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MICROBIAL COMMUNITY DYNAMICS IN *CLOSTRIDIUM DIFFICILE* INFECTION: CONNECTING THE DOTS

CANÁ ROSS[1,2], NUMAN OEZGUEN[1,2], SARA DANN[3], PRAPAPORN BOONMA[1,2], ANTHONY HAAG[1,2], JENNIFER K. SPINLER[1,2], and TOR SAVIDGE[1,2]

[1]Department of Pathology and Immunology, Baylor College of Medicine; [2]Texas Children's Microbiome Center, Texas Children's Children Hospital; and [3]Internal Medicine, University of Texas Medical Branch, Houston, Texas, USA.

SUMMARY

The Human Microbiome Project established a deep, molecular understanding of interactions between microbial communities and their hosts, and provided insight into how the host and epigenetic factors can influence intestinal ecosystems. In this context, we give a brief overview of the capacity for metagenomics and metabolomics to explore host-pathogen interactions over multiple life stages. Young children are naturally resistant to toxigenic *Clostridium difficile,* which forms part of the normal developing microbiome. By studying both paediatric and adult cases, we hope to discover why susceptibility to *C. difficile* changes as we age. Our mini-review considers current risk factors in *Clostridium difficile* infection and emerging treatment options, including faecal microbiota transplantation as promising therapy when antibiotics fail. Manipulation of the intestinal microbiota offers a relatively unexplored niche for targeted therapy, but incomplete genome coverage, bioinformatics discrepancies and inferred biological function highlight the need for a multi-'omic approach to complement next generation sequencing and provide a direct measure of the altered intestinal phenome.

INTRODUCTION

Antibiotic resistance among pathogenic bacteria is a major emerging public health threat with estimates of worldwide deaths reaching 10 million annually by 2050 (*Review on Antimicrobial Resistance*, 2014). Overuse of antibiotics and adaptability of bacteria have created a growing number of "super bugs" that are resistant to multiple antibiotics. *Clostridium difficile* is one such organism and is rapidly becoming one of the major public health threats of the 21st century. *C. difficile* is a spore-forming anaerobe that is the single leading cause of nosocomial infections in some hospitals (*Pant* et al., 2013; *Magill* et al., 2014). In the western world, it is by far the most deadly enteric pathogen triggering colonic disease due to the secretion of two potent exotoxins (Figure 1) (*Taylor* et al., 1981; *Savidge* et al., 2003; *Genth* et al., 2008; *Kuehne* et al., 2010). The incidence and severity of *C. difficile* infection (CDI) has risen dramatically in the United States since 2000 with almost half a million annual cases including 30,000 deaths in 2011(*Lessa* et al., 2015). Because of this perceived threat to patients, the Centers for

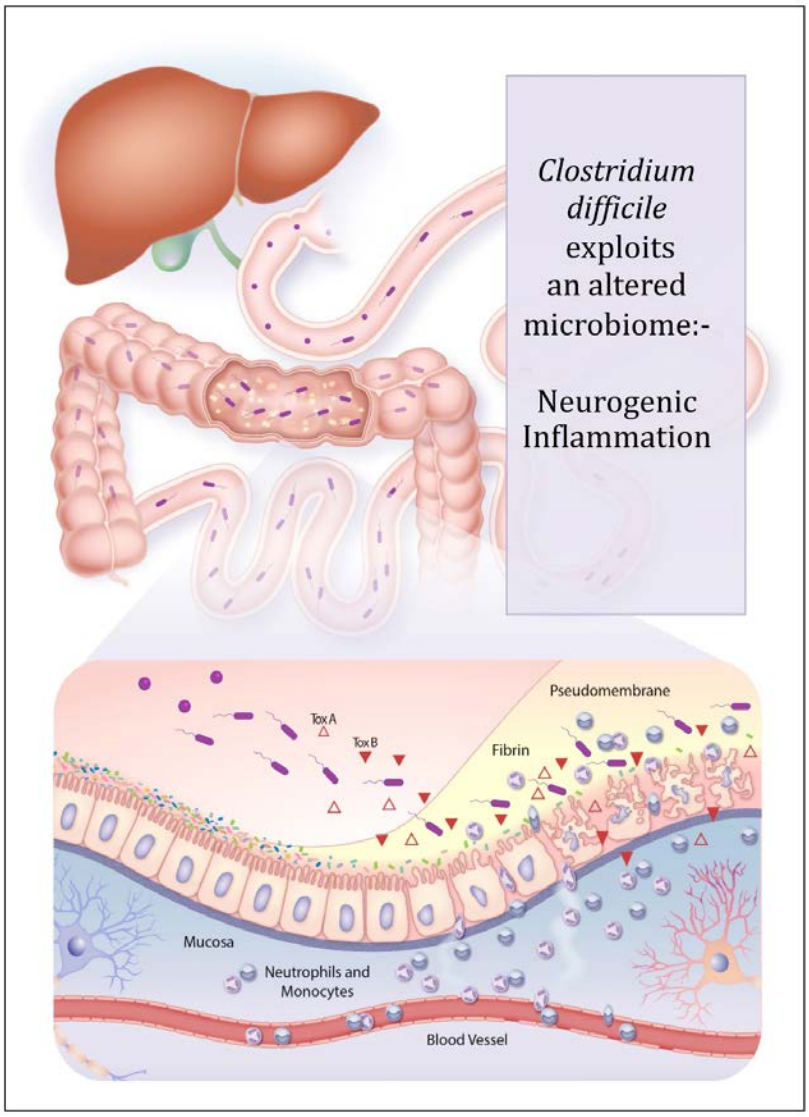

Figure 1: Schematic of *C. difficile* infection in a patient.

Disease Control and Prevention assigned an urgent hazard level (*Centers for Disease Control and Prevention*, 2013) to this pathogen and urged the scientific community to identify risk factors to better manage new, but expensive treatment (e.g. Dificid®).

Due to the growing international concern regarding both clinical management and dissemination of *C. difficile*, the United States and Europe now require hospitals to report symptomatic CDI cases. Because *C. difficile* is resistant to most antibiotics, first line therapy includes off-label use of metronidazole or oral vancomycin in clinically severe and recurrent cases (*Cohen* et al., 2010; *Goldberg* et al., 2015). However, up to 35% of CDI patients will experience a clinical recurrence following cessation of antibiotic use despite a favourable response to treat-

ment (*McFarland* et al., 1999; *Garey* et al., 2008; *Johnson*, 2009; *Kelly*, 2012; *Goldberg* et al., 2015). Of these patients, up to 50% will experience subsequent infective episodes, adding to patient morbidity (*Fekety* et al., 1997; *McFarland* et al., 1999, 2002). Notably, half of the recurrent episodes involve a new *C. difficile* strain, strongly suggesting that epigenetics and the intestinal ecosystem modulate host susceptibility to this pathogen (*Young* and *Schmidt*, 2004; *Garey* et al., 2008; *Antonopoulos* et al., 2009; *Rupnik* et al., 2009; *Centers for Disease Control and Prevention*, 2012; *Britton* and *Young*, 2012; *Peery* et al., 2012; *Theriot* et al., 2014). The heralded clinical success of faecal microbiota transplantation (FMT) in recurrent CDI cases (>90% efficacy) (*Burke* and *Lamont*, 2013; *McKinney*, 2013) is strongly supportive of host-microbe interactions being important in preventing CDI onset. The procedure involves single to multiple orogastric or intracolonic infusions of faecal bacteria originating from healthy donors. Typically, only a single treatment is necessary to eradicate disease in patients that previously experienced multiple recurrent CDI episodes. Although FMT is considered a medical triumph against recurrent CDI, it is also regarded as a treatment of last resort because of safety and social concerns, especially in children. After an initial 2013 FDA regulatory ruling (*McKinney*, 2013) FMT is now only available for recurrent CDI in a limited number of health centres. Due to limited access, self-administered FMT is becoming a common practice in the community, raising additional ethical and safety concerns.

CURRENT TREATMENTS FOR CDI

Current herapeutic options for *C. difficile* include metronidazole (currently the most common treatment), oral vancomycin and the newly approved fidaxomicin (Dificid®). Access to vancomycin and fidaxomicin is somewhat restricted due to high cost. However, vancomicin has increased market share with significant compounding occurring in hospital pharmacies, allowing capitalization of cheaper, generic formulations. Fidaxomicin is an important new antibiotic that reduces recurrent episodes with some strains of *C. difficile*. However, a critical liability may be a reduced effect on recurrence for the clinically important epidemic strains (24.4% vs. 23.6% for FXD and VA for ribotype 027) demonstrating a significant market opportunity for alternative approaches, notably microbial therapeutics with either defined bacterial communities or single non-toxigenic *C. difficile*.

It is proposed that toxin inactivation by the Merck antitoxin IgG monoclonal antibodies provide protection against recurrence. Merck recently advanced an antibody combination into Phase III clinical trials. Reported Phase II trial results demonstrated that single injections, when used with standard antibiotics, reduced disease recurrence to as low as 7%. A cogent rationalization for the reduction is lacking but Merck's investment provides evidence of substantial interest in a therapy directed toward reducing CDI recurrence. Other experimental therapies currently in development include vaccines, toxin-absorbing polymers, bile acid analogues and probiotics. Notably, toxin-binding resins also target bile acids. In support of a vaccine development program, antibodies against both toxins are protective in hamsters, and serum

anti-toxin antibodies in patients correlate with protection against symptomatic disease and recurrence. However, the scope of antitoxin vaccination remains uncertain since patients with severe CDI are usually elderly and critically ill. While effective in hamster CDI models, toxoid-based vaccines currently in Phase III trials have not proven as protective in patients and are associated with several inherent shortcomings, including batch-to-batch variations and potential residual toxicity. Given this critical need to generate predictors of clinical outcome, there is currently much interest in understanding the intestinal ecosystem that is vulnerable to *C. difficile* and epigenetic factors that promote antibiotic resistance in this pathogen.

ALTERED MICROBIAL COMMUNITY DYNAMICS IN CDI

Antibiotic exposure is a major risk factor for CDI development due to disruption of the indigenous microbiota (*Young* and *Schmidt*, 2004; *Jernberg* et al., 2007; *Dethlefsen* et al., 2008). However, the specific changes in microbiome structure that lead to increased risk are poorly defined. In an effort to increase our understanding of colonization resistance and identify microbial communities that are strongly associated with CDI risk, several investigators have characterized differences in the microbiomes of subjects with and without CDI (*Manges* et al., 2010; *Antharam* et al., 2013; *Vincent* et al., 2013; *Schubert* et al., 2014), as well as in patients before and after FMT (*Song* et al., 2013; *Dutta* et al., 2014; *Seekatz* et al., 2014). These studies primarily utilized three methods to assess the differences between the intestinal communities from groups of individuals: (1) alpha-diversity, which describes the microbiota community in terms of richness or diversity, (2) beta-diversity, a comparison of communities between samples, and (3) comparisons of the relative abundance of microbial taxa between individuals or clinical groups (*Schubert* et al., 2014). Results from these studies consistently illustrate that microbial communities in patients with CDI and antibiotic-associated diarrhoea (AAD) are less diverse and structurally different than those isolated from healthy subjects. These studies found that healthy stools were dominated by Bacteroidiaceae, Lachnospiraceae and Ruminococcaceae, while CDI and AAD patients typically exhibited an enrichment of Enterobacteriaceae, Enterococcaceae and Lactobacilliaceae (*Manges* et al., 2010; *Antharam* et al., 2013; *Song* et al., 2013; *Dutta* et al., 2014; *Schubert* et al., 2014; *Seekatz* et al., 2014).

In children, the clinical significance and outcomes associated with CDI remain poorly defined, even though the incidence is rising (*Nylund* et al., 2011). It is well established that young children are more refractory to CDI than adults despite the fact that microbiome studies clearly demonstrate that these populations exhibit similar low diversity (*Larson* et al., 1982; *Al-Jumaili* et al., 1984; *Jangi* and *Lamont*, 2010). Although colonization with toxigenic *C. difficile* is prevalent during the first two years of life, clinical disease is rare (*Larson* et al., 1982; *Sandora* et al., 2011; *Rousseau* et al., 2012). One study found that asymptomatic carriage rates increase in the first year of life and then drops to 6% by 24-36 months (*Rousseau* et al., 2012). While there is speculation regarding passive transfer of maternal antibodies (*Rolfe* and *Song*, 1995; *Dallas* and

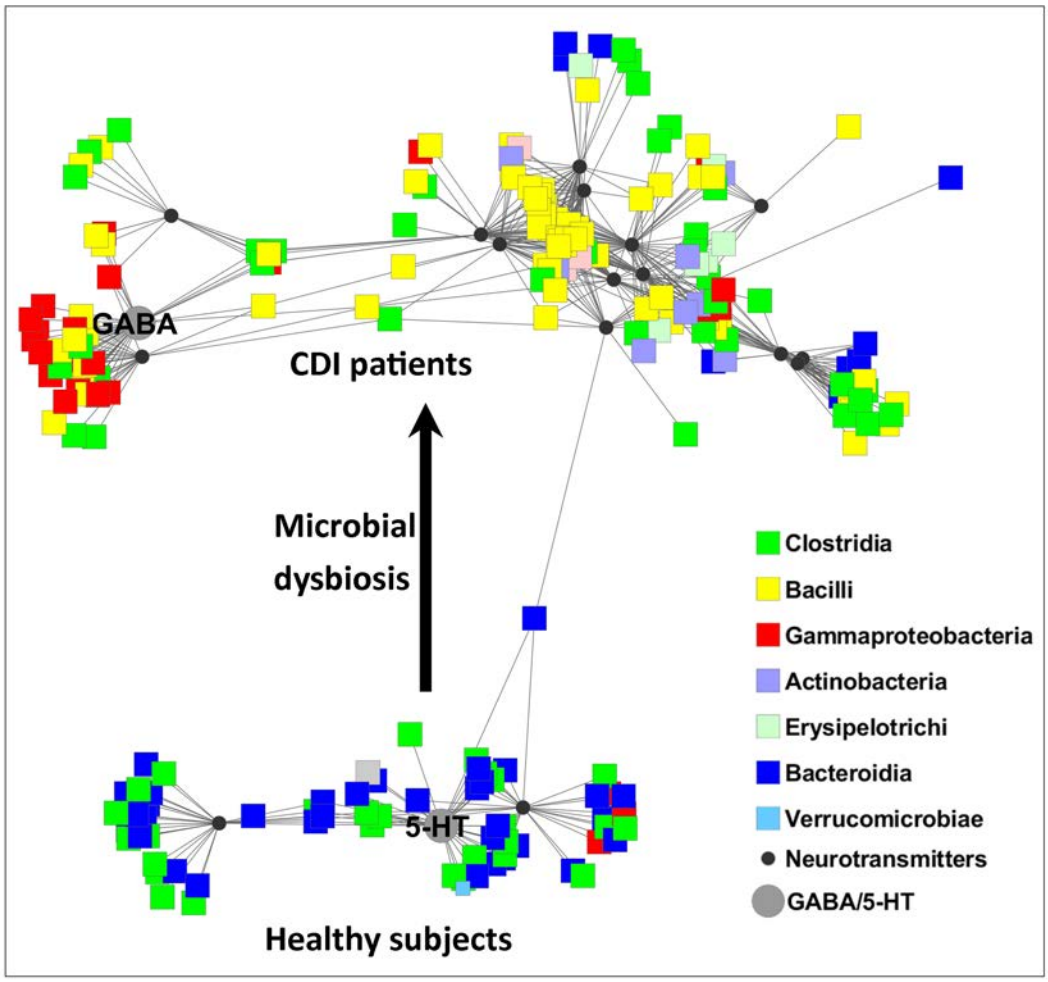

Figure 2: Operational taxonomical units (OTU)-Neurotransmitter correlation sub-networks: OTU's are shown as squares and coloured according to their assigned taxonomic class. The neurotransmitters are shown as black circles. The specific neurotransmitters, GABA and 5-HT, which are associated with disease and healthy subjects, respectively, are highlighted as larger gray circles.

Rolfe, 1998), a protective gut microbiome during infancy and a lack of toxin receptors (*Eglow* et al., 1992) have been proposed, the mechanism for asymptomatic *C. difficile* carriage in this population remains unknown. Not surprisingly, molecular diagnostics, which are more sensitive and specific, are problematic in this population. Aside from the >50% increase in CDI incidence, concerns about detection of colonization rather than true disease have been raised (*Gould* et al., 2013; *Longtin* et al., 2013; *Moehring* et al., 2013). A recent study by *Leibowitz* et al. (2015) found that hospitalized children aged 1-18 years (19% with diarrhoea and 24% without diarrhoea), tested positive for *C. difficile* by *tcdB*-specific PCR. Furthermore, high rates of *C. difficile* colonization have been reported in paediatric populations with additional co-morbidities, such as cancer (*Dominguez* et al., 2014) and IBD (*Hourigan* et al., 2013; *Pant* et al., 2013). As a result, the American

Academy of Pediatrics guidelines caution that testing for CDI should be exclusively performed on children who meet the clinical criteria (*Schutze* et al., 2013). They also note that test results for infants may be difficult to interpret due to high asymptomatic colonization rates and suggest that in children between 1 and 3 years of age, other causes of diarrhoea should be considered and tested for, even if the *C. difficile* test is positive. Notably, children with co-morbid conditions, such as IBD, are more susceptible to CDI, are more likely to recur and are prone to treatment failure. Moreover, CDI is known to affect IBD severity and is associated with higher rates of hospitalization (*Pant* et al., 2013; Kellermayer, 2015; *Sandberg* et al., 2015). The ability to identify symptomatic CDI in a population with high rates of asymptomatic *C. difficile* carriage would improve diagnostics and treatment for at-risk children. Furthermore, a better understanding of disease resistance in the paediatric population is likely to identify key host and microbial metabolic pathways, along with microbial species that may protect young children from developing clinical disease despite a lack of microbial diversity.

Through a re-examination of published microbiome data and our own metagenomics analyses, we found that in addition to the altered community dynamics described above, expansion of the Peptostreptococcaceae and Clostridiaceae families occur in adult and paediatric CDI populations, especially in recurrent cases. Bipartite correlation networks of stool operational taxonomical units (OTU) with neurotransmitter levels show that many OTUs associated with CDI development share identity with apparent γ-aminobutyric acid (GABA) producing Clostridiales, whereas OTUs associated with mucosal serotonin are lost (Figure 2). Both GABA and serotonin are potent neurotransmitters with important regulatory function in the intestine, including homeostasis of motility, mucosal barrier, blood flow and secretion, and modulating immune function. Preliminary global metabolomics profiling of CDI patient stool specimens demonstrate inverse correlations between GABA and serotonin that predict treatment failure in CDI patients (Figure 2). Intracellular recording of sensory neurons in the intestine confirm luminal GABA alone can induce action potentials that are likely enhanced by the *C. difficile* toxins. These findings are supported by potent GABA-producing Clostridiales yielding increased morbidity and mortality in animal models of CDI.

MICROBIAL NEUROTRANSMISSION IN THE INTESTINE

Our preliminary multi-'omics data are of interest because recent reports link serotonin receptor uptake inhibitors (SERT) and zolpidem (a $GABA_A$ receptor agonist) use with CDI development in at-risk patients. Therefore, microbial-derived neurotransmitters may represent novel druggable disease targets for therapeutic intervention in CDI although not all microbial-derived neurotransmitters potentiate CDI pathogenesis (Figure 3). Our laboratory recently demonstrated that luminal nitric oxide intermediary signals are capable of ameliorating CDI pathogenesis via a mechanism involving S-nitrosylation and inactivation of the *C. difficile* toxins (*Savidge* et al., 2011). The intestine is a rich source of nitric oxide and hydrogen sulphide neurotransmitters

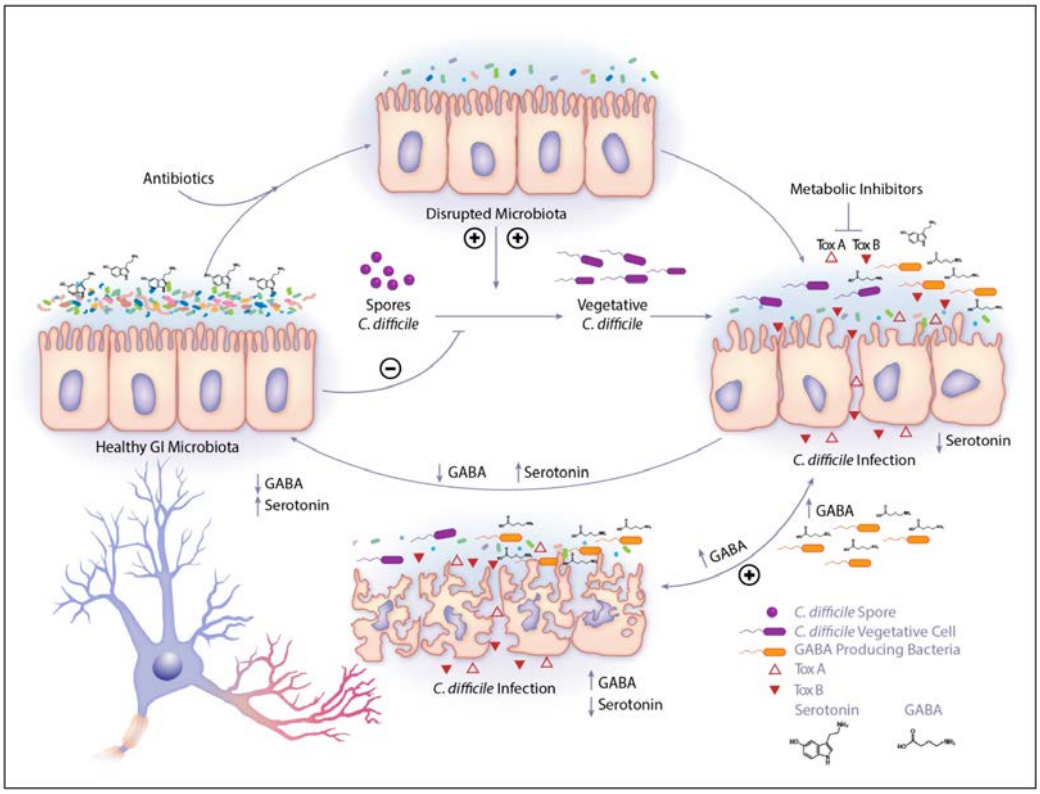

Figure 3: Antibiotic administration alters the intestinal microbiota, creating an environment that favours spore germination through reduced bile acid conjugation, and exacerbates *C. difficile* toxin activity by production of GABA and reduction of mucosal serotonin.

generated from bacterial conversion of dietary substrates such as nitrites. For example, *E. coli* is a prominent species that generates bioactive nitric oxide in both the small and large intestine using different oxygen dependent mechanisms. Because antibiotic-induced dysbiosis in CDI is associated with expansion of Enterobacteriaceae (*Song* et al., 2013; *Schubert* et al., 2014; *Seekatz* et al., 2014), this has the likely consequence of shifting the balance of nitric oxide generating bacteria in the colon. Our inability to culture the majority of microbial species in the intestine limits characterization of other neurotransmitter regulators in CDI. For example, expansion of antibiotic resistant Lactobacilliaceae in CDI patients may be associated with elevated histamine signalling, an important regulator of intestinal permeability, immune function and motility. Similarly, depletion of short chain fatty acid producing bacteria in CDI patients may exert epigenetic effects on neuronal signalling in the intestine resulting in altered immune responses, intestinal motility and luminal pH. At present, it is not clear whether alterations in microbial-derived neurotransmitters represent cause or consequence in CDI disease pathogenesis, but emerging data suggest further studies are needed to establish their role.

In conclusion, treatment options for patients experiencing recurrent CDI are limited and often involve long-term antibiotic administration, which poses a serious threat to development of

anti-bacterial resistance and reinfection by *C. difficile*. Because new antibiotic treatments are currently regarded as cost prohibitive as frontline treatment for CDI and efficacy decreases significantly with each recurrence, identifying at-risk patients for treatment failure is imperative. We expect that multi-'omics biomarkers may offer some candidate leads and provide insight into an alternative clinical cure that is not dependent on new antibiotics.

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