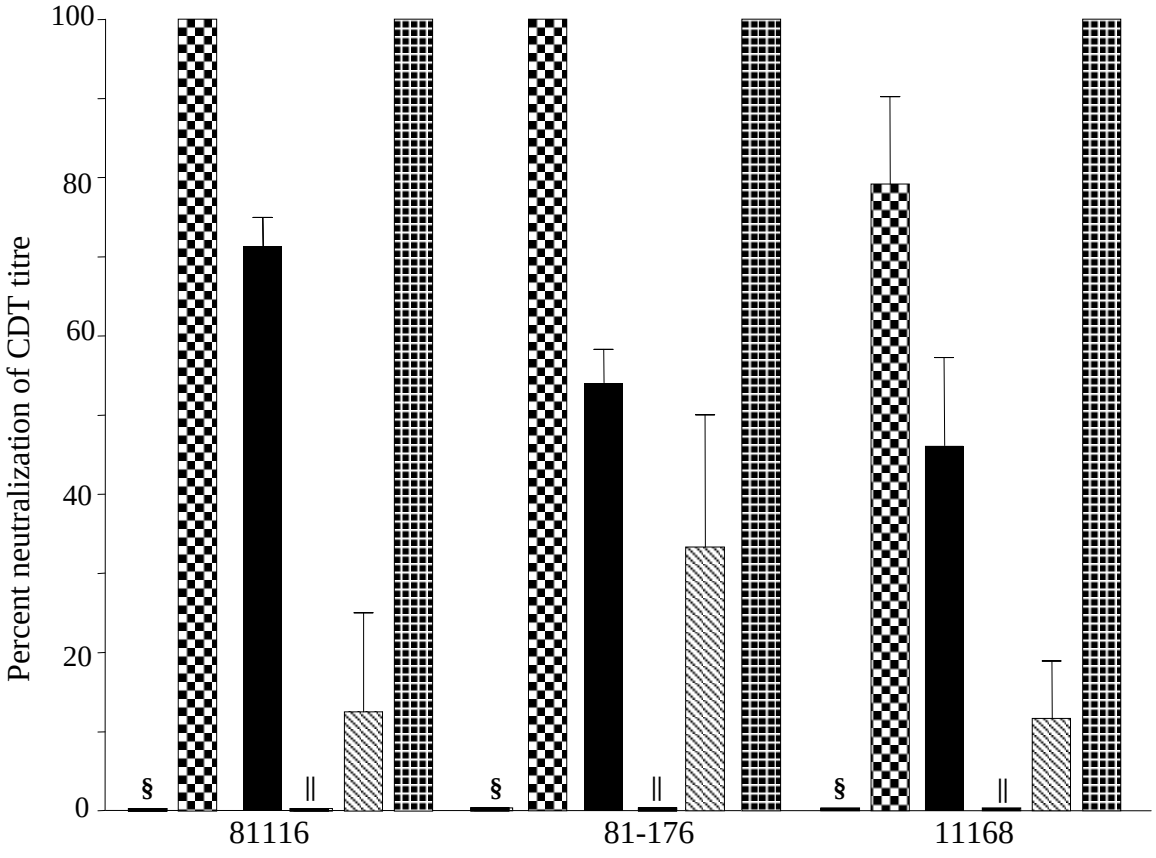

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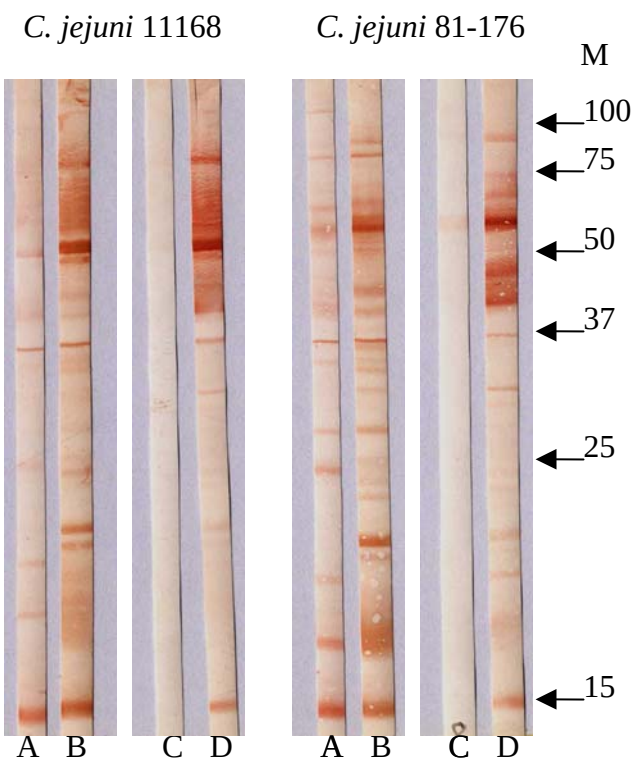

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