CLOSTRIDIUM DIFFICILE 027 DIARRHEA: HIGHER COUNTS, MORE TOXIN, MORE LACTOFERRIN

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ABSTRACT

C. difficile is the leading known cause of nosocomial antibiotic-associated diarrhea. Reports have linked ribotype 027 with worse outcomes, and worse outcomes with the presence of toxin. To identify potential effects of ribotype and microbial load we generated quantitative culture and analyte data using 48 anonymous, unlinked and already existing clinical samples, each from the same area of southwest Virginia and each containing a toxigenic ribotype of *C. difficile*. No clinical information was collected. Two groups, liquid samples (Bristol stool chart 7, n=18, 44% were 027) and 027 samples (n=14, 57% were liquid) had mean total (vegetative cells and spores) counts of ~10^5/g, about 10-fold higher than both solid samples (Bristol stool chart 1, 2 and 3) and non-027 samples. In 66% of liquid and 57% of 027 samples vegetative cells outnumbered spores; in only 15% of solid and 29% of non-027 samples did vegetative cells outnumber spores. In liquid samples the average levels of toxin A, toxin B and lactoferrin were respectively 78 ng/g, 122 ng/g and 250 µg/g. Levels in 027 samples were 165 ng/g, 187 ng/g and 373 µg/g. In solid samples the levels were lower, 54 ng/g 13 ng/g and 40 µg/g. They were significantly lower in non-027 samples, 35 ng/g*, 7 ng/g* and 91 µg/g* (*p<0.05). Semi-solid (Bristol stool chart 4, 5 and 6) analyte levels and counts were intermediate between those in liquid and solid samples. Overall, higher counts and vegetative growth were associated with higher levels of toxins, with 027, with liquid stool and with higher fecal lactoferrin. Our results suggest correlations during *C. difficile* diarrhea between stool consistency, toxin level, microbial burden, the relative abundance of vegetative cells, ribotype, and inflammation.

BACKGROUND

In a "more-is worse" model of untreated *C. difficile* infection higher fecal counts would mean higher GDH, more toxin, worse diarrhea, increased inflammation and worse outcomes.

Despite its simplistic appeal, the more-is-worse model does not fit all situations. It does not apply to newly born, milk-fed mammals who can, with no symptoms, carry *C. difficile* and its toxins. Their bowel ecology, distinct from that of adults, permits asymptomatic colonization rates of possibly up to 70%.

Several recent reports support aspects of a more-is worse model:
- •Akerlund et al (2006) showed fecal toxin titer with the number of stools per day and with pain, but not ribotype.
- •Planche et al (2013) showed, emphatically, that patients with fecal toxin have twice the 30 d mortality of those without.
- •Boone et al (2014) showed lactoferrin and mortality were linked to fecal toxin.
- •Polage et al (2012) associated fecal toxin with extended diarrhea and worse outcomes.
- •Su et al (2013) showed symptoms reflected the presence of fecal toxins.
- •LaSala et al (2102) saw higher levels of lactoferrin in toxin positive samples.
- •Huang et al (2104) confirmed the link between symptoms and fecal toxin levels and related both to counts.
- •Gyorke et al (2013) found 98% of those with fecal toxins were PCR positive, whereas only 58% of those without toxin were. PCR template concentrations were high in toxin positive feces.
- •Awad-el-Karim et al (2012) showed PCR positive, asymptomatically colonized individuals had fecal GDH but no toxins.
- •Naaber et al (2011) and Leslie et al (2012) have both linked higher template concentrations with higher counts and the presence of fecal toxins.

Some of these trends also correlated with ribotype 027, suggesting 027 may be more virulent than others. For example, the less virulent ribotype 014 is recovered from asymptomatically colonized patients more often than from diarrheic patients, a trend reproduced in hamsters (Sambol et al, 2001).

AIM

- Are higher counts of *C. difficile* related to higher levels of GDH, more toxins, and increased inflammation, i.e. Is more worse?
- Is there a role for specific ribotypes on top of any "more-is-worse" findings?

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MATERIALS AND METHODS

•Stool specimens: We used 48 already existing, anonymous and unlinked fecal samples, submitted in the Fall of 2013 from a single south western VA location for routine *C. difficile* testing. The Bristol Stool Chart was used to report consistency. All submitted samples were tested. None was discarded because it was a repeat, from an already treated individual or because the stool was solid.

•Glutamate dehydrogenase (GDH) was assayed by the TECHLAB *C. DIFF CHEK* ™ -60 test according to the Package Insert and quantitatively (ng/mL) using a modified TECHLAB *C. DIFF CHEK*™ -60.

•TcdA and TcdB (Toxins A and B respectively) were quantified using purified toxin standards and individual toxin-specific modifications of TECHLAB's *C. DIFFICILE TOX A/B II*™ test. Cytotoxic Toxin B was measured with the TECHLAB *C. DIFFICILE TOX-B TEST* and results were reported as Yes/No.

•Fecal lactoferrin was measured quantitatively using the TECHLAB *IBD - SCAN*® as instructed by the Package Insert and results were reported as µg/mL stool.

•Bacterial counts: We collected total and spore count/g feces on CCFA after 48 h incubation. Spores were selected by ethanol shock.

•PCR analysis and PCR ribotyping: DNA was extracted from broth cultures using the QIAamp Mini Kit (Qiagen, Valencia, CA) and amplified. Banding patterns were compared to TECHLAB's ribotype library. In addition we grouped ribotypes into four divisions (below) based on additional PCR testing for *tcdA*, *tcdB* and *cdtB* that were confirmed by toxin-specific immunoassays:

Non-toxigenic ribotypes
Other A+B+ toxigenic ribotypes
014
027

RESULTS

We focused on 027 because 027 was very common overall and significantly more common in toxin EIA positive than in negative stools. We focused also on 014 because it was the most common of the three other ribotypes that were significantly more common in toxin negative fecal samples (Table 1) .

Table 1. All significant differences between ribotype frequency in cytotoxic fecal samples that are toxin EIA positive or negative

Ribotype	GDH+ToxB+ABII+		GDH+ToxB+ABII-		Fisher exact
	n	% ot total	n	%	p
103	0	0	3	5	0.002
027	175	42	16	25	0.013
005	2	0	3	5	0.018
014	23	5	8	13	0.049

Our quantitative results (Table 2) are shown by ribotype (virulent 027, Other, 014 and non-toxigenic) and by stool consistency (liquid, semisolid, solid). They suggest two overlapping trends:

- Counts, analyte levels and sample consistency indicate a virulence spectrum within this clinical ecosystem, from low to high of non-toxigenic ribotypes, 014, Other (A+B+) ribotypes, culminating 027.
- More-is-worse (highest counts, toxin and lactoferrin levels were in liquid stools).

Table 2. *C. difficile* analyte and host inflammation by ribotype and by consistency

Ribotype	Mean total Cd/mL	Mean spore Cd/mL	Mean % spore	% with total > spore	Mean GDH (µg/mL)	Mean TcdA (ng/mL)	Mean TcdB (ng/mL)	Mean lactoferrin (ng/mL)	% liquid
Non-toxigenic	$10^{3.4}$	$10^{3.9}$	331	33	3	0**	0*	44**	19*
14	$10^{3.5}$	$10^{3.7}$	186	33	7	11*	1	39**	22*
Other toxic	$10^{4.7}$	$10^{4.8}$	206	36	7	44**	9**	110**	32
27	$10^{5.3}$	$10^{5.1}$	175	57	5	165	187	373	57

Stool consistency (Bristol stool #)	Mean total Cd/mL	Mean spore Cd/mL	Mean % spore	% with total > spore	Mean GDH (µg/mL)	Mean TcdA (ng/mL)	Mean TcdB (ng/mL)	Mean lactoferrin (ng/mL)	% 027
Solid (1,2,3)	$10^{4.0}$	$10^{4.4}$	290	24*	9	41	10	34**	12
Semi-solid (4,5,6)	$10^{4.6}$	$10^{4.8}$	367	31*	5	54	18	141	15
Liquid (7)	$10^{5.1}$	$10^{4.9}$	239	66	4	67	105	228	38

Significance vs. 027: * p < 0.05 ** p < 0.05

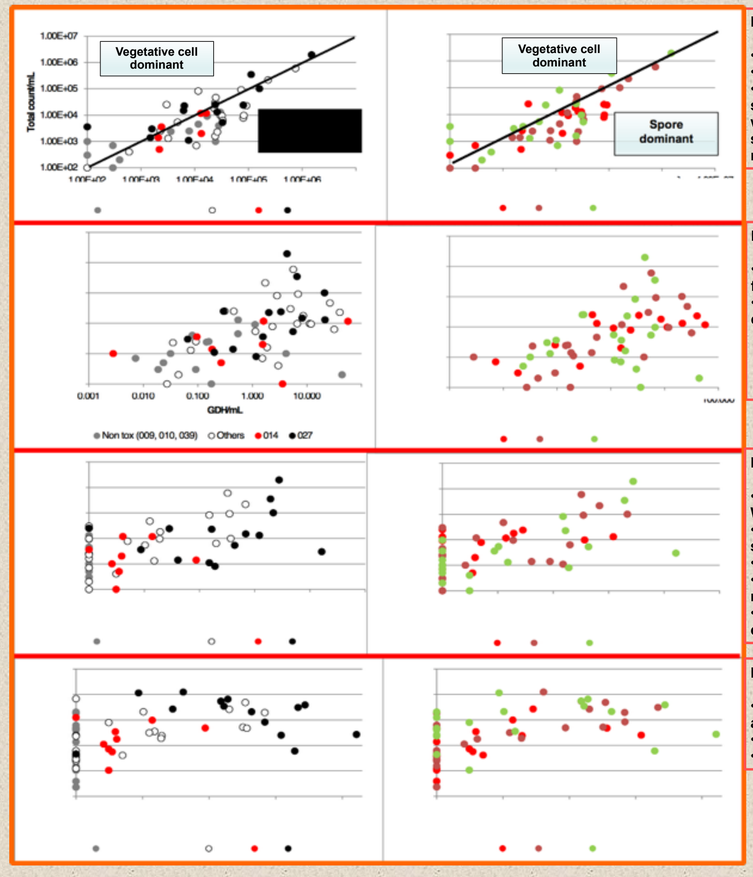

Figure 1. *C. difficile* counts in feces by: Left) ribotype and Right) consistency

•Counts were lowest in non-toxigenic samples and highest in 027 and Others.
•Spore count increased as total count rose regardless of ribotype.
•Only in primarily 027 and Other samples did vegetative cells ever outnumber spores; these samples tended to be liquid. This supports Akerlund et al (2006) who proposed, based on *in vivo* and *in vitro* data, that toxin production and sporulation are alternative, contradictory strategies for *C. difficile* related to nutrition.

Figure 2. GDH and total count by: Left) ribotype and Right) consistency

•We saw a trend from low count and low GDH to high count and high GDH running from non-toxigenic, 014, Other, to 027
•Although GDH rose with total count it was not related to consistency. A possible explanation may be:
- GDH is intracellular and is released when cells lyse.
- Sporulation (cell lysis) occurs when counts are high.
- Accordingly GDH rises once high levels of vegetative cells pass from making toxins to making and releasing spores.

Figure 3. TcdB and total count by: Left) ribotype and Right) consistency

•Higher levels of TcdA occurred in samples with higher levels of TcdB (not shown). We will focus on TcdB from this point.
•TcdB levels rose with total counts. Both were highest in 027 and Other fecal samples.
•Feces containing non-toxigenic isolates were free of toxin.
•The least TcdB was in the 014 samples. Levels were higher with the Other ribotypes and highest with 027.
•TcdB levels trended modestly with stool consistency. Even so, many solid stools contained toxin.

Figure 4. TcdB and lactoferrin by: Left) ribotype and Right) consistency

•Feces negative for TcdB or lactoferrin may be unrelated to *C. difficile diarrhea* and, in fact non-toxigenic ribotypes predominate this group
•Lower TcdB and lactoferrin levels occurred in samples containing 014.
•Others and 027 were associated with higher TcdB and lactoferrin levels

EFFECT OF TIME ON COURSE OF DISEASE AND INFECTION

Our samples were almost certainly collected at different time points in the course of infection. With time likely to prove important (see below), it is a significant flaw in our work.

For example, feces containing mostly vegetative cells might represent an early phase of infection during which cells grow rapidly, make toxin, and cause diarrhea. Later, those cells move into a sporulation phase with no new toxin formation but with the release of already formed toxins, GDH, DNA and a spore from each sporangium. Similar differences, vegetative growth and toxin production as opposed to spore formation with a reduction in toxin, occur as cells pass from a planktonic phase in the luminal digesta to a less metabolically active (i.e. less toxigenic) lifestyle in a biofilm. At first presentation therefore, patients might have liquid stools but later have stools that are more formed despite remaining culture and analyte positive albeit with the reduced levels that accompany milder symptoms. Su et al (2012) saw precisely that change in ~20% of patients whose initial symptom of diarrhea resolved without treatment within ~72 h.

CONCLUSIONS

•Counts and analyte levels were related to each other. Both reflected ribotype, stool consistency and host inflammation. They were highest in liquid samples and in those containing 027 samples and lowest in solid samples and those containing non-toxigenic or 014 isolates of *C. difficile*.

•High counts were accompanied by more toxin, more inflammation and were most frequently seen with liquid stool indicating a more-is-worse relationship especially in 027 infection.

•027 (>30% of all isolates) was virulent and 014 (9% of all isolates) was less virulent, possibly because 014 made less toxin or because it did not reach as high a count as did 027. The two findings may be related.

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