Detecting Toxins Associated with *C. difficile* Infection

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The bacterial pathogen *Clostridium difficile* (*C. difficile*) has become an urgent public health concern. With increased rates of infection and an associated increase in mortality, sponsors are seeking better ways to prevent, detect and treat *C. difficile*.

This whitepaper discusses the current impact of *C. difficile*, treatment options, and ongoing issues with testing as well as a range of tests for detecting these highly sensitive organisms and toxins.

The Impact of *C. difficile*

*C. difficile* is a spore-forming, gram-positive anaerobic bacillus that produces two exotoxins: toxin A and toxin B. As a common cause of antibiotic-associated diarrhea (AAD), it accounts for 15-25% of all episodes and is estimated to cause almost half a million infections in the United States. The infections can be fatal; in 2011, 29,000 people died within 30 days of the initial diagnosis. The most at-risk population is people who take antibiotics and receive medical care, especially older adults.

*C. difficile* infection (CDI) causes diarrhea, which may be mild and self-limiting, or fulminant and fatal. An analysis of stool specimens from individuals with CDI has demonstrated the presence of several bacterial proteins including glutamate dehydrogenase, toxin A and toxin B.

In the past decade, *C. difficile* has become a bacterial pathogen of global significance, spurring the need for a variety of treatments. Antibiotics such as vancomycin, metronidazole, fidaxomicin and rudimentary fecal transplants are being trialed as effective treatments. Antibiotic therapies either result in reduction of overall microbiome diversity or inhibition of bacteria other than *C. difficile*. They also have a failure rate between 12–27% depending upon the antibiotic used for treatment.

Fecal bacteriotherapy is used to treat relapsing or severe CDI that is refractory to treatment with antibiotics, and acts to restore balance to the gut microbiota to suppress *C. difficile* outgrowth. In addition to facing some regulatory issues, this specific treatment also needs to be further refined and standardized.

Several biotherapies are in the clinical trial pipeline to target the *C. difficile* major toxins, TcdA and TcdB, and include active and passive vaccine treatments to boost the immune response. Despite the progress and promise of emerging treatments, the need remains for a treatment that is more effective against *C. difficile* spores and less damaging to the resident gastrointestinal microbiome, while reducing recurrent disease.
The Rise of C. difficile and Recent Interests

The Centers for Disease Control and Prevention (CDC) cited C. difficile as an urgent public health threat in a 2013 report. This was the first time that the CDC prioritized bacteria in their report into one of three categories: urgent, serious and concerning.

The urgent category includes high-consequence antibiotic-resistant threats because of significant risks identified across several criteria. These threats may not be currently widespread but have the potential to require urgent public health attention to identify infections and limit transmission.

Over the past several years nationwide, states have reported increased rates of CDI, noting more severe disease and an associated increase in mortality. CDI remains a disease mostly associated with healthcare (at least 80%). Patients most at risk remain the elderly, especially those using antibiotics. Although the elderly are still most affected, more disease has been reported in traditionally “low risk” people such as healthy people in the community, and peri-partum women.

These changes may be largely due to the recent emergence of the current epidemic strain of C. difficile, known by its names assigned by various typing schemes as restriction enzyme analysis type BI, North American Pulsed Field type 1 (NAP1), or PCR ribotype 027. This strain spread widely after first being found responsible for outbreaks in Pittsburgh (2000), Atlanta (2001-2002), and Montreal (2003). The strain appears more virulent possibly due to its increased production of toxins A and B and its production of an additional toxin known as binary toxin, as well as other factors that are still under study.

In addition to being highly virulent, the strain is more resistant to a commonly used class of antimicrobials known as the fluoroquinolones. The hypervirulent strains account for 51% of CDI, compared to only 17% of historical isolates.

A Therapeutic Focus for Rare Disease Development

Looking across all the drugs in development for rare diseases as a function of therapeutic areas, Figure 1 reveals that infectious diseases are the number one cause of rare disease.

Figure 1: Rare disease drugs in development by therapeutic area.
As a therapeutic area, infectious disease clinical trials represent a significant fraction of the global drug development expenditures. More and more pharmaceutical companies are pursuing anti-infectives, vaccines and microbiome products to prevent or cure CDI.

As a CRO, Covance continues to field inquiries about microbiology testing services in this arena. These inquiries range from toxigenic *C. difficile* cultures and susceptibility, to rapid methods of *C. difficile* toxin detection, as well as questions about molecular capabilities for detection of the hypervirulent strain and detection of ribotype groups. In 2015 and 2016, we experienced a significant increase in the number of sponsor requests for many types of these tests, providing evidence of this growing area of focus.

Infectious disease also represents the leading cause of rare disease and as portrayed in Figure 2. A significant number of drugs are in development to address these infections related to *C. difficile*.

*Figure 2: Rare diseases with five or more drugs in development.*

![Image of bar chart showing rare diseases with five or more drugs in development including infections, malaria, tuberculosis, meningococcal, dengue fever, Salmonella, typhoid fever, typhus fever, brucellosis, typhus, leptospirosis, poliomyelitis, encephalitis, meningitis, and other infections.]
Technical Challenges with *C. difficile*

*C. difficile* toxin is very unstable. It degrades at room temperature and may be undetectable within two hours after collection of a stool specimen. False-negative results occur when specimens are not tested or promptly refrigerated until testing is performed. Maintaining specimen stability and preventing degradation of toxin is key to getting accurate results. However, patients usually collect their specimens at home and may lack the required tools, experience and motivation to follow the required criteria.

Understanding the Testing Options for *C. difficile* Organisms and/or Toxin Detection

Sponsors seeking testing options for *C. difficile* organisms and/or toxin detection have several options to consider through Covance. Currently, we have microbiology laboratories at four sites globally for Covance Central Laboratory Services; our primary lab is in Indianapolis and the others are in Geneva, Shanghai and Singapore.

*Table 1: C. difficile Testing Services Provided by Covance CLS*

<table>
<thead>
<tr>
<th>Method</th>
<th>Detects</th>
<th>Covance Central Laboratory Testing Site/Implementation</th>
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</thead>
<tbody>
<tr>
<td>Toxigenic culture</td>
<td><em>C. difficile</em> organism and toxin from either cultivated organism or raw stool</td>
<td>Indianapolis</td>
</tr>
<tr>
<td>Susceptibility testing</td>
<td></td>
<td>Indianapolis</td>
</tr>
<tr>
<td>RT-PCR (GeneXpert)</td>
<td>Detection of toxin B gene and presumptive detection of virulent strain</td>
<td>Indianapolis (current) Geneva (Q3 2017) Singapore (Q1 2018)</td>
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**Toxigenic Cultures**

- Anaerobic culture for *C. difficile* on selective agar media in conjunction with enrichment broth - *C. difficile* identification performed using a combination of biochemical tests and automated systems (e.g., Maldi-TOF & 16S sequencing)
- Cytotoxicity assay using a cell culture line for toxin production (suspect colonies of *C. difficile* or stool sample)

**Susceptibility Testing**

- Testing performed on *C. difficile* isolates
- This testing is performed by the agar dilution method
- Adherence to current CLSI guidelines for testing and interpretation of susceptibility results
Toxin Detection (rapid methods) Wampole® C. difficile QUIK CHEK COMPLETE® Test (TECHLAB)

- Rapid membrane enzyme immunoassay
- Simultaneous detection of C. difficile glutamate dehydrogenase antigen and toxins A and B in a single-reaction well
- Test detects C. difficile toxin and glutamate dehydrogenase antigen as a screen for the presence of C. difficile
- Approximately 5%-6% of these results are discordant (i.e., GDH-positive but toxin-negative or GDH-negative but toxin-positive). It is recommended by CDC that these discordant results be confirmed by NAAT. We utilize the PCR assay reference above as a confirmatory test (Table 1)

Cepheid GeneXpert® C. difficile/Epi Assay (RT-PCR)

- Qualitative in vitro diagnostic test
- Rapid detection of toxin B gene sequence and presumptive identification of 027/NAP1/B1 strains of toxigenic C. difficile
- The combined presence of the genes encoding toxin B, the binary toxin, and the tcdCD117 deletion have been associated with a hypervirulent C. difficile strain known as 027/NAP1/B1

Figure 3: CDC Recommendation and Covance’s Two-step Approach to Testing

Additional Testing Options to Consider

Beyond toxigenic cultures and toxin detection, common strain typing molecular-based methods can be employed, such as PCR-ribotyping, REA and PFGE. Microbiome analysis is another option for C. difficile. Fecal microbiota transplantation (FMT), often referred to as “fecal transplant,” is rapidly becoming acceptable as a viable, safe and effective treatment for recurrent CDI. This is likely due to the restoration of a disrupted microbiome. Changes in the microbiome population are also monitored during clinical trials to monitor the impact of the therapy to existing bacterial population, resulting in increased requests for microbiome analysis.
Considerations for Finding a Microbiology Testing Partner

Given that test results of *C. difficile* detection are used for a study’s inclusion/exclusion parameters, turnaround time is a critical element. Sponsors should ensure that their testing partner can deliver results rapidly and perform the testing within specimen stability.

For a global clinical trial, it is also important to work with a partner that has global coverage and can easily combine data from multiple sites for exceptional data consistency. Other factors to consider are the availability of comprehensive services to quickly scale with the ever-fluctuating demands of trials.

At Covance, we take pride in our track record of investing in solutions that improve a trial’s chances for success and ultimately generate robust data packages for regulatory submission. Our continued expansion of microbiology testing services offers automated and comprehensive capabilities to speed your research and help advance your novel therapies to the market. Together, we can confront the rise of this threat in vulnerable populations and deliver new *C. difficile* therapies to save lives and improve treatment options.

References:
4) Citeline’s Pharmaprojects®, data accessed November 2013