TECHLAB®

DIARRHEA DIGEST

DIARRHEA DIGEST is an irregular publication of TECHLAB® dedicated to the etiology, diagnosis, and therapy of diarrheal diseases and related aspects of intestinal ecology

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The *DIARRHEA DIGEST* is now green.

Just like previous paper issues, the green version will be an irregular publication and it will be available on our website. The green version may not be as easy to take to the bathroom, but by saving trees, the green version will help make sure that you don't run out of toilet paper.

C. difficile Testing … Basics 101

C. difficile is a spore-forming, gram (+) bacillus. Most toxigenic strains produce both toxin A and toxin B, although there are some clinical isolates that only produce a modified toxin B. Toxigenic strains cause *C. difficile*-associated diarrhea (CDAD) and colitis, with the severe stage known as pseudomembranous colitis. CDAD is an important clinical problem occurring predominantly after hospitalization and administration of broad spectrum antibiotics, especially in elderly patients.

Laboratory diagnosis of CDAD is usually done with a fecal test that detects the toxins, although some labs, particularly in Europe, still use toxigenic culture to detect the organism for diagnostic purposes. Endoscopic examination in patients with severe disease can show characteristic yellowish pseudomembranes, often with intervening normal-appearing mucosa. Gold standard tests include the tissue culture assay (also referred to as the cell cytotoxicity assay) and toxigenic culture. However, these methods are time-consuming. The tissue culture assay takes up to 48 hours to confirm a positive reaction and toxigenic culture, which includes isolation of the organism on cycloserine-cefoxitin fructose agar (CCFA) followed by growth in broth culture for 48-72 hours, can take a week. Both are inefficiently time-consuming for clinical use.

Enzyme immunoassays (EIAs) for the detection of toxins A and B have been implemented in many clinical labs because of their rapid turnaround time and relatively low cost. A number of reports have indicated decreasing performance for these tests in recent years. We suspect that this has occurred because a broader range of patients are being tested in earlier stages of the disease when toxin levels are below detectable limits. In addition, it is likely that levels of toxin have decreased in some patients because they have already begun receiving metronidazole or vancomycin. An EIA for antigen (glutamate dehydrogenase) is being used as a good surrogate test for the presence of *C. difficile* in fecal specimens. Earlier reports on antigen detection showed poor performance with the latex agglutination

and membrane tests. However, recent studies using more current microwell and membrane EIAs indicate excellent performance when compared with bacterial culture. The antigen is produced by all *C. difficile* strains, including toxigenic and nontoxigenic strains; therefore, the test does not differentiate between the two, and follow-up testing should be done with a toxin test.

In Dec, 2008, the FDA cleared the first commercially available real-time PCR assay (BD GeneOhm *Cdiff Assay*, BD Diagnostics, San Diego, CA) for the detection of the toxin B gene *tcdB* in fecal specimens as a marker for toxigenic *C. difficile*. In 2009, the Prodesse ProGastro Cd assay (now sold by Gen-Probe Prodesse, Waskesha, WI) and the Xpert *C. difficile* PCR (Cepheid, Sunnyvale, CA) became available. The ProGastro Cd test is similar to BD GeneOhm *C diff Assay*, directly detecting *tcdB* in fecal specimens on the SmartCycler. The Xpert *C. difficile* PCR test detects *tcdB*, the binary toxin gene, and *tcdC* deletion characteristic of ribotype 027 outbreak strain. There also are in-house real-time PCR tests (home-brews) that are starting to be used that have not gone through FDA review.

Real-time PCR methods have reported sensitivities ranging from 77% to 100% when compared to toxigenic culture. Two independent studies (Eastwood et al., 2009; Stamper et al., 2009) on the BD GeneOhm assay reported sensitivities of 94% and 84% with specificities of 99% and 98%, respectively. The Cepheid Xpert test has sensitivities of 94 to 100% with a specificity of 78% (Huang et al., 2009). The Prodesse ProGastro Cd assay, which uses Taqman probes on the Smart Cycler, has a reported sensitivity and specificity of 77% and 99%, respectively (Stamper et al., 2009). With home brew PCR tests, Peterson et al. (2007) reported a sensitivity and specificity of 93% and 97%, respectively, and Sloan et al., (2008) reported a sensitivity of 86% and specificity of 97%.

PCR tests detect as little as 10 copies of DNA in a PCR reaction. This raises the question of whether the tests may be too sensitive in some cases, leading to overtreatment of patients with diarrhea. There also are other concerns such as genetic drift of *tcdB*, resulting in negative results, and how costs for PCR will affect testing.

Recently, the *C. DIFF QUIK CHEK COMPLETE*® test (TechLab, Inc.) became commercially available. This test is a rapid membrane EIA for the detection of antigen and toxins A/B with one easy-to-use cartridge. Eastwood *et al.* (2009) and Swindells *et al.* (2009) reported a 100% agreement between the *C. DIFF QUIK CHEK COMPLETE*® antigen test and toxigenic culture. In other studies, a two-step algorithm using an antigen screen followed by a confirmatory test has been recommended to detect *C. difficile* infection. Using this approach, the *COMPLETE* can be used as a stand-alone test or as a screen for more extensive testing by PCR. For screening purposes, results are available in less than 30 minutes, and by using a simple rapid test for antigen and toxins A/B, results for more than 80% of specimens can be determined for samples that are negative for *C. difficile* or positive for *C. difficile* and its toxins. Our in-house studies show that DNA from fecal specimens is stable in the sample diluent of the *COMPLETE* assay and can be stored at 4ºC for up to 4 days after dilution. Therefore, samples that are questionable (e.g., antigen-positive, toxin-negative samples) can be confirmed by real-time PCR simply by using the fecal dilution prepared for the *COMPLETE* test, thus facilitating a more efficient workflow for the clinical lab.

-Li Chen

For more information on diagnosis, see the new guidelines for diagnosing, managing and treating *Clostridium difficile* disease, produced by a panel of experts from the Society for Healthcare Epidemiology (SHEA) and the Infectious Diseases Society of America (IDSA), to be published in the May issue of Infection Control and Hospital Epidemiology.

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PCR testing for C. difficile; what it may mean for the patient and the hospital

In our own little corner of the world --- Blacksburg, VA in case you wonder where our corner is --- the Roanoke Times ran an article entitled "Another infection surpassing MRSA" in the March 21, 2010 Sunday newspaper. This article was written by a writer for the Associated Press, and was printed in a number of newpapers around the country. The article covered much of what we in the *C. difficile* business were aware of but which needed to be brought to the Public's attention. Problems with hospital-acquired infections were the topic, and it's good for lay people to understand that even though you go to hospitals to get well, you can get an infection in the hospital that can make you sicker. *C. difficile* was the headline bug in the article.

C. difficile is passing MRSA, at least in some facilities, as the primary hospital-acquired infection. *C. difficile* has been in the news more often over the past several years simply because it has continued to grow as a huge problem in hospitals --- we are now talking billions of dollars for healthcare costs because of the infections caused by *C. difficile*. The disease continues to be the same persistent problem in elderly patients that we've known about for 30 years. If you treat patients with antibiotics that wipe out the intestinal flora they become susceptible, especially the older population, to a *C. difficile* infection. But now *C. difficile* disease has spread into the outpatient population and is being seen in younger adults. And nursing homes probably are an excellent source of carriers who are going back and forth into hospitals, shedding spores into hospital wards.

If *C. difficile* didn't form spores, then we probably would have a more even playing field in our battle against this organism (of course, if it didn't form spores, then it wouldn't be a *Clostridium*, would it!). But these spores, which are very hardy and whose levels continue to build up in hospitals over time, are transmitted to susceptible patients by every imaginable way, and probably by ways that we can't imagine. They infect the patient, and get to the large intestine where they grow and make the patient ill by causing diarrhea and colitis --- but, and this is an important but, although *C. difficile* does this often, it doesn't always cause disease.

This is where it gets confusing. Many --- but not all --- patients infected with *C. difficile* will get sick, be treated, and some time later after the treatment has stopped, relapse and the cycle starts again. Then there are some patients infected with *C. difficile* who will get sick, be treated, and that will be the end of it --- no more diarrhea or colitis. Interestingly, some of these patients will still carry *C. difficile*, most likely as spores, for extended periods of time but remain healthy. Occasionally, every couple of months or so,

some of these patients may relapse. Then there are the patients infected with *C. difficile* and who will carry the bug but not get sick. Why they are refractory, we don't know. Perhaps they have some innate resistance (no toxin receptors?) or perhaps they have antibodies against *C. difficile* and its toxins. In some hospitals there are more asymptomatic patients who are carrying *C. difficile* than those who actually get sick from the infection. Then there are the infants who very often carry *C. difficile*, even high levels of organism and toxin, and do not get sick. In fact, 50% or more infants in hospitals can carry this bug but not be any worse for wear because of it. You're probably getting the picture by now. This is a really mixed bag when it comes to figuring out who gets the disease, who doesn't, and who might get a severe form or relapse repeatedly with the disease. In other words, the presence of *C. difficile* does not always equal disease and the numbers of the organism certainly don't correlate with severity of disease.

There are several PCR tests on the market, and they are creating a buzz because of their exquisite sensitivity for detecting toxigenic *C. difficile* in fecal specimens. There's no question that this technology, which can detect the presence of just a couple of gene copies of the *tcdB* gene for toxin B, can do this. That's not the issue. The issue is, "what does a PCR-positive result actually mean and should all PCR-positive patients be treated"? Some facilities that switch to PCR testing will see their positivity rates for *C. difficile* increase by perhaps 50%, and it's going to look like an outbreak is occurring by switching to a new sensitive technology. But a legitimate question is whether all patients who might have been missed by previous tests, including the tissue culture assay which detects toxin B at picogram levels (that's why it is often considered the gold standard), should be treated.

This is what makes it so challenging for physicians. If everyone who is *C. difficile*-positive by PCR is treated --- and this can include, for example, people who have self-limiting diarrhea and who are carrying spores of *C. difficile* that are simply passing through the intestine --- then there will be patients who likely will be put at risk unnecessarily for a "true" case of *C. difficile* disease by unnecessary treatment. Of just as much concern is the possibility that treating all PCR-positive patients will result in overtreatment, leading to resistant hospital strains that don't respond to vancomycin or metronidazole. And PCR may pick up *C. difficile* spores, which are very common in hospital patients, so there are going to be a lot of patients who are positive.

Is there a way to help physicians identify PCR-positive patients who need immediate treatment? *C. difficile* disease can develop into a highly inflammatory disease and even death -- in pseudomembranous colitis, the pseudomembranes result from a very strong inflammatory component. Even in patients who haven't progressed to the pseudomembrane stage, there are high numbers of fecal leukocytes in patients who have colitis. We think that fecal lactoferrin, which is a biomarker for intestinal inflammation, serves as a very good indicator for patients who need immediate treatment. Lactoferrin can be used to help identify patients who are not only positive for toxigenic *C. difficile*, as indicated by PCR, but also positive for intestinal inflammation, and that immediate treatment (metronidazole or vancomycin) should be considered.

No diagnostic test is 100% accurate, but *C. difficile* and the manner in which it causes disease makes it especially challenging for physicians to determine the best approach for the patient. PCR testing will mean increased positivity rates for the hospital and for patients, more will be treated, some unnecessarily. A combination of tests, including ones that help determine severity, may help provide a more accurate diagnosis and approach to therapy.

First ribotype 027 … now ribotype 078

The recent increase in cases and possibly in severity of *C. difficile*-associated disease precisely parallels the emergence of *C. difficile* ribotype 027. This is the strain also variously called NAP1, REA type BI ("Bee eye"), and toxinotype III. The first 027 isolate, Cd196, appeared in 1986 in a French patient who had been in Montreal immediately before being ill in Paris. Before 2000, this ribotype was fairly uncommon, with all isolates being sensitive to moxifloxacin. Today ribotype 027 accounts for ca. 45% of North American isolates. Its incidence is lower in other countries. There are not many places where this particular strain is absent, although in Japan it is rare, and isolates are still mox-sensitive, like isolates before 2000.

You may have already seen another ribotype of *C. difficile* in the news. Ribotype 078 is Toxinotype V, REA type BK, and NAP 8. Ribotype 078 is the ribotype found in meats and in farm animals, especially pigs. Its frequency in humans is on the rise. We found this ribotype in samples collected in 2001 in Pittsburgh, in recent samples from at least two geographically distinct healthcare facilities, and in 20 year-old extraintestinal isolates from California. Perhaps 078 has the potential to be a zoonosis outbreak strain.

-Bob Carman

027 vs 078 …
What are the differences?

Property	027	078
Toxin A	Yes	Yes
Toxin B	Yes	Yes
Genetic defect in *tcdC*	A missing at position 117, causing a stop codon at 195	C at 184 replaced with T, causing a stop codon at 183
Deletion in *tcdC*	18 bp deletion	39 bp deletion
Binary toxin	Yes	Yes
Moxicillin	Resistant	Sensitive

Regarding ribotypes: In Germany, ribotype 001 is much more prevalent than ribotype 027 strains. Ribotype 001 strains frequently carry fluoroquinolone resistance genes (Zaiss et al., Emerg. Infect. Dis. 16:675-677, 2010).

STOOL NOTES

The giant montane pitcher plant of Borneo uses nectar to attract tree shrews and eats their droppings. Researchers used to think that the plant ate shrews since the "pitcher" was easily large enough to hold one. But they never found a shrew being eaten. Instead, researchers have proposed that the shrews are attracted to the plant nectar, and then the plant feeds on the droppings. A literal taming of the shrew.

More of the genes in our bodies are from our bacteria, in particular from our gut bacteria, than from our human cells. This is being referred to as the metagenome, a second genome within the human body. Researchers are looking at the gene sequences obtained from the feces of a pooled population of feces and hope to use this approach to study what the bacteria do instead of having to culture each species and analyze the DNA individually.

Sharks don't only eat other creatures, they eat poop --- shark tooth prints have been found in coprolites (fossilized feces). Perhaps the shark missed while trying to take a bite out of the backend of a victim.

A fungus found in the ancient droppings of mammoths has been used to raise an interesting theory that mammoths began to decline in number about 15,000 years ago due to a decline in the megafauna. By following the fecal fungus, researchers found that it

decreased, signaling a decrease in fauna. Environmental changes also accompanied the decrease.

Scientists used four genes, all involved with the junctions between the epithelial cells in the gut, to show that patients with ulcerative colitis possess leaky junctions between their epithelial cells compared to healthy persons. These defects may allow bacteria to leak back into the intestinal lumen, resulting in inflammation that is characteristic of ulcerative colitis.

Farm and meat animals make a lot of methane, which contributes significantly to greenhouse gas emissions. Probably about 6% is directly from the cows, especially cows grazing on wild grass. The methane comes both from belching and from the other end, and amounts to roughly 400 liters of methane per cow per day. The significance? Methane has a higher greenhouse effect than carbon dioxide.

We probably shouldn't ask this, but what do you do while on the toilet? In the UK, many read newspapers, books, and magazines, and a huge number talk on the phone or to family (hopefully through closed doors), and one in five sends texts. A good number don't bother to dry their hands after washing them. Don't be too hard on the UK folks. A study done at a U.S. microbiology meeting where you would expect pretty good hygiene, considering microbiology is the profession, showed that a significant number of bathroom users did not even wash their hands.

A cave in Oregon may provide more information on the migration pattern of early Americans. Fecal fragments in the cave were found to contain human mitochondrial DNA that resembled DNA from ethnic groups in Siberia and East Asia.

Mild to Severe C. difficile disease: How can we tell?

Clostridium difficile-associated disease (CDAD) involves a range of clinical presentations including mild to self-limiting diarrhea to life-threatening pseudomembranous ulcerative colitis and megacolon. Most cases are diagnosed based on clinical evaluations, history of antibiotic use and the presence of toxin in the stool (toxins A and B). Microwell and membrane EIAs are the most frequently used tests for detecting toxin in fecal specimens, with the gold standard being tissue culture combined with specific neutralization.

More recently, PCR tests have become available for determining the presence of *C. difficile tcdB* (*C. difficile* toxin B gene) and these are used as stand alone tests or in combination with the detection of glutamate dehydrogenase antigen (GDH) for ruling out *C. difficile*-negative patients and confirming *C. difficile*-positive patients. All of these assays are suitable for detecting the presence of *C. difficile* and its toxins as an aid to diagnosis but do not provide information regarding severity of disease. In general, about 30% of patients with CDAD present with fever, 50% have a slightly raised wbc (white blood cell count, leukocytosis) and 20% experience mild abdominal pain. Mild cases respond well to antibiotics and even to no treatment at all. Moderate to severe CDAD cases need early detection for a better outcome and for decreased mortality.

Stratifying CDAD patients based on severity isn't a new concept but it has gained attention because of the increase in incidence and frequent severity of CDAD over the past decade. In a study published by L. Kyne et al., the authors performed a detailed characterization of disease status in an outbreak of CDAD in Dublin, Ireland. This particular outbreak involved 14 patients who were stool cytotoxin positive but asymptomatic. Of the symptomatic patients, 25% had mild self-limiting disease with no antibiotic treatment, 35% had moderately severe CDAD responding to antibiotic treatment and 40% developed severe

disease with prolonged symptoms lasting between 11 to 36 days. A total of 8% of the severe CDAD patients progressed to severe colitis with pseudomembranes and toxic megacolon. The authors concluded that early indicators of disease severity are needed in order to lower the morbidity and mortality.

Currently, a combination of the clinical presentation plus various lab parameters have been proposed for stratifying patients by disease activity (mild to severe). White blood cell count, serum albumin level (indicator of leakage into the bowel), and creatinine level for monitoring kidney failure are the most commonly used lab indicators for disease activity for CDAD. Mild to moderate cases of CDAD usually present with a wbc ≤ 15,000/μL, normal serum creatinine (< 2.0 mg/dL) and albumin levels (≥ 2.5 g/dL). Symptoms range from less than 10 watery stools without blood per day to mild cramping lasting for up to an average of 4 days. The common treatment for patients with an initial episode of mild to moderate CDAD is 500 mg metronidazole 3 times daily for 10 days. Most cases resolve with no further complications but up to 25% of these cases may relapse and require a second round of antibiotics. Relapses are not limited to a single episode; patients can relapse multiple times.

Patients over the age of 65 are at a higher risk for CDAD and more often suffer from more severe disease leading to multiple relapses. Severe fulminant CDAD is characterized by having 11 or more liquid stools per day for more than 10 days. Fecal specimens often contain mucus and may be bloody. Defined lab parameters for fulminant *C. difficile* colitis are wbc ≥ 15,001/ μL, a rising serum creatinine (50% increase and levels ≥ 2.0mg/dL) indicating poor kidney function and albumin levels dropping below 2.5 d/dL showing loss of protein because of exudation of serum into the bowel. Clinical presentations may involve pseudomembranes on endoscopy, severe abdominal pain and cramping, and colonic thickening observed by CT scan. Toxic megacolon stemming from ileus may occur causing nausea, vomiting, severe dehydration and extreme lethargy. Treatment for severe cases of CDAD usually involves 125 mg vancomycin 4 times per day for 10 days.

Identifying disease activity for patients with CDAD is imperative for proper treatment and better outcome with decreased morbidity and mortality. A new diagnostic parameter for assessing severity in CDAD is the measurement of fecal lactoferrin as an indicator for intestinal inflammation. CDAD is an inflammatory disease involving the infiltration of activated neutrophils across the mucosa into the lumen causing colitis and in some cases, the formation of pseudomembranes.

Human lactoferrin is a glycoprotein that is present in most mucosal secretions and a primary component of the granules of activated neutrophils. During the onset of intestinal inflammation, activated neutrophils infiltrate the intestinal lumen causing an increase in fecal lactoferrin. Clinical studies involving patients with inflammatory bowel disease (IBD) and noninflammatory irritable bowel syndrome (IBS) have shown that lactoferrin levels in healthy person are similar to levels in IBS patients but increased in patients with active IBD. Changes in the level of fecal lactoferrin can be used to determine the clinical response to medical therapy and help predict a relapse of active disease. Recent clinical studies have shown that lactoferrin levels for IBD patients in confirmed remission return to baseline levels as seen in healthy persons. In addition, levels increase rapidly during active disease and even precede clinical symptoms by a mean period of 3 weeks. New studies are underway for evaluating a similar approach for assessing disease activity in patients with CDAD.

Fecal specimens are routinely collected for *C. difficile* testing; thus additional testing can be done easily to determine the level of fecal lactoferrin for determining the amount of intestinal inflammation and severity of disease. In a recent in-house study, fecal *C. difficile* glutamate dehydrogenase (GDH), toxins A and B, and fecal lactoferrin levels were measured in two subjects with *C. difficile* disease during antibiotic treatment. Both subjects had clinically confirmed *C. difficile* disease and were monitored for the

presence of glutamate dehydrogenase (GDH), toxins A and B and fecal lactoferrin by EIA. Specimen collection was initiated at the start of antibiotic treatment and was continued on a daily to weekly basis when possible. A symptom log was kept by each patient and all treatments were recorded during the test period. Both patients showed a rapid response to antibiotic treatment with fecal GDH, toxins A and B and fecal lactoferrin reaching baseline within 24 hours. Antigen, toxin and fecal lactoferrin remained negative during the antibiotic therapy.

Following the treatment, both patients experienced a clinical relapse and showed a rapid increase for all parameters. Following a second course of antibiotics, all parameters returned to baseline. At completion of the second course of antibiotics, all parameters increased rapidly in absence of clinical symptoms. Both GDH and toxin remained present for 3 to 4 weeks but fecal lactoferrin quickly returned to baseline. No antibiotics were administered since there were no clinical symptoms and the patients remained healthy.

In this evaluation, we observed that *C. difficile* GDH, toxin and fecal lactoferrin levels responded quickly to antibiotic therapy by returning to baseline (negative). More interestingly, both GDH and toxin were present without clinical symptoms and with no intestinal inflammation as determined by baseline lactoferrin. These results suggest a utility for fecal lactoferrin in determining which cases of CDAD may require no further treatment with antibiotics. Further investigation is needed to determine the role of fecal lactoferrin for monitoring *C. difficile* disease and for determining the significance of having *C. difficile* toxin without clinical symptoms. By determining the amount of intestinal inflammation using lactoferrin in CDAD patients along with symptom assessment, the stratification of patients for severity of disease may prove useful for optimizing treatment and patient care.

-James Boone

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The Joy of Travel?

"*Two roads diverged in a wood and I – I took the one less traveled by*." Robert Frost

Perhaps Mr. Frost spent most of his time in New England, and not quite as much traveling the back roads of our sister continents. It seems that some of the world's less traveled roads present diversions from your trip beyond an alternative view of life. Maybe a better suggestion would be to listen to your mother…"Never pick up hitchhikers".

In 2006, Freedman et al.[1] published a comprehensive report on diseases and conditions resulting from worldwide travel, as reported by 30 GeoSentinel sites located on six continents. The results provide a unique view of the potential ramifications of intercontinental excursions. It also highlights the significance of parasitic-related diseases acquired during international travel. The study revealed *Giardia* and *Entamoeba histolytica* as the most prevalent causes of acute diarrhea in returning travelers.

The GeoSentinel Report returned data for 17,353 patients reporting to the registered travel clinics from 1996 to 2004. In total, there were 1122 reports of disease, injury and/or other conditions per 1000 individuals (an average of 1.12 conditions per clinic patient). Of the patient reports, 30% were either acute (20%) or chronic (10%) diarrhea. For all regions studied except Southeast Asia, parasite-induced acute diarrhea was more common among ill returned travelers than bacterial-induced acute diarrhea. Parasitic diarrhea accounted for 35% of all acute diarrheal cases, with *Giardia* and *E. histolytica* accounting for 49% and 34% of the parasitic diarrhea cases, respectively. The infection rate for each of these two parasites individually was roughly equivalent to the infection rate of *Campylobacter*, *Shigella*, and *Salmonella* infections combined.

So, we wish you the safest of travels. But, whether you chose Mr. Frost's advice or not, listen to your mother and stay away from hitchhikers – macroscopic and microscopic.

-Joel Herbein

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[1]Freedman DO, Weld LH, Kozarsky PE, Fisk T, Robin R, von Sonnenburg F, Keystone JS, Pandey P, and Cetron MS for the GeoSentinel Surveillance Network. 2006. "Spectrum of Disease and Relation to Place of Exposure among Ill Returned Travelers". *The New England Journal of Medicine*. 354(2):119-130.

Intestinal Protozoan Parasites. In a Category All Their Own?

Actually, no. The intestinal protozoan parasites *Giardia lamblia*, *Cryptosporidium parvum/hominis*, and *Entamoeba histolytica* are part of a larger group known as "Category B Priority Pathogens". Categorized in 2002 by the National Institute of Allergy and Infectious Disease's Blue Ribbon Panel on "Biodefense and Its Implications for Biomedical Research", *Giardia*, *Cryptosporidium* and *E. histolytica* are considered a threat due to their possible dissemination through compromised food and water supplies in the United States (www.niaid.nih.gov). The Blue Ribbon Panel placed an emphasis on the development and implementation of diagnostic tests and therapies specific for these pathogens. The three parasites are significant pathogens in the United States and share several common traits that would readily permit spreading of an infectious dose to susceptible populations.

Organism	Rate per 100,000 in the U.S.	Chlorine Resistance	Infectious Dose	Environmental Stability
Giardia	9.5	Moderate	Low (<100)	High
Cryptosporidium	1.4	High	Low (<100)	High
E. histolytica	1.2	Moderate	Low (<100)	High

C. difficile CDT (Binary Toxin) in Feces of Humans with Diarrhea

CDT is a binary toxin produced by ribotype 027 and other isolates of *C. difficile*. It is the most famous example of a very highly conserved class of clostridial binary toxins. Type E isolates of *C. perfringens* make iota toxin while the helically-coiled *C. spiroforme* makes the iota-like, S toxin. Each binary toxin consists of two (hence binary) unrelated, separate gene products.

Iota toxin consists of ia (the enzymatic mono-ADP-ribosyltransferase) and ib (cell binding/membrane translocation component), [8,9]. The enzymatic and binding components of CDT and S toxins are similarly designated cdtA and Sa and cdtB and Sb. Neither the enzyme nor the binding component by itself has toxic activity. Sb and ib are secreted as propeptides requiring proteolytic activation by clipping at an Ala:Ala link to achieve their mature forms. Activation of pro-iB to ib and of pro-Sb to Sb occurs readily during *in vitro* growth. The predicted amino acid sequence of pro-CDTb reveals no Ala:Ala linkage between the pro-peptide and mature CDTb such as is found in pro-ib and pro-Sb and at which the propeptidase cuts [2]. The *in vitro* activation of pro-CDTb requires supplementary trypsin that apparently cuts the immature peptide at an Arg:X bond almost immediately adjacent to the dysfunctional clip site [2]. It is entirely feasible that host proteases activate pro-CDTb *in situ*. Mature ib rapidly oligomerizes on the surface of an exposed cells where it forms a heptameric, donut-shaped channel through which ia, the enzyme, enters the cell following endosome acidification [10]. Once in the host cell, ia mono-ADP-ribosylates globular actin. This ultimately kills the cell by disrupting filamentous actin formation and the cell cytoskeleton [1, 3].

Clostridial binary toxins are biologically active. They kill mice when injected intraperitoneally and are dermonecrotic when injected intradermally into the flanks of guinea pigs [4]. The binary toxins are enterotoxic and cause fluid accumulation in ileal loops in rabbits [5]. Clostridial binary toxins are cytotoxic [6]. All of these activities are neutralized by antiserum to one or both of the binary components. Additionally, since each component is also cross neutralized by antiserum to the equivalent component of the other binary toxins, anti-iota toxin neutralizes S toxin and anti-Sb will neutralize *C. perfringens* iota toxin, etc. These results confirm the high degree of structural similarity between these binary toxins. It should come as no surprise, therefore, to learn that chimeric toxins (i.e. Sb mixed with ia, ib with Sa, etc.) are biologically active [7]. Schwan *et al.* [18] has recently ascribed a novel biological function to CDT with implications for virulence. CDT induced the formation of microtubules protruding from the intestinal epithelial cells. These microtubules formed a dense mesh, rich in specific capture proteins that ensnared *C. difficile,* extending its period of contact with the host's epithelium.

Popoff and his group were the first to describe CDT [13]. They reported an ia-like ADP-ribosyltransferase activity in the culture fluids of *C. difficile* 196, an historical ribotype 027 isolate from a human with diarrhea. They went on to show, the production of enzymatic and binding components for a binary toxin that they called CDT and showed it to be very similar to both iota and S toxins in terms of activity, antigenicity and genetics [14]. They also showed that in *C. difficile*, expression of cytotoxic levels of CDT was approximately forty-fold lower than that for *C. perfringens* iota toxin, a finding since confirmed by others [2]. A low level of transcription rather than any reduction in specific activity, explained the shortfall [13]. While *cdtR* has been shown to regulate CDT expression in *C. difficile* [15], the gene is absent from *C. spiroforme* and *C. perfringens.* In fact, each binary toxin locus has a unique sequence upstream of the gene for the enzyme, distinct from the other two loci. This may point to three distinct mechanisms of regulation.

In about 2000, resistance among *C. difficile* isolates to moxifloxacin began to burgeon. Though many different strains have become resistant, ribotype 027 was the dominant epidemic strain in several outbreaks worldwide [16,17] that followed. Even if resistance to fluoroquinolones helps explain the increased incidence of *C. difficile*

diarrhea, it has surely less to say about the rise in the severity of *C. difficile* 027 disease reported by some, though not all. Any possible link between increased severity and 027 infections would thus require another explanation. There is the deregulation of toxins A and B expression during early log-phase growth in the test tube that is assumed to translate to increased toxin production *in vivo*. This is caused not by the widely reported deletions in the down regulator, *tcdC*, but by the less well-known stop codon resulting from a point mutation in *tcdC*. Increased, or at least, earlier toxin A and B production may indeed help explain the observed rise in severity of symptoms.

So might CDT. Some isolates, most notably ribotype 027, carry and express the entire CDT locus consisting of *cdtR, cdtA*, *cdtB*. Other isolates carry a ghost locus, consisting of the entire *cdtR* but only parts of c*dtA* and *cdtB*. These do not make CDT. A third set of isolates lack the locus altogether. Though many researchers have, using PCR, identified isolates that carry the CDT locus, very few have shown *in vitro* production of CDT. Fewer still have looked for CDT in feces. IS 58, an otherwise non-toxic isolate lacking genes for toxins A and B, made low levels of CDT [2]. It was enterotoxic. However, hamsters infected with IS 58 did not have diarrhea, though whether there was CDT in the hamsters stool is not known. Nevertheless, this evidence suggests no role for CDT in disease. Whereas, in an unpublished study, TechLab used an enzyme immunoassay to detect cdtB, the binding component of CDT, in antibiotic-associated diarrheal samples from humans and from which *C. difficile* was cultured. The estimated levels of fecal cdtB in 12 of 19 positive samples exceeded 100 ng/mL. If correct, these 12 samples contained a level of CDTb that, had it been Sb, would have been lethal to mice [12] and exceeded the level of Sb in cecal digesta from clearly diseased, diarrheic rabbits [11]. So, even if CDT does not have a direct role in *C. difficile*-induced diarrhea, it may reach levels that contribute more indirectly to the overall clinical picture, in a complementary way not seen with otherwise non-toxigenic isolates like IS 58.

In summary, while a role for iota and S toxins in causing diarrhea is clear, any similar role for CDT remains unproven. Fecal levels from cases of human disease do, however, on occasions match the levels of S toxin in diarrheic animals.

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