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Casey M. Theriot, Joshua R. Fletcher

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Commentary

Clostridioides difficile is a significant public health threat, and diagnosis of this infection is challenging due to a lack of sensitivity in current diagnostic testing. In this issue of the *JCI*, Robinson et al. use a logistic regression model based on the fecal metabolome that is able to distinguish between patients with non–*C. difficile* diarrhea and *C. difficile* infection, and to some degree, patients who are asymptomatically colonized with *C. difficile*. The authors construct a metabolic definition of human *C. difficile* infection, which could improve diagnostic accuracy and aid in the development of targeted therapeutics against this pathogen.

Human fecal metabolomic profiling could inform *Clostridioides difficile* infection diagnosis and treatment

Casey M. Theriot and Joshua R. Fletcher

Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, USA.

Clostridioides difficile **is a significant public health threat, and diagnosis of this infection is challenging due to a lack of sensitivity in current diagnostic testing. In this issue of the *JCI*, Robinson et al. use a logistic regression model based on the fecal metabolome that is able to distinguish between patients with non–*C. difficile* diarrhea and *C. difficile* infection, and to some degree, patients who are asymptomatically colonized with *C. difficile*. The authors construct a metabolic definition of human *C. difficile* infection, which could improve diagnostic accuracy and aid in the development of targeted therapeutics against this pathogen.**

Antibiotics and the rise of CDI

Clostridioides difficile is a spore-forming anaerobic bacterial pathogen. There are approximately 500,000 cases of *C. difficile* infection (CDI) and 29,000 deaths per year in the United States, making CDI an urgent public health threat (1). Patients with CDI experience a range of clinical disease, from being asymptomatic to experiencing mild to moderate diarrhea, pseudomembranous colitis, more severe colitis, toxin megacolon, and death. Antibiotic use continues to be a major risk factor for acquiring CDI; however, the first line of treatment is the antibiotic vancomycin and/or fidaxomicin, with an average relapse rate of 20% (2, 3).

Antibiotics are important for human health but can also cause collateral damage to the indigenous gut microbiota, thereby decreasing colonization resistance and allowing pathogens like *C. difficile* to colonize the gut (4). Increased precision and resolution of mass spectrometry technology have begun to reveal how antibiotics not only alter the gut microbiota and consequently the metabolome, the compilation of small molecules in a biological system. Metabolites represent the biochemical footprint in the gut that encompasses host-associated, bacterial-associated, and exogenous (such as those from diet) small molecules (5).

Each stage of the *C. difficile* life cycle requires specific metabolites to drive metabolism in vitro. The host-associated bile acids taurocholate (TCA) and cholate (CA) act as germinants for *C. difficile* spores (6), while the gut microbiota–derived secondary bile acids deoxycholate (DCA), lithocholate (LCA), and ursodeoxycholate (UDCA) can inhibit vegetative growth and in some cases suppress toxin expression and activity (7, 8). In order to successfully colonize the gut, *C. difficile* requires specific nutrients to grow, as it is auxotrophic for six amino acids: cysteine, isoleucine, leucine, proline, tryptophan, and valine (9). Thus, *C. difficile* has to acquire these amino acids from the surrounding environment. Certain classes of anaerobic bacteria can generate ATP through the paired oxidation and reduction of these amino acids, a process known as the Stickland reaction (10).

Once the *C. difficile* population in the gut reaches high cell density and has depleted these and other nutrients, the bacteria produces two toxins, toxin A and toxin B, the primary virulence factors in CDI. Toxin expression is exquisitely sensitive to changes in nutrient availability, tightly linking metabolism and pathogenesis (11). Metabolomics have been used to interrogate changes in mouse models of CDI and have shown that susceptibility to *C. difficile* is associated with an increase in host-derived primary bile acids, which are required for *C. difficile* spore germination, and amino acids, which are required for growth (12, 13). Human studies have focused on the bile acid metabolome and have indicated that recovery and clearance of CDI after fecal microbiota transplantation (FMT) is associated with a return of gut microbiota–derived secondary bile acids and a decrease in the host-derived primary bile acids that *C. difficile* can use for germination (14, 15). Far fewer studies have investigated the gut metabolome throughout the course of CDI in humans, and no studies to date have included patients with asymptomatic carriage. The paucity of studies on asymptomatic carriers could be due to the lack of sensitivity in current diagnostic tests, making collection of fecal samples from these patient populations challenging (16).

CDI-associated metabolites

In this issue, Robinson et al. collected stool samples from three different patient populations based on current diagnostic testing results and defined the fecal metabolomic features in patients diagnosed with toxigenic culture–positive (Cx^+) CDI, toxin enzyme immunoassay–positive (EIA^+) CDI, patients asymptomatically colonized

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with *C. difficile* (Cx^+/EIA^-), and patients with non–CDI diarrhea (Cx^-/EIA^-) (17). The aims of this study were to understand the relationship between the intestinal metabolome and CDI in humans, evaluate if metabolomics could be used to aid in better diagnostic approaches to improve sensitivity, and create a metabolomic model that will aid in the treatment of CDI.

Robinson and colleagues used untargeted and targeted mass spectrometry approaches that spanned multiple platforms, including gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–tandem mass spectrometry (LC-MS/MS), to capture a range of metabolites in patient fecal samples (17). They detected 2463 distinct features (metabolites) and applied statistical modeling to define only the key features that were able to differentiate samples between patients with CDI and patients without CDI or with non–CDI diarrhea. Nine metabolites, including two short chain fatty acids (SCFAs), one amino acid, one bile acid, one lipid, three carbohydrates, and one aromatic alcohol, were able to distinguish among these groups. One of the strongest CDI-associated metabolites was the SCFA 4-methylpentanoic acid (4-MPA), which is the byproduct of leucine metabolism through Stickland amino acid fermentation. Eighty percent (8/10) of Stickland reaction products were frequently detected in CDI patient stool, which could be due to *C. difficile* metabolic activity in the human gut. Using targeted GC-MS, the authors also detected the isoleucine diastereomer *allo*-isoleucine in CDI patients, a novel metabolite that is now associated with *C. difficile* pathogenesis in humans.

Other metabolites, including bile acids, cholenic acid (CE), and monohydroxycholenoic acid (MHCE), were able to discriminate between CDI-positive and CDI-negative samples. Additional LC-MS/MS analysis showed that CDI associated with bile acids with decreased sulfation, dehydroxylation, and unsaturation. Robinson and colleagues also looked at carbohydrates and found an association between decreased monosaccharides, disaccharides, and sugar alcohols and CDI. Fructose had a negative association that could be due to *C. difficile* metabolism. The authors revisited the hypothesis that trehalose is a favored substrate of more virulent *C. difficile* strains. Using stable isotope labeling to detect trehalose in stool samples, they found no association with the presence of trehalose among these groups, as it was detected in 115 of 189 (61%) samples. There was also no association between trehalose and colonization with *C. difficile* strains from ribotype 027.

Next, Robinson et al. tested if metabolomics could be used to distinguish among the three different patient populations. The authors used the most discriminating metabolites, the Stickland reaction product 4-MPA, and the bile acid community analysis to construct a logistic regression model of the CDI metabolome. When this model was applied to the fecal metabolome of asymptomatically colonized patients, only 38% had a metabolome that resembled the CDI-associated metabolome. This result suggests that metabolomic profile and this model might be able to increase the sensitivity of current diagnostic tests by distinguishing patients with asymptomatic carriage versus CDI.

It is not surprising that Stickland reaction products were associated with CDI. *C. difficile* is well known for its ability to ferment amino acids using this process, both in vitro and in vivo. The metabolism of *C. difficile* is strongly linked to expression of the toxin genes through the activity of a variety of nutrient-sensing transcriptional repressors, including the allosteric regulator CodY, which represses toxin gene expression when bound by GTP or branched chain amino acids. The finding of 4-MPA as a biomarker for patients who were *C. difficile* culture–positive and toxin EIA–positive is consistent with *C. difficile* actively fermenting leucine and the subsequent de-repression of toxin gene expression as a consequence of less branched chain amino acids, including leucine, being available. However, there are other anaerobic gut bacteria that produce Stickland reaction products in addition to *C. difficile*, as seen in the non-CDI diarrheal fecal samples.

Metabolomic studies using antibiotic-treated mouse models of infection have strongly implicated the Stickland reaction as an important factor for *C. difficile* colonization and growth, although proline fermentation is more often highlighted in these studies (18, 19). Robinson et al. did detect proline and 5-aminovalerate in CDI samples; however, it was not as strong of a signal as leucine and 4-MPA. This could be due to differences in human and mouse gut microbial ecosystems, but it also shows the value of the mouse model in approximating the human CDI metabolome. Diet, class of antibiotic, and how these factors alter the gut microbiota are also very important factors in CDI. For example, production of 5-aminovalerate from proline fermentation is a strong signal of active *C. difficile* metabolism in antibiotic-treated mice. However, in this model, the gut microbiota is significantly depleted and thus few or no species are present that are able to ferment proline except for *C. difficile* (18).

The bile acid metabolomic data correlate nicely with what others have found in both mouse models and human studies. The lack of secondary bile acids in patients with CDI could be due to the altered gut microbiota and lack of commensal *Clostridia*, which aligns with much of the literature. Robinson et al. use the whole community of bile acids, not just one or two bile acids, as a predictive value. The focus on sulfated bile acids is interesting as they have not been reviewed as much as other bile acids, and could provide a new class of metabolites to target in CDI (20).

Carbohydrates were not as predictive as the amino acids and bile acids, and this could be due to the fact that *C. difficile* can utilize carbohydrates, but they are not required for growth. The lack of association with trehalose in the gut of patients with CDI is not surprising as trehalose is not required by *C. difficile*, but can be utilized (21, 22). It is not clear what carbohydrates *C. difficile* preferentially utilizes in the gut, but it is clear that it has many to choose from.

Conclusions

As metabolite detection technology improves, metabolomics will be a valuable tool in the field of biomedical research. Attention to specific metabolomic platforms is needed, as the results will vary by methodology. Since the gut microbiota is responsible for the production of Stickland reaction products and secondary bile acids, it is important to use complementary -omic approaches to define the community of bacteria that are present via 16S rRNA sequencing or metagenomics (23). This could add to the sensitivity of the diagnostics and also lead to the creation of novel

therapeutics based on an individual's existing microbiome and metabolome. If we know what metabolites *C. difficile* requires for germination, colonization, and toxin production, we can rationally select specific bacteria that are able to convert primary to secondary bile acids, and compete for Stickland reaction amino acids that *C. difficile* requires for colonization.

Finally, the use of computational and statistical modeling to inform diagnostics and treatment of CDI and other diseases is an important tool. Can we model *C. difficile* Stickland reaction flux and control toxin expression in vivo? Can we target the amino acids and bile acids in the gut to control colonization and toxin production, thereby preventing disease? By leveraging a data-driven modeling approach to define the CDI microbiome and metabolome, we can start to answer these questions.

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Address correspondence to: Casey M. Theriot, Department of Population Health and Pathobiology, College of Veterinary Medicine, Research Building 406, North Carolina State University, 1060 William Moore Drive, Raleigh, North Carolina 27607, USA. Phone: 919.513.0711; Email: cmtherio@ncsu.edu.

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