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Digging Deep in the Microbiome to Diagnose *Clostridioides difficile* infection

Alison Laufer Halpin[1], **L. Clifford McDonald**[1]

[1]Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention, Atlanta, GA

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Clostridioides (formerly *Clostridium*) *difficile* accounts for close to 500,000 infections and 14,000 deaths each year in the United States (1). However, toxin gene-positive *C. difficile* can also be found in colonized patients, some of whom have diarrhea from other causes, leading to diagnostic confusion, especially in the era of molecular diagnostics. It was quickly realized that detecting toxigenic *C. difficile* alone via anaerobic culture was not specific, leading to early reliance on the functional detection of toxin via the cell cytotoxin neutralization assay. However, this test was cumbersome with a slow turnaround. Toxin enzyme immunoassays (EIAs) eventually replaced the functional assay but never achieved its sensitivity and concerns surfaced over missed diagnosis of CDI with poor outcomes. It was hoped that development of nucleic acid amplification tests (NAATs) for *C. difficile* toxin genes could advance the rapid, sensitive diagnosis of CDI. Yet *C. difficile* infection (CDI) remains a clinical diagnosis based upon a consistent history (e.g., often, but by no means uniformly, previous antibiotic use), symptoms (e.g., unexplained diarrhea, cramping), and physical findings (e.g., fever, abdominal tenderness), that is confirmed as being caused by *C. difficile* through a positive *C. difficile* diagnostic assay performed on the stool.

There is now increasing concern that the use of NAATs may lead to over-diagnosis of CDI and many patients who are NAAT-positive, but negative for toxin by EIA, may not have true CDI (i.e., positive for *C. difficile* by culture and for toxin by EIA). Several studies suggested disease and outcomes are more severe in toxin EIA-positive patients (2). However, another recent study suggests that even sensitive, quantitative detection of toxin in stool cannot differentiate diarrhea caused by *C. difficile* from that due to other causes in the presence of colonization by the toxigenic organism (3). Accurate diagnosis is critical as unnecessary exposure of patients to CDI-specific antibiotic therapy contributes significantly to an already disrupted lower intestinal microbiome, resulting in both recognized and potential outcomes

Corresponding Author: Alison Laufer Halpin, 1600 Clifton Road, MS H17-5, Atlanta, GA 30329; 404-639-1776; ALaufer@cdc.gov;.

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of prolonged diarrhea, colonization with multidrug-resistant organisms, bacteremia, and sepsis (4).

The advent of next generation sequencing technology presents an opportunity to identify new targets for diagnosis, as well as to improve our understanding of pathogenesis and appropriate treatment. This includes whole genome sequencing of isolates, as well as microbiome-based approaches. However, the focus, to date, has been on using 16S ribosomal RNA (rRNA) amplicon sequencing to define the composition of the microbiome, or "who is there". 16S rRNA sequencing is relatively easy to perform but provides limited data on strain-level differences, function, or pathogenesis. Some tools have been developed that link composition and function using predictive metagenomics to make a best guess at what roles the bacterial residents in a community are performing (e.g., PICRUSt; picrust.github.io/picrust/). However, this approach lacks the ability to discern functions that are unique to different strains of the same genera or even the same species. Strain-level differences are critical for understanding pathogenesis. Moving beyond 16S amplicon sequencing, true shotgun metagenomics provides a comprehensive view of the genetic content, with direct insight on the functional genes present. However, like 16S, this approach lacks the ability to uncover the active functions and processes microbes are undergoing. Metabolomics advances this further by characterizing the metabolites, or small molecules, produced in a system under a specific state. In comparison to 16S and metagenomic sequencing, true elucidation of function via metabolomics is less common, in part due to the technical expertise required and the relatively recent effort to combine the necessary chemistry tools to perform metabolomics.

Leveraging this next generation approach, Robinson, et al. designed a multidisciplinary research project to identify new targets, that are both sensitive and specific, for accurate diagnosis of true CDI (5). In the design of their study, the authors used strict inclusion and exclusion criteria to select a clean subset from all symptomatic samples submitted for *C. difficile* testing. This allowed for critical comparisons between the metabolomes from symptomatic patients highly likely to represent true CDI (i.e., both culture positive for *C. difficile* and toxin EIA-positive), patients more likely only colonized with toxigenic *C. difficile* (i.e., without detectable toxin production by EIA), and uninfected (and non-colonized) controls.

Although they demonstrated that each individual's metabolome is unique, analytes (Robinson et al. refer to these as 'features') were found that consistently differentiated true infection from non-colonized controls. Their major findings included identification of a handful of features positively associated with true CDI. The short chain fatty acid (SCFA), 4-methylpentanoic acid (4-MPA), was most strongly associated with and predictive of current, true CDI. As 4-MPA can be produced by *C. difficile* itself through Stickland metabolism of leucine, their findings suggest this *C. difficile* pathway may be more intensive during true infection. They also identified amino acid, bile acid, lipid, carbohydrate, and aromatic alcohol features associated with true infection, but in their models, 4-MPA alone was as good a diagnostic as six of these features together. Through this approach, they have added critical information to our understanding of pathogenesis of *C. difficile* infection. In addition, Robinson et al. confirmed previous results that secondary bile acids inhibit *C.*

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difficile. Notably, their findings stemmed from identification of less commonly studied bile acids that are often overlooked: cholenoic acid and monohydroxycholenoic acid. This led to further efforts which uncovered multiple networks of primary and secondary bile acids. Their metabolomic model of CDI, comprising a 4-MPA:leucine ratio and characteristic bile acid profile, distinguishes true infections from non-colonized controls in a manner that is highly sensitive and specific.

These findings are substantial; however, an area that continues to be a challenge and was not fully resolved by this effort is the ability to distinguish between likely colonization (culture-positive/toxin-negative) and true infection. The authors note that colonized fecal metabolomes were diverse and profiles could be similar to uninfected stool, infected stool, and anything in between. Robinson et al. propose that leveraging their metabolomic model of CDI with existing diagnostics might decrease the false-positive rate of culture (or NAAT) alone, and the false-negative rate of EIA toxin test alone. Future metabolomic efforts may resolve this issue, potentially resulting in the development of rapid detection of one or more metabolites in stool or urine as a supplementary diagnostic assay. The pathway to CDI and pathogenesis of *C. difficile* remain a complex interplay between the pathogen, the host, and the microbial community present. Thus, this may require further interdisciplinary approaches.

In addition, metabolites encompass all small molecules in an environment, irrespective of their source (e.g., bacteria, host) and thus their metabolomic analyses include a component of the host status and response. However, left largely untouched is the immunological response, a critical component in dictating the severity of infection. The ability to predict whether *C. difficile* infection will be limited to mild diarrhea or progress to severe outcomes, including pseudomembranous colitis or toxic megacolon, would further improve our ability to both diagnose, and treat (e.g., by directing more aggressive therapy to those who need it most) CDI.

Robinson et al. completed an elegant and powerful analysis of a complex ecologic system of metabolites contributing towards improving diagnosis of CDI, a disease with significant morbidity and mortality (1). Extensive additional development, testing, and validation is necessary before these findings can be implemented in the clinical laboratory setting. The equipment and infrastructure to perform these assays are already well-established in many clinical laboratories for diagnosis of metabolic disorders. This infrastructure would support the implementation of metabolomic assays for CDI, such as the targeted GC-MS assay for 4-MPA:leucine ratios proposed in the manuscript. Irrespective of improved diagnostic tools developed with these or other infection-specific targets, it will remain imperative that each step of the diagnostic process is managed appropriately, including maximizing pretest probability, such as testing only unformed stool in patients with ≥3 episodes of diarrhea in 24 hours (2). In this sense, diagnostic stewardship, or the oversight of appropriate use of diagnostic tests across a patient population, will remain foundational for protecting patients, preserving antibiotics, and saving lives.

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Abbreviations:

EIAs	Enzyme immunoassays
NAATs	Nucleic acid amplification tests
CDI	*Clostridioides difficile* infection
rRNA	Ribosomal RNA
SCFA	Short chain fatty acid
4-MPA	4-methylpentanoic acid

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