Molecular biology in the diagnosis of *Clostridium difficile*

*Michel Delmée*
Clostridium difficile

C. difficile is the main cause of hospital acquired diarrhea

epidemiology of CDI changed in 2003
Clostridium difficile

anaerobe

toxins

spores
CDAD: Physiopathology

- Disturbance of intestinal flora
  - Neonate
  - AB (other)

- Colonization by CD
  - Endogenous (no toxin)
  - Exogenous (toxins A/B)

- Asymptomatic
- Diarrhoea, PMC
- Relapses

CD Antwerp 13 Dec 2011
North America outbreak

New cases of nosocomial and community-acquired *Clostridium difficile*-associated diarrhea (CDAD; diagnosed by positive cytotoxin assay result) reported by the microbiology laboratory at the Centre hospitalier universitaire de Sherbrooke.

Valiquette et al. CMAJ 2004 171: 27-29
CDI incidence and severity

Table 1: Reports of increases in incidence and severity of *Clostridium difficile*-associated diarrhea (CDAD)

<table>
<thead>
<tr>
<th>Source</th>
<th>Period</th>
<th>Outcome measure</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK Health Protection Agency Communicable Disease Surveillance Centre(^9)</td>
<td>1986–2001</td>
<td>Voluntary laboratory reporting system of stools positive for <em>C. difficile</em> toxin</td>
<td>From &lt; 2000 positive test results per year in 1986/87 to &gt; 12 000 per year in 2000/01</td>
</tr>
<tr>
<td>Oregon(^1)</td>
<td>1994–2000</td>
<td>All-cause 90-day mortality among patients with CDAD</td>
<td>3.5% in a previous 10-year cohort v. 15.3% in 1994–2000 cohort</td>
</tr>
<tr>
<td>Pittsburgh(^1)</td>
<td>1989–2000</td>
<td>Fulminant colitis</td>
<td>From 0% to 3.2% (mean 1.6% over 10 years)</td>
</tr>
</tbody>
</table>

Valiquette et al. CMAJ 2004 171: 27-29
### CDI: Mortality

#### Table 2. Age-Specific Incidence and Mortality Attributed to *Clostridium difficile*-Associated Diarrhea.

| Age (yr) | No. of Cases | No. of Cases/1000 Admissions* | Attributable 30-Day Mortality Rate%
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;40</td>
<td>76</td>
<td>3.5</td>
<td>2.6</td>
</tr>
<tr>
<td>41–50</td>
<td>85</td>
<td>11.2</td>
<td>1.2</td>
</tr>
<tr>
<td>51–60</td>
<td>191</td>
<td>20.0</td>
<td>3.2</td>
</tr>
<tr>
<td>61–70</td>
<td>272</td>
<td>24.4</td>
<td>5.1</td>
</tr>
<tr>
<td>71–80</td>
<td>523</td>
<td>38.3</td>
<td>6.2</td>
</tr>
<tr>
<td>81–90</td>
<td>458</td>
<td>54.5</td>
<td>10.2</td>
</tr>
<tr>
<td>&gt;90</td>
<td>114</td>
<td>74.4</td>
<td>14.0</td>
</tr>
</tbody>
</table>

* Values are based on 1719 episodes of nosocomial *C. difficile*-associated diarrhea.
† Values are based on data from 1703 patients with nosocomial *C. difficile*-associated diarrhea.

Loo et al. NEJM, 2005; 353:2442-49
NAP1/027 isolate

- Toxinotype III
- 18bp deletion \( tcdC \)
- Increased production tox A and B
- Ribotype 027/ PFGE NAP1
- Binary toxin
- FQ resistance
Clostridium difficile hospital discharge rates in Belgian hospitals, 1999-2006.

Source: database of hospital discharges, Ministry of Public Health. ICD-9 codes 845
**Clostridium difficile** mortality rates
Brussels and Flanders, 1998-2006

Source: death registry. ICD-10 code A047

CD Antwerp 13 Dec 2011
Age-specific rates of *Clostridium difficile* related mortality
Flanders and Brussels, 1998-2007

Source: death registries, Flanders and Brussels. ICD-10 code A047 (total mentions)
C. difficile isolates sent for typing, by type of strain and city, Belgium, Jan 2006- Oct 2008

Total N of CD typed : 2864
N of 027 : 679 (23.7 %)

centers with 027 strains (N)
- 1-5
- 5-15
- >15

centers without 027 strains (N)
- 1-5
- 6-20
- >20

---

C. difficile isolates sent for typing, by type of strain and city, Belgium, Jan 2006- Oct 2008

Total N of CD typed : 2864
N of 027 : 679 (23.7 %)
Laboratoire de référence

La figure représente le nombre de hôpitaux qui ont envoyé des souches de bactéries sur une période de 4 ans (2006 à 2010). Les différentes barres colorées représentent le nombre d'hôpitaux avec des souches spécifiques: 027, 014 (UCL 16), et 078 (UCL 3).
% Lab’s

Year:
2006 S2
2007 S1
2007 S2
2008 S1
2008 S2
2009 S1
2009 S2
2010 S1
2010 S2
% strains
Mulligan et al. 1979
- env. contamination <24 h. after admission
- persist > 8 w.

National *C. difficile* standards group.

« *CD* is transmitted between patients, healthcare workers and the environment... in most studies correlation can be shown between environmental contamination and infection rate »
Accurate and rapid diagnosis is essential:

- to improve patient outcome
- to prevent spread

CD is the main cause of hospital acquired diarrhea

Environmental contamination by spores causes outbreaks

GOAL: same day diagnosis
# Role of the laboratory

<table>
<thead>
<tr>
<th></th>
<th>CD requested by physician</th>
<th>CD not requested</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N. of stools</strong></td>
<td>258</td>
<td>138</td>
</tr>
<tr>
<td><strong>CD toxin +</strong></td>
<td>22 (8.5%)</td>
<td>11 (8%)</td>
</tr>
<tr>
<td><strong>enteric pathog.</strong></td>
<td>1 (Shigella)</td>
<td>0</td>
</tr>
</tbody>
</table>

Testing for *Clostridium difficile* Without Physicians Request Improves Diagnosis of *C. difficile*-Associated Diarrhea N.Vaessen¹, E.van de Vorm², H.Endtz³, I.Spijkerman¹, H.Gerritsen¹, Ed J.Kuijper¹. ICAAC 2004, Washington DC
Recommendation 1

• We should test every diarrhoeal stool with one of these conditions
  • history of AB therapy
  • hospital acquired
  • > 65 y.
  • history of CDAD
Laboratory Tests

- Cytotoxicity assay 6-48 h
- Culture 24-48h
Cytotoxicity assays

- moderate sensitivity (50-70%)
- the most specific
- slow (6 to 48 h. + neutralization)
- supply of monolayers required
- fresh specimen needed
- not very well standardized
Culture

- very sensitive
- allows the recovery of positive samples with negative toxin tests
- allows epidemiological investigations

- not very specific (due to non toxigenic isolates)
- slow

*Cycloserine Cefoxitine Fructose Agar*

*Home-made CCFA or commercial*

CD Antwerp 13 Dec 2011
What Is Toxigenic Culture?
1997 – 2004 Results

<table>
<thead>
<tr>
<th>Condition</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cul - Tox -</td>
<td>9427</td>
<td>89.3%</td>
</tr>
<tr>
<td>Cul - Tox +</td>
<td>5</td>
<td>0.1%</td>
</tr>
<tr>
<td>Cul + Tox +</td>
<td>460</td>
<td>4.4%</td>
</tr>
<tr>
<td>Cul + Tox -</td>
<td>589</td>
<td>5.6%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>10481</td>
<td></td>
</tr>
</tbody>
</table>

\[ Cul + = 10\% \]

\[ iv\text{ tox} + : 348 (3.3\%) \]

\[ iv\text{ tox} - : 241 (2.3\%) \]

Recommendation 2

Toxigenic culture should be used as ‘gold standard’ in the evaluation of new diagnostic tests
Laboratory Tests

Reference tests

- Cytotoxicity assay  6-48 h
- Toxigenic culture  24-48 h

Rapid tests

- Toxin EIA (A/A+B)  15-60 min
- GDH EIA  15-60 min
- PCR  2-3 h
Toxins A/B EIA

Easier and Faster (20-40min) than
- cytotoxicity assays
- culture

Sensitivity $\geq$ CTA
BUT still 60-70%

well-type

membrane

CD Antwerp 13 Dec 2011
### Multicenter evaluation of tox A+B test

<table>
<thead>
<tr>
<th>Faecal cytotoxin</th>
<th>Tox A&amp;B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pos N = 23</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td>Neg N = 344</td>
<td>9</td>
<td>335</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tox A&amp;B</td>
<td>91.3 %</td>
<td>97.4 %</td>
<td>70.0 %</td>
<td>99.4 %</td>
</tr>
</tbody>
</table>

vs. toxigenic culture

<table>
<thead>
<tr>
<th>Tox A&amp;B</th>
<th>cytotoxicity as.</th>
</tr>
</thead>
<tbody>
<tr>
<td>79</td>
<td>70</td>
</tr>
<tr>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>98</td>
<td>97</td>
</tr>
</tbody>
</table>

van den Berg et al., JCM 2005, 43:5338-40
# GDH as a Screening Test

<table>
<thead>
<tr>
<th></th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDH positive</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>GDH negative</td>
<td>2</td>
<td>161</td>
</tr>
</tbody>
</table>

**Sensitivity**: 88.9 %  
**Specificity**: 88.5 %  
**PPV**: 43.2 %  
**NPV**: 98.8 %

Personal results; unpublished
GDH as screening test

<table>
<thead>
<tr>
<th></th>
<th>toxigenic culture</th>
<th>stool cytotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDH</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GDH</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>145 108</td>
<td>39 35</td>
</tr>
<tr>
<td>-</td>
<td>0 186</td>
<td>1 291</td>
</tr>
</tbody>
</table>

- **Sensitivity**: 100 %
- **Specificity**: 63.3 %
- **PPV**: 57.3 %
- **NPV**: 100 %
- **Sensitivity**: 97.5 %
- **Specificity**: 90.5 %
- **PPV**: 52.7 %
- **NPV**: 99.7 %

GDH as screening test

- very rapid
- excellent NPV
- bad PPV
# PCR vs toxigenic culture

<table>
<thead>
<tr>
<th></th>
<th>Cytotoxicity assay</th>
<th>Tox A&amp;B EIA</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>sensitivity</td>
<td>70%</td>
<td>79%</td>
<td>88%</td>
</tr>
<tr>
<td>specificity</td>
<td>100%</td>
<td>99%</td>
<td>99%</td>
</tr>
<tr>
<td>PPV</td>
<td>100%</td>
<td>90%</td>
<td>88%</td>
</tr>
<tr>
<td>NPV</td>
<td>97%</td>
<td>98%</td>
<td>99%</td>
</tr>
</tbody>
</table>

van den Berg et al., JCM 2005, 43:5338-40
## Molecular biology tests

<table>
<thead>
<tr>
<th>Test</th>
<th>Method</th>
<th>Assay</th>
<th>Automation</th>
</tr>
</thead>
<tbody>
<tr>
<td>GeneOhm</td>
<td>RT-PCR</td>
<td>tcdB</td>
<td>Semi-automated 2h</td>
</tr>
<tr>
<td>GenXpert</td>
<td>RT-PCR</td>
<td>tcdB, binary toxin, tcdC deletion</td>
<td>Automated 1h</td>
</tr>
<tr>
<td>Illumigene*</td>
<td>LAMP</td>
<td>tcd A</td>
<td>Semi-automated 1h30</td>
</tr>
</tbody>
</table>
BD GeneOhm™

RT-PCR tox B on SmartCycler®

Stool specimen
Specimen Prep
Lysis - DNA Extraction
Reconstitution
Real-time PCR Analysis on the SmartCycler® Instrument
Definitive On-screen Results

Results in < 2 Hours Lab Time
Cepheid Xpert C. difficile®

RT-PCR on SmartCycler®
fully automated

tcdB gene
deletion tcdC
binary toxin

epidemic (027) identification

CD Antwerp 13 Dec 2011
Cepheid Xpert C. difficile®

5 Easy Steps
Total hands-on time: 2 Minutes

1. Insert swab into Sample Reagent vial and break
2. Vortex and dispense Sample into Port 5
3. Dispense Reagent 1 into Port 1
4. Dispense Reagent 2 into Port 2
5. Insert Cartridge and start assay
Meridian Illumigene®

Loop-Mediated Isothermal Amplification
The MBI Molecular C diff assay contains primers that amplify an ~204 base pair sequence within the 5’ region of the Toxin A (tdcA) gene.

This region is found in all cytotoxin positive (A+/B+, A-/B+) strains, and is not present in cytotoxin negative strains (A-/B-).
## Illumigene® vs cytotoxicity assay

<table>
<thead>
<tr>
<th></th>
<th>cult -</th>
<th>cult +</th>
<th>sens</th>
<th>spec</th>
<th>PPV</th>
<th>NPV</th>
<th>correl</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTA -</td>
<td>423</td>
<td>15</td>
<td>69.4</td>
<td>100</td>
<td>100</td>
<td>96.6</td>
<td>96.8</td>
</tr>
<tr>
<td>CTA+</td>
<td>0</td>
<td>34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Illum -</td>
<td>419</td>
<td>4</td>
<td>91.8</td>
<td>99.1</td>
<td>91.8</td>
<td>99.1</td>
<td>98.3</td>
</tr>
<tr>
<td>Illum +</td>
<td>4</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

## BD GeneOhm® vs EIA

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>sens</th>
<th>spec</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tox A/B Premier</strong></td>
<td>600</td>
<td>80.8</td>
<td>97.5</td>
<td>89.4</td>
<td>95.1</td>
</tr>
<tr>
<td><strong>Tox A/B Quik chek</strong></td>
<td>600</td>
<td>74.4</td>
<td>98.9</td>
<td>96.9</td>
<td>93.6</td>
</tr>
<tr>
<td><strong>BD Gene-Ohm</strong></td>
<td>558</td>
<td>88.5</td>
<td>95.4</td>
<td>85.2</td>
<td>97.5</td>
</tr>
</tbody>
</table>

## Xpert® vs EIA

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>sens</th>
<th>spec</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tox A/B EIA</td>
<td>1023</td>
<td>67,5</td>
<td>92</td>
<td>62,6</td>
<td>93,5</td>
</tr>
<tr>
<td>Xpert</td>
<td>1023</td>
<td>94,1</td>
<td>93,7</td>
<td>74,6</td>
<td>98,8</td>
</tr>
</tbody>
</table>

# Molecular biology tests

<table>
<thead>
<tr>
<th>author</th>
<th>Test</th>
<th>N. samples</th>
<th>Preval.</th>
<th>SENS</th>
<th>SPEC</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barbut 2009</td>
<td>BD GeneOhm</td>
<td>300</td>
<td>8.6</td>
<td>93.9</td>
<td>97.9</td>
<td>83.8</td>
<td>99.2</td>
</tr>
<tr>
<td>Tenover 2010</td>
<td>GeneXpert</td>
<td>2296</td>
<td>10.8</td>
<td>93.5</td>
<td>94.0</td>
<td>73.0</td>
<td>98.8</td>
</tr>
<tr>
<td>Van Broeck 2010</td>
<td>GeneXpert</td>
<td>231</td>
<td>9.7</td>
<td>87.0</td>
<td>96.7</td>
<td>74.1</td>
<td>98.6</td>
</tr>
<tr>
<td>Barbut 2010</td>
<td>Illumigene</td>
<td>470</td>
<td>10.2</td>
<td>94.1</td>
<td>100</td>
<td>68</td>
<td>99.8</td>
</tr>
<tr>
<td>Van Broeck 2011</td>
<td>Illumigene</td>
<td>296</td>
<td>7.4</td>
<td>85.7</td>
<td>99.6</td>
<td>94.7</td>
<td>98.9</td>
</tr>
</tbody>
</table>
Molecular tests

- very rapid
- excellent sensitivity

- Moderate PPV
- expensive

<table>
<thead>
<tr>
<th></th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>44</td>
<td>7</td>
</tr>
<tr>
<td>PCR</td>
<td>10</td>
<td>204</td>
</tr>
</tbody>
</table>

Sensitivity : 81.5%
Specificity : 96.7%
PPV : 86.3%
NPV : 95.3%

ICDS Bled 2010
# Molecular biology tests

<table>
<thead>
<tr>
<th>author</th>
<th>Test</th>
<th>N. samples</th>
<th>Preval.</th>
<th>SENS</th>
<th>SPEC</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barbut 2009</td>
<td>BD GeneOhm</td>
<td>300</td>
<td>8.6</td>
<td>93.9</td>
<td>97.9</td>
<td>83.8</td>
<td>99.2</td>
</tr>
<tr>
<td>Tenover 2010</td>
<td>GeneXpert</td>
<td>2296</td>
<td>10.8</td>
<td>93.5</td>
<td>94.0</td>
<td>73.0</td>
<td>98.8</td>
</tr>
<tr>
<td>Van Broeck 2010</td>
<td>GeneXpert</td>
<td>231</td>
<td>9.7</td>
<td>87.0</td>
<td>96.7</td>
<td>74.1</td>
<td>98.6</td>
</tr>
<tr>
<td>Barbut 2010</td>
<td>Illumigene</td>
<td>470</td>
<td>10.2</td>
<td>94.1</td>
<td>100</td>
<td>68</td>
<td>99.8</td>
</tr>
<tr>
<td>Van Broeck 2011</td>
<td>Illumigene</td>
<td>296</td>
<td>7.4</td>
<td>85.7</td>
<td>99.6</td>
<td>94.7</td>
<td>98.9</td>
</tr>
</tbody>
</table>

- culture +
- history of CDI
- true false neg

CD Antwerp 13 Dec 2011
Molecular tests

- very rapid
- automated
- excellent sensitivity
- moderate PPV
- expensive
Two-step algorithm

GDH ➔ - ➔ ≠ CDAD

CTA or EIA ➔ + ➔ ≠ CDAD

= CDAD

≠ CDAD

CD Antwerp 13 Dec 2011
Two-step algorithm

Van Broeck et al. ECCMID Helsinki poster
Three-step algorithm

GDH → - ≠ CDAD

EIA → - toxigenic culture → - ≠ CDAD

= CDAD

= CDAD
Three Step Algorithm

1. GDH
   - 244
   + 51
   incl. 3 TC+

2. Tox A&B
   -
   +
   +
   16
   incl. 15 TC+
   -
   +
   +
   = 10 TC+
   25
Quik-Chek Two Step Algorithm

GDH and Tox A&B

+ +

- -

+ -

- +

Report POS

Report NEG

CULTURE or PCR

+ 

- 

Report POS

Report NEG

CD Antwerp 13 Dec 2011
Quik-Chek Two Step Algorithm

GDH and Tox A&B

50
incl. 48 TC+

482
incl. 6TC+

73

0

RT-PCR

31
Incl. 28 TC+

42

Sens  92,6 %
Spec  99 %
PPV   93,8 %
NPV   98,8 %
<table>
<thead>
<tr>
<th>Author</th>
<th>test</th>
<th>conference</th>
<th>year</th>
<th>N</th>
<th>Gold st.</th>
<th>sens.</th>
<th>spec.</th>
<th>sens.</th>
<th>spec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van Broeck</td>
<td>BD-GeneOhm</td>
<td>ECCMID Vienna</td>
<td>2010</td>
<td>605</td>
<td>TC</td>
<td>91,7</td>
<td>96,5</td>
<td>90,5</td>
<td>99</td>
</tr>
<tr>
<td>Van Broeck</td>
<td>GeneXpert</td>
<td>3° iCDS Bled</td>
<td>2010</td>
<td>231</td>
<td>TC</td>
<td>87</td>
<td>96,7</td>
<td>82,6</td>
<td>98,1</td>
</tr>
<tr>
<td>Van Broeck</td>
<td>illumigene</td>
<td>Icaac Chicago</td>
<td>2011</td>
<td></td>
<td>TC</td>
<td>85,7</td>
<td>99,6</td>
<td>81</td>
<td>99,6</td>
</tr>
<tr>
<td>Vandecandelaeere</td>
<td>GeneXpert</td>
<td>ECCMID Milano</td>
<td>2011</td>
<td>1137</td>
<td>TC</td>
<td>-</td>
<td>-</td>
<td>96,2</td>
<td>99,8</td>
</tr>
<tr>
<td>Goldenberg</td>
<td>BD-GeneOhm</td>
<td>JHI</td>
<td>2010</td>
<td>500</td>
<td>TC</td>
<td>-</td>
<td>-</td>
<td>94</td>
<td>99</td>
</tr>
<tr>
<td>Larson</td>
<td>home-made</td>
<td>JCM</td>
<td>2010</td>
<td>699</td>
<td>TC</td>
<td>97,5</td>
<td>99,7</td>
<td>83,8</td>
<td>99,7</td>
</tr>
<tr>
<td>Novak</td>
<td>GeneXpert</td>
<td>JCM</td>
<td>2010</td>
<td>428</td>
<td>TC</td>
<td>94,4</td>
<td>96,3</td>
<td>86,1</td>
<td>94,4</td>
</tr>
</tbody>
</table>

**CD Antwerp 13 Dec 2011**
Conclusion

• Molecular tests are very sensitive and fast but have a moderate PPV and are expensive
• We need more data about the significance of the false positive
• They can be used in multistep algorithms
• Culture is still usefull (necessary !)
Other molecular application in CD

- **Ribotyping** by capillary electrophoresis on ABI 3130 sequencer

Intergenic 16S-23S space amplification
GenoType Cdiff®

Examples:

Example 1: non-pathogenic moxifloxacin-sensitive *C. difficile* strain

Example 2: virulent moxifloxacin-resistant *C. difficile* strain

Example 3: virulent moxifloxacin-resistant *C. difficile* strain (e.g., ribotype 001, 042, 046, 070, 077, 081, 087)

Example 4: hypervirulent moxifloxacin-sensitive *C. difficile* strain (e.g., ribotype 078, 126)

Example 5: hypervirulent *C. difficile* ribotype 027