Molecular analysis of relapses or reinfections of *Clostridium difficile*-associated diarrhea

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*Clostridium difficile* is the most common cause of nosocomial antibiotic-associated diarrhea (Johnson and Gerding, 1998). Treatment with metronidazole and vancomycin has proved effective but approximately 15-20% of patients have recurrences (Wilcox and Spencer, 1992) that may occur in up to 42% of patients in high-risk groups such as the elderly (Borriello and Larson, 1981). Recurrences may be explained by endogenous persistence of *C. difficile* spores (relapse) or by the acquisition of a new strain from an exogenous source (reinfection). This increases the costs of treatment, the risks of cross-infection and the duration of hospitalization. The aim of our study was to analyze *C. difficile* isolates obtained from patients with recurrent *Clostridium difficile* infection (CDI) and collected over a period of 1 year (May 2009-April 2010) were included in this study (Table 1). A recurrence rate of 41% was found during the period of observation. Before CDI diagnosis, all patients were treated with proton pump inhibitors (PPI) a known risk factor for recurrences (Cadle et al., 2007). Multiple *C. difficile*-positive faeces (at least two) were obtained from each patient during hospitalization. These

Recurrence is a major complication of *Clostridium difficile*-associated diarrhea and occurs in 15 to 20% of patients after discontinuation of therapy. Strains from 53 patients with *Clostridium difficile* recurrences were fingerprinted by PCR ribotyping. Reinfection with a different strain occurred in 15 out of 53 patients (28,3%), while 38 patients relapsed. These data suggest the need to perform molecular typing for implementation of infection control procedures and for a more appropriate therapeutic strategy.

**KEY WORDS:** Clostridium difficile, Recurrences, RAPD

**SUMMARY**

A total of 130 *C. difficile* strains isolated from 53 patients presenting recurrence of *Clostridium dif-

<table>
<thead>
<tr>
<th>Patient population</th>
<th>No. of patients</th>
<th>Time range between recurrences</th>
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<tbody>
<tr>
<td>Total</td>
<td>53</td>
<td>2-5 weeks</td>
</tr>
<tr>
<td>With relapse</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>With reinfection</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>34:19</td>
<td></td>
</tr>
<tr>
<td>Ward</td>
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<td></td>
</tr>
<tr>
<td>Cardiology</td>
<td>18</td>
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</tr>
<tr>
<td>Medicine</td>
<td>22</td>
<td></td>
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<tr>
<td>Cancer</td>
<td>13</td>
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</tbody>
</table>
patients were inpatients at a long-term facility (ASP Istituti Milanesi Martinitt e Stelline e Pio Albergo Trivulzio, Milan; Italy). Recurrences were defined as patients with a resurgence of symptoms after cessation, at least 10 days after the first episode. All faecal specimens from patients suspected of having CDI were tested for toxin A/B using the enzyme-linked immunosorbent assay (ImmununoCard Toxins A&B; Meridian) according to the manufacturer’s instructions.

Toxins A and B positive stools were plated on selective cycloserine-cefoxitin-fructose agar plates (CLO agar; bioMérieux, Italy) and incubated in an anaerobic atmosphere for 48 h. Species identity was confirmed using an automatic system (Anaerobic Card-Vitek; bioMérieux, Italy). Strains were fingerprinted using PCR ribotyping (Bidet et al., 1999) and toxinotyping (Rupnik et al., 1997).

DNA was extracted from a single C. difficile colony using a Chelex resin-based commercial kit (InstaGene Matrix; Bio-Rad) as recommended by the manufacturer.

For PCR ribotyping, amplification reactions were performed in a 50 µl volume containing 1x reaction buffer; 1.5 mM MgCl₂, 200 µM of each dNTP (Sigma), 50 pmol of each primer; 1.25 U of Taq polymerase (Sigma) and 5 µl of DNA extract (or distilled water as negative control). After a denaturation of 5 min at 94°C, the DNA was amplified by 35 cycles of 1 min at 94°C, 1 min at 57°C, and 30 s at 72°C. At the end, samples were held at 72°C for 7 min.

Amplification products were electrophoresed in an 3% agarose (SeaKem LE; Lonza) and visualized under UV light following ethidium bromide staining. PCR ribotype profile analysis was carried out using BioNumerics software (version 4.0; Applied Maths). Strains were considered to be “indistinguishable” when showed ≥97% similarity and no banding differences. We compared the patterns with those obtained by the use of clinical strains with known ribotypes. For toxinotyping we followed the methods reported in the toxinotyping home page (http://www.mf.uni-mb.si/mikro/tox).

The determination of toxin genes were performed by PCR as previously described (Kato et al., 1998) using 5 µl of crude DNA extract. Because the spread of hypervirulent C. difficile strains, ribotype 027 (Loo et al., 2005; McDonald et al., 2005) and 078 (Goorhuis et al., 2008), the presence of binary toxin was also investigated by PCR, as previously described (Stubbs et al., 2000).

Ages of patients ranged from 76 to 91 years (86±4 years). All the isolates were confirmed as being toxin A and B producers, and to belong to toxinotype 0; no strain harboured binary-toxin gene.

During the period of study, the strains circulating in the wards of the long-term facility belonged to 5 different ribotypes; the most frequent type, ribotype 018, accounted for 78% of all the strains, followed by ribotype 002 (10%), ribotype 106 (8%), ribotype 014 (2%), and ribotype 001 (2%). Fifteen of the 53 recurrences (28.3%) were due to reinfection with exogenous C. difficile strains, while 38 (71.7%) were relapses (Figure 1). Our results are in agreement with those obtained by Alonso et al. (2001).

Ribotypes found during the first episode of reinfection were: 018 (66.7%), 002 (20%), 106 (6.7%) and 014 (6.7%). Strains isolated from the next episodes belonged to ribotypes 018 and 004. For the relapses, 26 (68.5%) samples belonged to ribotype 018, four (10.6%) to ribotype 002, four to ribotype 106 (10.6%), two (5.3%) to ribotype
false
inhibitors with outcomes in Clostridium difficile colitis. Am. J. Health. Syst. Pharm. 64, 2359-2363.


