EUROPEAN PROSPECTIVE STUDY OF CLOSTRIDIUM DIFFICILE STRAINS: PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF THE ISOLATES FROM DIFFERENT CLINICAL STATUS: INTERIM RESULTS


for the European Study Group on Clostridium difficile (ESGCD)

16th ECCMID, Nice, France, 2-4 April 2006
Main objectives:

- To establish a well defined European collection of *C. difficile* strains
- To study and to compare the phenotypic and genotypic markers of isolates (*prevalence of toxinotype III ?*)
- To get an estimation of the incidence of *C. difficile* infections

Other objectives:

- To correlate clinical presentations with phenotypic and genotypic features *C. difficile*.
- To get the baseline characteristics of *C. difficile* strains isolated in 2005 making further investigations possible to follow trends in antimicrobial susceptibility, serogroups, genotypic patterns...
MATERIALS AND METHODS

• Design
  – Prospective European-wide study on C. difficile strains isolated during a 2-month period with phenotypic and genotypic characterization of isolates

• Requirements for participating hospitals:
  ▪ to have an on-site laboratory of microbiology
  ▪ to systematically perform culture each time a test for C. difficile is requested.
  ▪ to be able to detect toxins A or B from stools or from strains
  ▪ to fill out a questionnaire (=data form) for each isolated CD strain (whatever the toxin result is)
GENERAL ORGANIZATION OF THE STUDY

COUNTRY 1

- Hosp. 1
- Hosp. 2
- Hosp. 3

Local coordinator
- check the data and the corresponding strains

Main Coordinator (FB)
- enters data
- duplicates strain collection

Data forms + strains

Strain characterization (reference labs)

ESGCD executive committee

Analysis of data

Global and national results

Global results Data base from country 1

- Toxins A and B (Pr M. Delmée)
- Binary toxin (Dr F. Barbut)
- Antimicrobial susceptibility (Dr P. Mastrantonio)
- Epidemiological markers: Toxinotyping, PCR ribotyping (Dr Barbut)
MATERIALS AND METHODS

• Inclusion criteria:
  All the *C. difficile* strains (including the non toxigenic strains) isolated from inpatients (with community or nosocomial diarrhea)

• Exclusion criteria:
  Strains from outpatients or day-care patients.
  Strains from children under 2 years old

• Length of the study:
  a 2 month-period (or 1 month for every hospital which reaches 30 *C. difficile* strains at the end of the first month) between April and June.
• Clinical data form:
  • Clinical symptoms
  • Biological parameters
  • Risk factors (antimicrobial treatment, gastrointestinal procedures, previous hospitalization ……)
  • Endoscopic or radiologic examinations
  • Treatment and outcome
**MATERIALS AND METHODS**

- Phenotypic detection of toxins A and B
  (Pr M. Delmée, J. Van Broeck)
  - **Toxin A:**
    - *C. difficile* toxin A (Oxoid, UK)
    - suspension of 10 colonies in PBS
  - **Toxin B**
    - Cytotoxicity assay (Vero cells)
    - Supernatant of a BHI broth incubated 4 days
• Detection of binary toxin by PCR (Dr Barbut, L. Bonné, B. Burghoffer)
  • DNA extracted with Instagene*
  • Primers (Stubbs et al., FEMS Microbiol. Lett 2000, 186, 306-607)
    - CdtA pos 5'-TGAACCTGGAAAAAGGTGATG-3' 0.353 kb
    - CdtA rev 5'-AGGATTATTTACTGGACCATT TG-3'
    - CdtB rev 5'-ACCGGATCTCTTGCTTCAGTC-3'
    - CdtB pos 5'-CTTATTGCAAGTAAATACTGAG-3' 0.490 kb
  • CD 196 was used as positive control for detection of cdtA and cdtB.
MATERIALS AND METHODS

• **Toxinotyping (Dr Barbut, L. Bonné, B. Burghoffer)**
  - PCR-RFLP based method for analysing the polymorphism of the Paloc region compared to VPI 10463 as referred toxinotype 0
  - Amplification of A3 (A3C-A4N) and B1(B1C- B2N) fragments restricted by EcoR1 (A3) and Acc1 and Hinc2 (B1)
  - Toxinotyping scheme: [http://mf.uni-mb.si/mikro/tox/](http://mf.uni-mb.si/mikro/tox/)

• **PCR for tcdC (Van den Berg, 2005)**
  - tcdCfor : 5’ CATATCCTTCTTCTCCTCCTC-3’ 159 bp
  - tcdCrev : 5’ AATTGTCTGATGCTGAACC-3’
MATERIALS AND METHODS

• **PCR ribotyping** (Dr Barbut, L. Bonné, B. Burghoffer)
  - Method described by Bidet et coll. (J CM 2000, 38, 2484-87)
  - Primers
    - CD1 : 5’-GTCCGGCTGGATCACCTCCT63’
    - CD2 : 5’- CCCTGCACCCTTAATAACTGGACC-3’
  - Electrophoresis through Resophor Agarose 3%

• **Antimicrobial susceptibility** (P. Mastrantonio, P.Spigaglia, F. Barbanti)
  - Etest method on Brucella blood agar supplemented with hemin and Vit K
  - Vancomycin (VA), Metronidazole (MZ), Erythromycin (EM), Clindamycin (CM), Moxifloxacin (MX), Tetracycline (TC)
  - Breakpoints used:
    - VA $\geq$ 8 mg/l; MZ $\geq$ 8 mg/l; MX $\geq$ 4 mg/l
    - TC $\geq$ 8 mg/l; EM $\geq$ 4 mg/l; CM $\geq$ 4 mg/l
### Participating Countries

14 countries, 38 hospitals, 486 strains

<table>
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<th>Country</th>
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Distribution of participating hospitals
Strains and clinical data

- 486 strains and 464 clinical charts but an imperfect correspondance....
  - 30 clinical charts w/out strains
  - 43 strains w/out clinical charts
  - 435 complete files (strain + clinical charts)

- But ....
  - Some patients were children under 2 y.
  - Two countries sent a lot of repetitive isolates

- Therefore,
  - The data base needs to be cleaned
  - A last call for strains and clinical charts will be done
Brief overview on clinical data (n=464)

- **Age (excluding children <2)**:
  - 63+20 y. (68)
  - 57% of patients are older than 65 y.
- **Sex**:
  - male: 48.6%
  - female: 51.4%
- **Wards**:
  - Med: 72.9%
  - Surgical: 18.6%
  - ICU: 8.5%
  - Obstetrics: 0%
- **Previous ATB**: 76.4%
- **Endoscopy**: 11.9%
- **PMC**: 3.8%
Brief overview on clinical data

Incidence varies widely (23 hosp.):

\[ 2.45 \pm 1.8 \text{ CDAD/10,000 patient-days} \]
(range : 0.14- 7.1)

Hospitals N : 0.13-0.14 CDAD/10,000 patient-days
Hospital F: 0.5-1.2 «
Hospitals G, B: 2-7 «

USA : 12.1 CDAD /10,000 patients days (range 3-25.1)
(Sohn, ICHE 2005)
USA : 5 /10,000 patient-days
(Archibald, CDC, J ID 2004)
Québec : 12.8 /10,000 patients-days
(INSPQ report, 2005)
Toxigenicity

486 strains

414 toxigenic (85.2 %)

72 NON toxigenic (14.8 %)

Toxinotype 0308 (74.4%)

Toxinotype I-XXIV 106 (25.6%)

Geric B., J MM 2004: toxinotype = 12%
Distribution of toxin variant strains (n=106)

- III: 24%
- IV: 7%
- V, VII: 3%
- VIII: 31%
- others: 8%

USA (Geric, J MM 2004): III (17%), V (6%), VIII (12%)
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<th>ATB</th>
<th>MIC50 (mg/l)</th>
<th>MIC90 (mg/l)</th>
<th>Range (min. max)</th>
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<td>% S EM+CM+TC+MX</td>
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Toxinotype III

- 25 strains isolated in 6 different countries
- 2 types of toxinotype III isolates by PCR-ribotyping:
  - «027» (n=20): AII13, AII6, AII9, AII12, AII25, AII27, AIII2, AIII3, AIII5, AIII8, AIII14, GII1, GII3, GII5, GII6, GII7, GII8, GII9, GII10, DII12
  - Non «027» (n=5): AII16, KIII3, BII1, MII27, MII23
- Epidemic «027» strains were clustered in 2 countries (Netherlands and Belgium) and isolated once in Ireland.
- Non «027» toxinotype III isolates were found once in France, Belgium, Spain and Sweden (2 strains),
PCR-ribotyping of toxinotype III isolates

Strains from Belgium

American Epidemic strain (1067)

French strain tox III

Belgian strain

American Epidemic strain (1067)
PCR-ribotyping of toxinotype III isolates

Strains from the Netherlands
PCR ribotyping of toxinotype III isolates

Epidemic clone « PCR ribotype 027 »

Non epidemic strains
Toxinotype III: antimicrobial susceptibility

- Epidemic «027» strains (n=20):
  - MIC EM > 256 mg/l
  - MIC MX ≥ 12 mg/l (except 1 strain with MIC = 6 mg/l)(AIII8)

- Non «027» toxinotype III strains (n=5):
  - MIC EM = 0.064->256 mg/l (1 strain R)
  - MIC MX < 2 mg/l

- Resistance to MX is not specific of the epidemic clone: 31% of strains different from toxinotype III are resistant to MX
TcdC polymorphism

<table>
<thead>
<tr>
<th>Ld. 10463 (0)</th>
<th>AI10 (VI)</th>
<th>BI3 (V)</th>
<th>BI12 (V)</th>
<th>8864 R9367</th>
</tr>
</thead>
</table>

*159 bp* (undeleted)

*141 bp* (-18 pb)

*120 bp* (-39 bp)
Toxinotype VIII

- 34 strains isolated in 6 countries
- All the strains were A-B+
- High prevalence in countries:
  - Poland (HII) : 80% (4/5)
  - Poland (HI) : 46.7 (5/11)
  - Ireland (DI) : 30.7% (4/13)
  - Sweden (MI) : 25% (3/12)
  - Germany (CIII) : 14.2% (7/49)
  - Ireland (DII) : 15.9% (3/19)
- PCR-ribotyping is under investigation
Toxinotype V

• This toxinotype is widely distributed (9 countries)
• It is more prevalent in some countries:
  – France: 17% (9/53) (in 3 hospitals)
  – Greece: 29% (7/24) (in 2 hospitals)
• Binary toxin was found in 72 strains (17.4% of toxigenic strains)
• Binary toxin was not detected in non toxigenic strains

Stubbs (Wales): 6.4%
Goncalves (France): 6%
Rupnik (Asia): 1.6%
Geric (USA): 6.1%
Conclusion

• So far, the epidemic strain toxinotype III, PCR-ribotype «027» has been isolated in 3 countries
• In 4 hospitals, this clone represents 30% to 70% of all the toxigenic isolates
• Resistance to MX is not a good screening method for detecting the epidemic clone
• There is a polymorphism among toxinotype III strains
• Correlation between toxinotypes and antimicrobial resistance patterns
• Analysis of clinical data: correlation between severity of disease and toxinotypes and/or binary toxin
• Correlation between previous antimicrobial treatment and antimicrobial susceptibility of isolates
• PCR ribotypes of toxinotype VIII and V
• ....
Acknowledgments to all participants involved in data and strain collection

- Belgium: C. Nonhoff, M. Delméé, V. Verhaegen
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- Switzerland: C. Balmelli
- Poland: G. Nutzynska, H. Pituch,
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