Molecular Diagnosis of *Clostridium difficile*

*It’s About Time!*....
*And a Few Other Things!*

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The Johns Hopkins University School of Medicine
Disclosures

• Research funds
  – BD Diagnostics, Inc.
  – Nanosphere, Inc.

• Scientific Advisory Boards
  – NanoMR, Inc.
  – Quidel, Inc.
  – Roche Molecular, Inc.
Objectives

After attending this session, participants will be able to:

• Discuss the epidemiology of *C. difficile* infections and its impact on laboratory testing

• Know the features and performance characteristics of the various molecular tests available for the diagnosis of *C. difficile*

• Gain insight on the impact of molecular testing on hospital epidemiology and antimicrobial management
Importance of *Clostridium difficile*

- *C. difficile* causes 25-30% of antibiotic-associated diarrhea and > 95% of cases of pseudomembranous colitis
- Most common cause of healthcare associated diarrhea
  - incidence has doubled since 1996
    - hospital outbreaks are common
  - estimated costs: > $796 million
- *C. difficile* associated mortality has increased
  - increasing virulence/resistance of *C. difficile* strains
  - increasing host vulnerability

Emergence of Epidemic Toxin Variant Strain of *Clostridium difficile*  

US-8 facilities/6 States reported outbreaks in 2001  
- 50% of 187 isolates were clonal—PFGE (NAP-1)/ribotype 027 (baseline < 1%)  
- Toxin variant strain—toxinotype III  
- Deletions in *tcdC*  
  - 18 bp deletions at nucleotides 330-347  
- Binary toxin positive  
- Fluoroquinolone resistant  

Quebec study—12 hospitals  
- 30-day attributable mortality was 6.9% (1.5% baseline)  
- 33 patients required colectomy (1.9%)  
- More pts. received quinolone antibiotics  


Global Spread of Ribotype 027/NAP1/Toxinotype III Strains


Other Virulent Strains of Global Importance

- Ribotype 017 (Toxinotype VIII, J4 subtype)
  - A-, B+ strain
  - Resistant to MLS and quinolone antibiotics
  - More common in Asia—assoc. severe disease
  - Heteroresistance to metronidazole described

- Ribotype 078
  - Causes disease in animals and humans
  - Also has deletions in tcdC
  - Binary toxin positive
Clostridium difficile Testing
Available Testing Methods for Clostridium difficile

- Toxin A/B EIAs
- Glutamate dehydrogenase antigen (GDH)  
  – metabolic enzyme expressed at high levels by all strains of C difficile  
  – EIA Screen  
  – GDH/Toxin  
    - Lateral Flow, Membrane Assay
- Cell culture cytotoxin neutralization assays (CCCNA)
- Toxigenic Culture (Culture + Cytotoxin Assay)  
  – “Gold Standard”
- Molecular methods
## Performance Characteristics of Various Test Methods for *C. difficile*

<table>
<thead>
<tr>
<th>Method/Assays</th>
<th>Performance Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity (%)</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>Toxigenic Anaerobic Culture</td>
<td>31-99</td>
</tr>
<tr>
<td>Enzyme immunoassays</td>
<td>67-86</td>
</tr>
<tr>
<td>Cell culture neutralization assays</td>
<td>71-100</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>77-100</td>
</tr>
</tbody>
</table>

Two step *Clostridium difficile* Testing Algorithm

**GDH negative**

- Report as: *C difficile* antigen not detected

**GDH positive**

- *C difficile* antigen detected. The presence of antigen may not correlate with disease. Toxin assay will be performed.

  - *C difficile* cytotoxicity neutralization assay.

  - **Negative**
    - Reported as: *C difficile* toxin assay negative

  - **Positive**
    - Reported as: Positive by *C difficile* neutralization assay
C DIFF QUIK CHEK COMPLETE™

- Manufactured by TechLab, Blacksburg VA
- Combines GDH testing and toxin testing (A&B) into one device
- TAT 30 min
- 2 published studies compared to toxigenic culture:

<table>
<thead>
<tr>
<th></th>
<th>GDH</th>
<th>Toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>100</td>
<td>61-78</td>
</tr>
<tr>
<td>Specificity</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td>PPV</td>
<td>82</td>
<td>100</td>
</tr>
<tr>
<td>NPV</td>
<td>100</td>
<td>95</td>
</tr>
</tbody>
</table>


Combined sensitivity and specificity in Sharp et al (*J Clin Microbiol* 2010;48:2082) compared to toxigenic culture 60% and 99.6%, respectively.

Negative test
Test positive for antigen and toxin
Glutamate Dehydrogenase: A Meta-analysis


• 13/21 manuscripts met inclusion criteria
• Original research publications: used “gold standard” CCCNA or toxigenic culture
• Summary receiver operating characteristic (SROC) analysis was used
• Results: high accuracy of GDH for detection of the presence of *C difficile* in stool
  – Sensitivity, specificity > 90% compared to culture
Toxigenic Culture

- Methods are not standardized
- JHH procedure
  - Spore enrichment using heat
  - Inoculate stool to:
    - Pre-reduced CCFA-HB
    - CCMB-TAL broth
  - Incubate 5 days
  - Confirm ID
  - Determine toxin production by CCCNA

Toxigenic culture performed after negative direct toxin test increased yield by 23%

Molecular Testing for *Clostridium difficile* Diagnosis

- Early reports using PCR appeared in the literature in 1991
  - end detection
  - cumbersome extractions
  - cross reactivity with other *Clostridium* species

- Decade later
  - DNA extraction methods from fecal samples improved (e.g. QIAamp DNA stool MiniKit (Qiagen, Valencia, CA; Infectio Diagnostic Inc.))
  - Real-time platforms became available
  - Analytical sensitivity compared to CCCNA (10-100 times more sensitive ~ 10 genome copies per PCR)
  - Analytical specificity also improved

Available FDA – Cleared Platforms
BD GeneOhm™ Cdiff Assay Procedure Overview

Stool Specimen → Specimen Preparation → Lysis - DNA Extraction → Reconstitution Of Reagents → Real-time PCR Analysis on the SmartCycler® → Definitive On-screen Results

Results in < 2 Hours

Compliments of Beth Billyard BD-GeneOhm

Target: tcdB
BD GeneOhm™ Cdiff Assay
Published Performance Characteristics

- Eleven publications to date
- Comparisons to CCCNAs, GDH, Toxigenic Culture and other NAATS
- Overall ranges:
  - Sensitivity: 84-98%
  - Specificity: 91-100%
  - PPV: 59-100%
  - NPV: 97-100%
- Unresolved rates: 0.5-7.3%
- Disadvantage: manual extraction
- Advantage: less costly than other platforms
Prodesse ProGastro™ Cd Assay

- Stool sample is added to S.T.A.R. buffer
- Internal control is added to clarified stool
- Isolation and purification of DNA occurs using bioMerieux NucliSENS easyMag automated extractor
- Targets *tcdB* based on Taqman chemistry
- PCR amplification using Cepheid SmartCycler II instrument
- Qualitative result
## Prodesse proGastro™: Published Performance

<table>
<thead>
<tr>
<th>Reference</th>
<th>#</th>
<th>Prev. (%)</th>
<th>Comp. Method</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stamper, et al 2009. JCM 47:3846</td>
<td>272</td>
<td>10</td>
<td>CCCN Tox Culture</td>
<td>83.3</td>
<td>95.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>77.3</td>
<td>99.2</td>
</tr>
<tr>
<td>Karre, et al. 2011. JCM 49:725-7</td>
<td>346</td>
<td>10.7</td>
<td>In-house PCR method*</td>
<td>91.9</td>
<td>99</td>
</tr>
<tr>
<td>Selvaraju SB, et al. DMID 2011;71:224</td>
<td>200</td>
<td>100</td>
<td>Tox Cult.</td>
<td>100</td>
<td>93.4</td>
</tr>
<tr>
<td>Chapin KC, et al. J Mol Diagn 2011;13:395</td>
<td>81</td>
<td>32.1</td>
<td>Other PCR assays</td>
<td>88.5</td>
<td>100</td>
</tr>
</tbody>
</table>

* Discrepant analysis performed by culture
Cepheid Gene Xpert® *C difficile* Assay

Place swab with stool into buffer vial, vortex, pipette into cartridge.

Close top and place into instrument.

- Early assay termination can report positive results as soon as 29 minutes
- <1 minute specimen processing
- Moderate complexity - FDA cleared for *C. difficile tcdB* and for Ribotype 027 (C.diff/EPI)

Compliments of Ellen Jo Baron, PhD, Cepheid Diagnostics
## Published Performance of the Cepheid GeneXpert® *C difficile* Assay

<table>
<thead>
<tr>
<th>Reference</th>
<th>#</th>
<th>Prev (%)</th>
<th>Comp Methods</th>
<th>Sens (%)</th>
<th>Spec (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swindells, et al JCM 2010; 48:606</td>
<td>150</td>
<td>12.7</td>
<td>Toxigenic culture; CCCNA</td>
<td>100</td>
<td>99.2</td>
</tr>
<tr>
<td>Sharp SE, et al JCM 2010; 48:2082</td>
<td>284</td>
<td>14.8</td>
<td>Toxigenic culture</td>
<td>93.5</td>
<td>99.6</td>
</tr>
<tr>
<td>Huang, et al JCM 2009;47:3729</td>
<td>220</td>
<td>ND</td>
<td>CCCNA ; Toxigenic culture*</td>
<td>97.1</td>
<td>93.0</td>
</tr>
<tr>
<td>Novak-Weekley SM JCM 2010; 48:889</td>
<td>432</td>
<td>ND</td>
<td>Toxigenic culture</td>
<td>94.4</td>
<td>96.3</td>
</tr>
<tr>
<td>Pancholi P, et al JCM 2012;</td>
<td>200</td>
<td>21.5</td>
<td>CCCNA Other NAAT</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Babady NE, et al JCM 2010; 48:4519</td>
<td>487</td>
<td>12.3</td>
<td>GDH-Cytotoxin;Toxigenic culture*</td>
<td>99.7</td>
<td>98.6</td>
</tr>
</tbody>
</table>

*Discrepant analysis only
## Xpert C diff vs. GDH in Clinical Trials for 027 vs. Non-027 Isolates

### Toxigenic C. difficile 027 ONLY

<table>
<thead>
<tr>
<th>Site</th>
<th>027 only</th>
<th>Xpert Toxigenic C. diff 027</th>
<th>GDH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>POS</td>
<td>NEG</td>
</tr>
<tr>
<td>14</td>
<td>9</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>48</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

# GDH (027) v GDH (Not 027): p < 0.001

### Toxigenic C. difficile, NOT 027

<table>
<thead>
<tr>
<th>Site</th>
<th>NOT 027</th>
<th>Xpert Toxigenic C. diff</th>
<th>GDH</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>POS</td>
<td>NEG</td>
</tr>
<tr>
<td>14</td>
<td>26</td>
<td>24</td>
<td>2</td>
</tr>
<tr>
<td>48</td>
<td>10</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>33</td>
<td>3</td>
</tr>
</tbody>
</table>

+Xpert (Not 027) v GDH (Not 027): p <0.001.

Modified from: Tenover FC et al. 2010; JCM 48:3722. Compliments of Susan Novak-Weekly, PhD
# Sensitivity of Xpert vs. EIA Assays by Ribotype in Clinical Trials

<table>
<thead>
<tr>
<th>Ribotype</th>
<th>n</th>
<th>Xpert Pos</th>
<th>Xpert Neg</th>
<th>Sensitivity</th>
<th>EIA Pos</th>
<th>EIA Neg</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>100.0%</td>
<td>6</td>
<td>2</td>
<td>75.0%</td>
</tr>
<tr>
<td>002</td>
<td>13</td>
<td>13</td>
<td>0</td>
<td>100.0%</td>
<td>2</td>
<td>11</td>
<td>15.4%</td>
</tr>
<tr>
<td>017</td>
<td>11</td>
<td>10</td>
<td>1</td>
<td>90.9%</td>
<td>7</td>
<td>4</td>
<td>63.6%</td>
</tr>
<tr>
<td>027</td>
<td>74</td>
<td>74</td>
<td>0</td>
<td>100.0%</td>
<td>58</td>
<td>16</td>
<td>78.4%</td>
</tr>
<tr>
<td>053</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>100.0%</td>
<td>8</td>
<td>4</td>
<td>66.7%</td>
</tr>
<tr>
<td>078</td>
<td>11</td>
<td>9</td>
<td>2</td>
<td>81.8%</td>
<td>7</td>
<td>4</td>
<td>63.6%</td>
</tr>
<tr>
<td>104</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>83.3%</td>
<td>3</td>
<td>3</td>
<td>50.0%</td>
</tr>
<tr>
<td>106</td>
<td>16</td>
<td>12</td>
<td>4</td>
<td>75.0%</td>
<td>3</td>
<td>13</td>
<td>18.8%</td>
</tr>
</tbody>
</table>

Tenover FC et al. 2010; JCM 48:3722
Lack of Effect of Strain Type on Detection of \textit{C. difficile} by GDH and PCR


- Used GDH EIA (C. diff Chek-60, Techlab, Blacksburg, VA) and PCR (GeneXpert, Cepheid, Sunnyvale, CA)

- Previously isolated strains from symptomatic patients
  - 027 (n=14); 10 each of ribotypes: 002, 106, 078, 023, and 005.
  - Isolates were grown on CCFA, suspended in sterile water to a 0.5 McFarland std. then diluted 1:100 to 1:800

- Results
  - PCR was positive for all isolates at all dilutions
  - GDH detected all isolates at the 0.5 McFarland conc. and 75% of all isolates (irrespective of ribotype) at the 1:100 dilution
Xpert® CD/Epi Assay

• Multiplex real-time PCR
• Detects $tcdB$, binary toxin gene $cdt$ and $tcdC$ deletion at nt 117—markers for 027/NAP1/BI
• Pancholi et al (JCM 2012) – all 027 strains were positive for all 3 markers (n=36)
• Kok, et al (JCM 2011)-false positive results related to non-027 with unusual mutations
• Babady et al (JCM 2010)-agreement between Xpert PCR and ribotyping was 93% (42/45)
illumi
gene™ Product Review:
Assay Protocol – Sample Preparation

• **Loop mediated Isothermal amplification (LAMP)**

• **Target:** 204-bp sequence within tcdA region of the Pathogenicity locus

• **11 minute sample extraction with <1 minute of hands on time.**

• **No centrifugation required.**

Compliments of John Kohl, Meridian Diagnostics
illumi
gene™ Product Review:
Assay Protocol – Sample Preparation

- illumipro-10 provides walk-away amplification and detection.
- No precision pipetting (3 x 50μl pipetting steps)
- Amplicons contained in sealed & locked illumigene™ device
- Total assay:
  - ~ 2 min hands on time
  - ~ 70 minutes to result
  - No centrifugation or precision pipetting

Compliments of John Kohl, Meridian Diagnostics
# illumigene™ Published Assay Performance

<table>
<thead>
<tr>
<th>Reference</th>
<th>#</th>
<th>Prev (%)</th>
<th>Comp Methods</th>
<th>Sens (%)</th>
<th>Spec (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lalande V, et al J Clin Microbiol 2011;49:2714-16</td>
<td>472</td>
<td>10.4</td>
<td>Tox Culture CCCNA*</td>
<td>91.8</td>
<td>99.1</td>
</tr>
<tr>
<td>Boyanton Bl, et al J Clin Microbiol 2012;50:640</td>
<td>145</td>
<td>15</td>
<td>BD assay GDH/EIA; EIA Tox Culture+</td>
<td>95.2</td>
<td>96.6</td>
</tr>
</tbody>
</table>

* Sensitivity of CCCNA—69.4%; † Discrepant analysis only
**illumigene™ Assay: Advantages and Disadvantages**

- Theoretical risk of missing *tcdA*-negative, *tcdB*+ strains
- Actual
  - False positives have been noted
  - Invalid test results—2.8% to 2.9%
    - Occurred early in implementation in one study
    - Possibly related to too heavy an inoculum
  - Not amenable to large volume testing
- Easy to perform—no molecular expertise required
- Rapid
- No upfront expensive equipment purchase

<table>
<thead>
<tr>
<th>Assay</th>
<th>FDA Cleared</th>
<th>Targets</th>
<th>Extraction</th>
<th>Internal Control</th>
<th>TAT</th>
<th>Cost/test</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD GeneOhm®</td>
<td>Yes</td>
<td>tcd B</td>
<td>Manual</td>
<td>Yes</td>
<td>75-90 min</td>
<td>$25-$49*</td>
</tr>
<tr>
<td>Prodesse ProGastro™</td>
<td>Yes</td>
<td>tcd B</td>
<td>Easy Mag</td>
<td>Yes</td>
<td>180 min</td>
<td>$25*</td>
</tr>
<tr>
<td>Gene Xpert®</td>
<td>Yes</td>
<td>tcdB Cdt nt 117 del tcdC</td>
<td>Automated (Infinity platform)</td>
<td>Yes</td>
<td>29-45 min</td>
<td>$45*</td>
</tr>
<tr>
<td>illumigene™</td>
<td>Yes</td>
<td>tcdA</td>
<td>Manual</td>
<td>Yes</td>
<td>70 min</td>
<td>$25-$49</td>
</tr>
</tbody>
</table>

Time to perform *C. difficile* assays

NAATs for Diagnosis of *C difficile*

Caveats and Questions

• Practical concerns
  – Assays detect genes encoding toxins and not toxin
  – Impact on CDI rates when switching from EIA to PCR
  – Contamination
  – Platforms are expensive

• Theoretical concerns
  – Genetic drift of *tcdB* or other gene targets in regions of primer/probe binding
  – Lack of toxin gene expression
    • Do we need to monitor? How to monitor?
  – Effect of strain variation on test results

• Other questions
  ‣ Will use of NAATs lead to reductions in *C difficile* transmission?
  ‣ Are NAATs cost-effective?
Appropriate Use of NAATS

- Testing should be limited to patients with clinical suspicion and true diarrhea
  - Specificity will drop if clinical presentation is not considered
  - Adverse effects of false positives
    - Unnecessary treatment
    - Prolonged contact precautions
    - Inflated rates of CDI rates reported to public health organizations

- Test of cure not recommended

- Definition of diarrhea
  - At least 3 diarrheal bowel movements in 24 h
  - Loose or soft stools that take the shape of the container

- Bristol Stool Chart type 6 or 7

3-Step Algorithms

Modified from Swindells, et al JCM 2010;48:608
## Cost-Effectiveness Analyses Using Multi-step Algorithms

<table>
<thead>
<tr>
<th>Study</th>
<th>Algorithm/ Comparator</th>
<th>Same day Report</th>
<th>Cost per positive US $</th>
<th>Cost per test US $</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doing DMID 2010;66:129</td>
<td>GDH + PCR, PCR alone</td>
<td>100%</td>
<td>N/A</td>
<td>71.00, 38.00</td>
</tr>
<tr>
<td>Larson JCM 2010;48:124</td>
<td>GDH + toxin EIA, GDH + PCR, GDH + toxin EIA + PCR</td>
<td>486, 425, 368</td>
<td>18.05, 35.22, 40.68</td>
<td></td>
</tr>
<tr>
<td>Sharp JCM 2010;48:2082</td>
<td>GDH + EIA + PCR, PCR alone</td>
<td>100%</td>
<td>N/A</td>
<td>11.50 (88%), 44.88 (12%), 33.38</td>
</tr>
</tbody>
</table>
Potential Disadvantages to Multi-Step Algorithms

- Time to detection—Impact on patient care?

- Maintenance of multiple test methods

- Cost/re-imbursement issues

- Variability in reported GDH assay performance

# Effect of Various Laboratory Algorithms on Infection Control

<table>
<thead>
<tr>
<th>Algorithm/Test</th>
<th>False Neg.</th>
<th>False Pos.</th>
<th>Pts. in isolation with CDI</th>
<th>Pts. in isolation, no CDI</th>
<th>Pts. with CDI, not in isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reflex to NAAT for GDH+, EIA-</td>
<td>15</td>
<td>55</td>
<td>Day 1, 55; Days 2-5, 85</td>
<td>Day 1, 54; days 2-5, 55</td>
<td>Day 1, 45; days 2-5, 15</td>
</tr>
<tr>
<td>NAAT alone</td>
<td>5</td>
<td>36</td>
<td>Days 1-5, 95</td>
<td>Days 1-5, 36</td>
<td>Days 1-5, 5</td>
</tr>
</tbody>
</table>

Assumptions: 1000 pts. 10% prevalence (100 TP, 900 TN); 5 d LOS; once/day testing; isolation continues until results are available.
Impact of Molecular Testing on Infection Control

- Cantanzaro and Cirone. 2011; Am J Infect Control
- 6 month retrospective study comparing impact of switch from ToxA/B EIA and PCR
- Post implementation significant decrease in:
  - Healthcare associated CDI
  - Patient isolation days
  - Tests ordered
  - Metronidazole Rx for pts. with negative C diff tests
- Several manuscripts report doubling of C difficile rates initially when making the switch from EIA to PCR
- After period of increase, rates declined, suggesting better case detection leading to decreased transmission

Fong KS et al 2011; ICHE 32:932; Goldenberg SD 2012; 1231-32.
Laboratory Diagnosis of *Clostridium difficile*: Summary

- It is time to simplify!
- EIA’s and CCCNA’s: too insensitive
- Multi-step algorithms: unreliable, complicated
- Toxigenic culture: not timely
- Molecular assays
  - There are numerous FDA-platforms—all with advantages and disadvantages.
  - Combine accuracy, speed, convenience in one test method
  - May be economical
    - Eliminate need for retesting
    - May reduce transmission
    - Remove patients from isolation more quickly
    - Reduce unnecessary treatment
“The clinical laboratory can place the perpetrator (\textit{C. difficile}) and the weapon (toxin B) at the scene of the crime, but only the clinician can establish whether a crime (CDI) has taken place.” Ferric C. Fang