Clostridium difficile PCR ribotype 027/PFGE type NAP1 – why is it emerging?

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Running title: C. difficile PCR ribotype 027 in Sweden

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Clostridium difficile PCR ribotype 027 comprised 0.2% of Swedish isolates 1997-2001 (n=3/1325). Except for higher moxifloxacin MICs in recent epidemic types, there was no difference in tcdC sequence or toxin yield among the 027 isolates. Type 027 produced 3- to 13-fold more toxin than major Swedish types. One epidemic strain (027/NAP1a) sporulated more than other 027 isolates, a feature that should contribute to its survival and spread.
A high-level toxin producing strain of *Clostridium difficile* (PCR ribotype 027 and PFGE type NAP1) has in recent years been associated with increased infection rate, outbreaks and severe disease (10, 12, 15, 16, 17, 18, 19, 22, 25, 26, 31, 34). Its high toxin yield may in part be caused by a frame-shift mutation in *tcdC* (5), encoding a negative regulator of TcdR and part of the *C. difficile* pathogenicity locus (20, 21). Type 027 has been associated with fluoroquinolone use, especially moxifloxacin and gatifloxacin (3, 4, 8, 27, 32) that promote *C. difficile* growth and toxin production in an animal model (1). The role of these factors for the world-wide expansion of type 027/NAP1 is obscure. We compared historical and epidemic isolates of PCR ribotype 027 with respect to antibiotic susceptibility, *tcdC* sequence, toxin yield, S-layer and sporulation.

*C. difficile* isolates were obtained from a collection of 1325 strains isolated 1997-2001 in central and south Sweden (23, 29, 30, 35, 36, 37), the recent US-Canadian epidemic (US1067 and US1165, representing PCR ribotype 027/PFGE types NAP1a and NAP1b, respectively) and the Culture Collection, University of Göteborg, Göteborg, Sweden (CCUG 19125 [VPI 10463], CCUG 37783, and CCUG 20309 [8864]). For PCR ribotyping, see reference 30. Antibiotic susceptibility was determined by Etest (AB Biodisk, Solna, Sweden) using IsoSensitest Agar (Oxoid Ltd, Basingstoke, United Kingdom) supplemented with 5% defibrinated horse blood and 20 mg/l of β-nicotinamide adenine dinucleotide (cf. Swedish Reference Group for Antibiotics; http://www.srga.org/). Isolation of bacterial DNA, PCR and sequencing were performed as previously described (24) using primers for *tcdC* (28). For growth experiments, overnight cultures were diluted $10^6$-fold into triplicate tubes containing...
peptone-yeast without cysteine or glucose (PYo), that were further grown for 48 h (13, 14). Sampling, separation of intra- and extracellular fractions, sonication and toxin measurements (by EIA) were performed as previously described (13, 14). Vegetative and sporulated cells were scored by microscopy using a Bürker chamber; 10 squares containing 5-15 cells were counted per isolate and the values averaged. Two-dimensional gel electrophoresis was performed as described in reference 13; duplicate 24-h intracellular protein samples of US1067, US1165, and T-378 and single sample of Ö99-1670 were focused on 180 mm linear 4-7 IPG strips (Amersham Biosciences, Uppsala, Sweden). For identification, protein spots were excised from Coomassie-stained gels and processed using the Montage In-Gel Digestion Kit (Millipore, Billerica, MA, USA) together with a vacuum manifold unit. Two µl eluted peptides were loaded on an Anchor-Chip-plate (Bruker Daltonics Inc., Bremen, Germany) and covered by 1 µl of α-cyano-4-hydroxycinnamic acid. Peptide mass mapping of tryptic peptides was performed by a Bruker Daltonics Reflex IV MALDI-TOF apparatus, equipped with a nitrogen laser (337.1 nm) and operated in a reflective positive mode. Spectra calibration was performed by internal use of the trypsin fragments 842.510, 1045.564 and 2211.105 Da and the external use of a 1000-4000 Da peptide calibration standard (Bruker Daltonics Inc.). Identified peptide masses were analyzed in the Mascot search engine at http://www.matrixscience.com/

By using two C. difficile PCR ribotype 027 reference strains, three type 027 isolates were identified in the Swedish national database representing 0.2% of strains collected 1997-2001. (PCR ribotype 027 corresponded to type SE10 according to the Swedish nomenclature.) The 027-infected patients had developed moderate to severe CDAD (Table 1) and like other historical 027 isolates (22) the Swedish ones had lower MICs for moxifloxacin compared to the recent epidemic type 027/NAP1 (Table 2). In addition, the MICs for metronidazole were about 3-fold lower in the Swedish isolates. The tcdC sequence was identical in the 027-
isolates Ö99-1670, US1067 and US1165, including the characteristic 18-bp deletion and the frame-shift mutation at position 117, while isolates representing the major Swedish types SE20 and SE30 had a wild-type \textit{tcdC} allele (Fig. S1). The toxin yield was similar in historical and epidemic type 027 isolates but 3- to 13-fold higher than in isolates representing types SE20, SE30 and SE21 (Fig. 1).

Three of the 027 isolates had a sporulation frequency of about 25\% at 48 h (Fig. 2). T-378 was morphologically different from the other 027 isolates, showing elongated doublet cells usually containing a single spore (not shown). Thus, the actual sporulation frequency per unit cell was about 2-fold lower than the scored one, i.e. closer to 25\% than 45\% (black and hatched bar, Fig. 2). Strain US1067 (NAP1a) sporulated more (60\%, p<0.001, Fig. 2) and had a smaller cell and colony size compared to the other 027 isolates. Excluding the low-sporulating strains VPI 10463 and 8864, US1067 also had a 20\% higher OD at 24 h compared to all other isolates (p<0.001, not shown), i.e. consistent with characteristics of the epidemic 027 type (34). Despite the differences in sporulation frequency and morphology, the expressed protein patterns of US1067, US1165 and Ö99-1670 were similar (Fig S2). However, T-378 expressed an additional S-layer (Fig. S2, Table S1), possibly causing its unique morphology.

The “hypervirulence” of \textit{C. difficile} 027/NAP1 (16, 25, 26) has been ascribed to its about 20-fold higher toxin yield \textit{in vitro} compared to toxinotype 0 strains (34), caused by loss of \textit{TcdC} function (5, 21). However, the moderate 3-fold higher toxin yield of type 027 (defective \textit{tcdC}) compared to types SE30/SE20 (wild-type \textit{tcdC}) indicates that also other factors may affect the course of infection. For example, the nutritional sensor CodY may further affect the range of toxin levels (6). That strains VPI 10463 and 8864 yielded few spores but superior amounts of toxin during stationary phase was in accord with the inverse relation between spore and toxin yield generally found among \textit{C. difficile} isolates (2). No clinical isolate of the
same PCR ribotype as strain 8864 and only two isolates sharing type and characteristics with VPI 10463 were found in our national database, suggesting that high-level toxin producers with low sporulation capacity have a poor transmission rate. Although antibiotics that promote growth and toxin production by resistant *C. difficile in vivo* are major risk factors for developing CDAD (1, 7, 9, 11, 33), our data showed that certain PCR ribotype 027 strains have different morphology and growth characteristics as well as high capacity for both toxin and spore production, features that may contribute to disease severity, therapy failure, relapse, and spread.

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**REFERENCES**


Table 1. Clinical data for Swedish patients infected with C. difficile PCR ribotype 027.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Year</th>
<th>Age</th>
<th>Stools per day</th>
<th>Temp. (°C)</th>
<th>Leukocytes (1000/mm³)</th>
<th>Underlying disease</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-378</td>
<td>1997</td>
<td>53</td>
<td>&gt;10</td>
<td>&gt;39</td>
<td>17.9</td>
<td>None</td>
<td>penicillin V</td>
</tr>
<tr>
<td>Ö99-1670</td>
<td>1999</td>
<td>85</td>
<td>5-10</td>
<td>38</td>
<td>31.9</td>
<td>Lung cancer</td>
<td>amoxicillin</td>
</tr>
<tr>
<td>A177:1</td>
<td>2001</td>
<td>92</td>
<td>5-10</td>
<td>&gt;38</td>
<td>31.6</td>
<td>None</td>
<td>piperacillin/tazobactam metronidazole/ciprofloxacin</td>
</tr>
</tbody>
</table>

*Indicates severe underlying disease. The patient infected with T-378 recovered while the others died within 3 months after CDAD diagnosis.

*Used within 2 months prior to CDAD onset; piperacillin/tazobactam treatment was directly followed by oral metronidazole/ciprofloxacin. After CDAD diagnosis, all patients were treated with metronidazole.

Table 2. MICs of antibiotics for C. difficile isolates (Etest, µg/ml).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>T-378⁴</th>
<th>Ö99-1670⁴</th>
<th>A177:1⁴</th>
<th>US1067⁵</th>
<th>US1165⁵</th>
<th>8864⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>0.5</td>
<td>2</td>
<td>1.0</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>1.0</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Metronidazole⁵</td>
<td>0.053 (3)</td>
<td>0.125 (3)</td>
<td>0.084 (3)</td>
<td>0.33 (3)</td>
<td>0.25 (3)</td>
<td>0.19 (2)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.5</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

⁴Historical Swedish PCR ribotype 027 isolates.
⁵Recent epidemic PCR ribotype 027/PFGE type NAP1 isolates.
⁶Reference strain (toxin A⁺B⁺) obtained from CCUG, Göteborg, Sweden.
⁷Averages of independent experiments (n). Means were 0.088 and 0.31 µg/ml for historical and epidemic isolates, respectively (p<0.015, t-test using unequal variance).
Figure legends

**Fig. 1.** Toxin yields of 24-h *C. difficile* cultures. Isolates T-378, Ö99-1670, A177:1, US1067 and US1165 are type SE10, i.e. PCR ribotype 027. Isolates representing major Swedish PCR ribotypes, CCUG 37783 (SE20, i.e. PCR ribotype 001), Ö99-1751 (SE30), Ö99-0304 (SE21) and two high-level toxin producing reference strains, VPI 10463 (SE20c) and 8864 (SE10b), were included for comparison. Values are averages of duplicate cultures and bars indicate standard error. Statistics were calculated using logarithmic average values of total toxin for “All SE10”, CCUG 37783, Ö99-1751, Ö99-0304, VPI 10463 and 8864 using ANOVA and Bonferroni post-hoc compensation for multiple comparisons (p-value for comparison of “All SE10” and strain Ö99-1751 is shown).

**Fig. 2.** Sporulation frequencies of *C. difficile* cultures. For further explanation of strains, see legend to Fig. 2. Bars indicate standard error. Statistics were calculated using ANOVA and Bonferroni post-hoc compensation for multiple comparisons. P-value for comparison of US1067 and US1165 is indicated. As T-378 formed doublet-cells, the total frequency scored (filled + hatched area) and an estimate of the true sporulation frequency per unit cell (filled area) are shown. For details, see text.