CLOSTRIDIUM DIFFICILE BINARY TOXIN (CDT) IN ANTIBIOTIC ASSOCIATED DIARRHEA

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Abstract

We used an EIA, calibrated with purified recombinant antigen, to quantify cdtB which acts as the binding component of Clostridium difficile binary toxin (CDT). This work was done with feces from 80 confirmed human cases of C. difficile diarrhea and culture fluids of isolates recovered from 53 of the same fecal samples. By using PCR, we ribotyped isolates and probed each for the three genes (cdtR, cdtA, cdtB) that make up the CDT locus. CDT is best known as a toxin associated, though not exclusively so, with the ribotype 027 isolates that are central to the recent worldwide rise in the incidence of C. difficile diarrhea. We found CDT(+) isolates in 50/80 (63%) fecal samples; cdtB was present in only 38% of that 50. In contrast, 90% of all CDT(+) isolates we recovered made cdtB protein in broth culture. Ribotype 027 isolates were 86% of all CDT(+) isolates. Six other ribotypes accounted for the 7 non-027, CDT(+) isolates; 6 of the 7 made cdtB in vitro. 25/80 (31%) isolates carried the ghost CDT locus, i.e. an intact cdtR but only fragments of cdtA and cdtB. 5/80 (6%) lacked all or any part of the CDT locus. No CDT- isolate was recovered from an EIA negative stool sample. Levels of cdtB in the fecal samples were on average nearly twenty-fold greater than the corresponding isolates achieved in vitro. We saw, however, no direct relationship between toxin concentrations in feces and in vitro cultures. Over half of the EIA positive samples contained >100 ng cdtB/mL, a level that reached in stools matches other closely related clostridial binary toxin diarrheas. This information suggests that when there is 100 ng/mL or more of cdtB present, the level of CDT may be sufficient to cause diarrheic symptoms, possibly complementing the known enteric activities of Toxins A and B.

Background

Clostridium difficile is an important nosocomial pathogen associated with about 20% of all antibiotic-associated diarrheas. *C. difficile* is also the most common causative agent of pseudomembranous colitis, a potentially life threatening form of disease in the colon. The main virulence factors of *C. difficile* are two toxins, A and B, which are large single-chain proteins 308 and 270 kDa, respectively. It has been shown that in addition to Toxins A and B several *C. difficile* strains produce a third, binary toxin, CDT. CDT is composed of two independent, unlinked polypeptides, CDTa and CDTb. CDTa is an enzyme, an actin-specific ADP-ribosyl transferase. Though enzymatically active by itself, CDTa is only toxic when internalized via a CDTb pentamer docked on the surface of host enterocytes. CDTb forms the oligomer even in the absence of CDTa. Only together do CDTa and CDTb have cytotoxic and enterotoxic activity.

At genetic, immunologic and toxicologic levels CDT is very closely related to two other clostridial binary toxins, iota toxin of *C. perfringens* type E, and iota-like toxin of *C. spiroforme*. Expression in *C. perfringens* and *C. spiroforme* is 10 or more-fold greater than in *C. difficile*, suggesting a different regulation for *C. difficile*. In fact, *cdtR*, a gene absent from the plasmid in *C. perfringens* that carries the iota toxin genes or from the iota-like gene locus on the chromosome of *C. spiroforme*, regulates CDT production by *C. difficile*. Production of CDT by *C. difficile* is positively regulated by CDTr, encoded by *cdtR*.

We screened samples from patients with *C. difficile* diarrhea for the presence of CDT using an ELISA. We isolated C. difficile from these samples and screened the isolates for the CDT gene locus using PCR and for the *in vitro* production of CDTb.

Aims

- To assay stool from patients diagnosed with *C. difficile* diarrhea for the presence of CDTb, the binding component of *C. difficile* binary toxin.
- To recover *C. difficile* from patient stool and to assay these isolates for the three genes, *cdtA*, *cdtB* and *cdtR*, that make up the CDT locus.
- To assay recovered isolates for the *in vitro* production of CDTb.

Materials and methods

Patient feces : Already existing, anonymous and unlinked fecal samples from patients diagnosed by the presence of GDH and toxins A or B or both as having *C. difficile* diarrhea were used.
Bacterial isolates: Feces were plated onto Cycloserine cefoxitin fructose agar, a selective and differential medium for the selective growth of *C. difficile*. The inoculated plates were incubated anaerobically at 37 C for 48 to 72 h. Individual colonies were subcultured and stored in chopped meat broth until required.
Bacterial DNA: DNA was extracted using the QIAGEN DNA Mini Kit. In summary, 24 h brain heart infusion broth cultures were centrifuged and the pellets digested with a lysozyme buffer. After incubation, proteinase K and an additional lysis buffer was added, followed by a second incubation. The mixture was centrifuged and DNA was washed and collected by alcohol precipitation in QIAamp spin columns. Collected DNA was stored at -20ºC until ready for analysis.
PCR: See table below.
ELISA for CDTb: A prototype ELISA using monoclonal and monospecific polyclonal antibodies against CDTb was used. Feces were assayed with no added dilution being needed. *In vitro* cultures were concentrated 10 or 20-fold before testing. This was necessary since, as we and others have previously noted, levels of detectable CDT in broth medium are 10 or 20 fold lower than the equivalent levels in levels in *C. perfringens* Type E and *C. spiroforme* and, in the event, about 10 or more fold lower than the levels found in fecal samples from which isolates were recovered. Concentrations of CDTb were established by comparison with a curve prepared with rCDTb standards.

PCR conditions and primers for detection of *cdt* locus

Gene amplified	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Thermocycling Conditions
cdtR (regulatory gene)	AGTCTATAATTAT TGTTAAATAATTC TTC	AGTTGCGCAGCAT GCATCTAAATCTG GTAAAG	30 cycles 94 C 30 sec 50 C 30 sec 72 C 1 min
cdtA (ADP ribosyl transferase)	TGAACCTGGAA AAGTGGATG	AGGATTATTTACT GGACCATTTG	35 cycles 94 C 30 sec 50 C 1 min 72 C 1 min
cdtB (binding component)	GATTCACAGCTA ATGTAACTA	TATAGTCTGACCA ACTATTTC	35 cycles 94 C 30 sec 50 C 1 min 72 C 1 min

Primers for the cdtR gene were taken from Carter et al (2007). Primers for the *cdtA* gene were taken from Stubbs et al. (2000). Primers for *cdtB* were generated from the complete sequence using the Primer3 web-based primer selection program.

References

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Stubbs S, Rupnik M, Gibert M, *et al*. 2000. FEMS Microbiol Lett **186:** 307-12.

Fig. 1. Agarose gel analysis of PCR amplicons. Lanes 1 and 2 represent isolates negative for *cdtA*. Lanes 3, 4, and 5 represent positive isolates. Lanes 6 and 7 are negative control isolates and lane 8 is a positive control isolate.

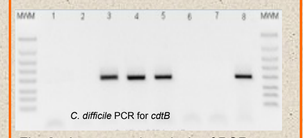

Fig. 2. Agarose gel analysis of PCR amplicons. Lanes 1 and 2 represent isolates negative for *cdtB*. Lanes 3, 4, and 5 represent positive isolates. Lanes 6 and 7 are negative control isolates and lane 8 is a positive control isolate.

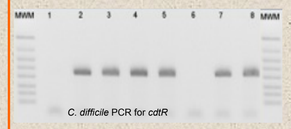

Fig. 1. Agarose gel analysis of PCR amplicons. Lane 1 is an isolate negative for *cdtR*. Lanes 2 - 5 represent positive isolates. Lane 6 is a negative control isolates and lanes 7 and 8 are positive control isolates.

Table 1. CDTb and *cdtb* in *C. difficile* diarrhea

Sample #	Fecal CDT	PCR for *cdtA*	PCR for *cdtB*	PCR for *cdtR*	*In vitro* CDTb production
1	-	-	-	-	
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	-	-	-	-	-
6	-	-	-	-	-
7	-	-	-	-	-
8	-	-	-	+	-
9	-	-	-	+	-
10	-	-	-	+	-
11	-	-	-	+	-
12	-	-	-	+	-
13	-	-	-	+	-
14	-	-	-	+	-
15	-	-	-	+	-
16	-	-	-	+	-
17	-	-	-	+	-
18	-	-	-	+	-
19	-	-	-	+	-
20	-	-	-	+	-
21	-	-	-	+	-
22	-	+	+	+	+
23	-	+	+	+	+
24	-	+	+	+	+
25	-	+	+	+	+
26	-	+	+	+	+
27	-	+	+	+	+
28	-	+	+	+	+
29	-	+	+	+	+
30	-	+	+	+	+
31	-	+	+	+	+
32	-	+	+	+	+
33	-	+	+	+	+
34	-	+	+	+	+
35	-/+	+	+	+	+
36	-/+	+	+	+	+
37	+	+	+	+	+
38	+	+	+	+	+
39	+	+	+	+	+
40	+	+	+	+	+
41	+	+	+	+	+
42	+	+	+	+	+
43	+	+	+	+	+
44	+	+	+	+	+
45	+	+	+	+	+
46	+	+	+	+	+
47	+	+	+	+	+
48	+	+	+	+	+
49	+	+	+	+	+
50	+	+	+	+	+
51	+	+	+	+	+
52	+	+	+	+	+
53	+	+	+	+	+

Results

A total of 53 fecal samples from patients diagnosed with *C. difficile* diarrhea were tested for the presence of CDTb using a prototype immunoassay.

Samples 1 to 7 (13% of 53) were all negative in the CDT assay. The recovered isolates lacked all three genes of the CDT locus. None made CDT *in vitro*.

Samples 8 to 21 (26%) were all negative in the CDT assay. The recovered isolates carried *cdtR* but were negative in the PCR assay for *cdtA* and *cdtB*. None made CDT *in vitro*.

Samples 22 to 34 (26%) were all negative in the CDT assay despite all the recovered isolates carrying the entire CDT locus and expressing CDTb *in vitro*. Detection of CDTb however, required the concentration of culture fluids by 10 or 20 fold.

In the CDT assay samples 35 and 36 (4%) gave positive elevated signals well above the background seen with true negatives. Their isolates carried the entire CDT locus. They made CDTb *in vitro* but once more it was only detected after concentration.

Fecal samples 37 to 53 (30%) had CDTb in them, 5 of them at levels over 1 µg/mL (data not shown). Their isolates carried the entire CDT locus. They made CDTb *in vitro* but again it was only detected after concentration.

Discussion

The CDT immunoassay detected CDTb in about 2/3rds of the samples that reasonably might be expected to contain CDTb. Its minimum level of detection might be further improved with refinement, though it may be as much a question of expression levels or prior metronidazole or vancomycin therapy as shortcomings in the assay. Even so, CDTb concentrations in at least 5 samples were of the same order as iotaB in an enterotoxic dose of purified iota toxin from *C. perfringens* Type E (Carman *et al*., 1986).

All *C. difficile* isolates carrying the full CDT locus produced CDT, though at only very low levels compared with other binary toxin producers. Low levels of CDTb have been reported before (Popoff *et al*., 1988, Geric *et al*., 2006) and it may possibly reflect two or more regulatory mechanisms among *C. difficile*, *C. perfringens* Type E and *C. spiroforme*.

Isolates carrying the 5' end of *cdtA* and the 3' end of *cdtB* but not the central spanning sequence have been known for some time (Chang & Song, 2001). They are said to carry ghost genes. All known examples have recently been shown to also carry *cdtR* (Carter *et al*., 2007). In this study these are represented by the isolates from samples 8 to 21. They predictably did not produce CDTb; none of the corresponding fecals was positive in the immunoassay.

A direct role for CDT in *C. difficile* disease caused by isolates producing neither toxin A nor B but only CDT has been questioned (Geric *et al*., 2006), though some of the levels of CDTb we saw may warrant reconsideration.

We are continuing our work by improving the immunoassay. We are also working on the complementation of toxin A and B by the addition of CDT (of special concern with the epidemic Ribotype 027 isolates that produce all three *C. difficile* toxins) and collecting data on the relationship between CDTb levels and the degree of intestinal inflammation indicated by lactoferrin levels.

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