Molecular characterization of *Clostridium difficile* clinical isolates in a geriatric hospital

Isabelle Poilane,1 Christel Humeniuk-Ainouz,1 Isabelle Durand,1 Claire Janoir,2 Philippe Cruaud,1 Michel Delmée,3 Michel R. Popoff4 and Anne Collignon1,2

1AP-HP, Hôpital Jean Verdier-René Muret, Service de Microbiologie, Bondy, France  
2Université de Paris-Sud-XI, Faculté de Pharmacie, USC INRA EA3534, Châtenois-Malabry, France  
3Université Catholique de Louvain, Unité de Microbiologie, Brussels, Belgium  
4Institut Pasteur, Unité des Bactéries Anaérobies et du Botulisme, Paris, France

The discriminatory potential of a combination of various typing methods was evaluated on a set of 21 *Clostridium difficile* isolates obtained from symptomatic patients hospitalized in a geriatric unit and 7 non-toxigenic isolates from the same hospital. Isolates were firstly serotyped and toxinotyped. Of the 28 isolates, 19 belonged to serogroup A. PCR-ribotyping and PCR-RFLP on the *fliC* and *slpA* genes were then applied to these 19 isolates. The results suggest that the combination of PCR-ribotyping with PCR-RFLP analysis of *slpA* could be more discriminatory and suitable for studying *C. difficile* epidemiology.

INTRODUCTION

*Clostridium difficile* is recognized as the main enteric pathogen involved in nosocomial diarrhoea. Many risk factors have been identified, including antibiotic treatment, duration of hospital stay, increasing age and underlying diseases (Bignardi, 1998). Consequently, elderly patients hospitalized in long-stay units are particularly susceptible to developing *C. difficile*-associated disease. Many typing methods have been evaluated for *C. difficile* epidemiological studies (Brazier, 2001). Phenotypic methods are mainly based on serogrouping (Delmée et al., 1985), which determined 10 major serogroups (A, B, C, D, F, G, H, I, K and X) and 20 subserogroups in group A (A1–A20). Genotyping has also been developed for studying *C. difficile*. Toxinotyping is based on the polymorphisms observed in both toxin genes *tcdA* and *tcdB* (Rupnik et al., 1998). Arbitrarily primed PCR, PFGE and PCR-ribotyping have been applied to *C. difficile* strains with success (Gal et al., 2005; Wullt et al., 2003). PCR-ribotyping has been widely used and offers good typeability, reproducibility and discriminatory power. In the UK, Stubbs et al. (1999) applied this method to 2030 strains and differentiated 116 PCR-ribotypes.

Other typing methods have been proposed for *C. difficile*. Tasteyre et al. (2000) studied the variability of the flagellin *fliC* gene and compared the RFLP groups to serogroups of 10 reference strains. They described a good correlation between the two methods and proposed PCR-RFLP on the *fliC* gene as an additional typing method. Previous reports have also described the variability of the S-layer protein (SlpA) among *C. difficile* isolates (Calabi et al., 2001; Kato et al., 2005; McCoubrey et al., 2003). Karjalainen et al. (2002) suggested that genotyping based on *slpA* gene studies could be useful for typing strains. DNA and deduced amino acid sequences of the *slpA* variable region were 100% identical within a given serogroup whereas variability appeared greater among subserogroup A strains. The subserogroup specificity of highly flagellated serogroup A could be attributable to the flagella. Consequently, these PCR-RFLP methods could be of interest for subtyping.

The aim of this study was to characterize by various typing methods *C. difficile* isolates from a population of elderly patients hospitalized in a geriatric hospital in order to better understand the local *C. difficile* epidemiology. We used serotyping and toxinotyping first to discriminate the *C. difficile* isolates. We then applied PCR-ribotyping and PCR-RFLP analysis of the *slpA* and *fliC* genes to the serogroup A isolates in order to test these methods with clinical isolates.

METHODS

Clinical isolates and reference strains. Twenty-one *C. difficile* isolates were obtained over a period of 12 months (November 2001–November 2002) from symptomatic patients with a diagnosis of *C. difficile*-associated disease on the basis of a positive *C. difficile* culture, and toxin A and B detection. These patients were hospitalized in a long-stay hospital (René Muret Hospital, Assistance Publique-Hôpitaux de Paris, Sevran, France). In addition, seven non-toxigenic...
isolates from the same hospital and two reference strains of specific group A (ATCC 45594 and TO005 for serogroups A1 and A10, respectively) were also analysed as unrelated control strains.

**Serogrouping.** The serogroup of each strain was determined as previously described by Denecke et al. (1985).

**Toxinotyping.** DNA was extracted from 1 ml of an overnight anaerobic culture (Trypticase Glucose Yeast broth) using the protocol provided in the Instagene kit (Bio-Rad). Genetic identification of tcdA and tcdB profiles was determined by the PCR method described by Rupnik et al. (1998). Fragments A1, A2 and A3 and B1, B2 and B3 were amplified, and fragments B1 and A3 were digested by HindIII or AccI and by EcoRI or SpeI, respectively. The profiles were compared to those described by Rupnik et al. (1998).

**DNA preparation for PCR-typing.** DNA templates were prepared from 48 h anaerobic cultures with the PSP Spin Swab Kit (Invitrek) according to the manufacturer’s instructions. PCR amplification of target DNA was performed using PuRe Taq Ready-to-Go PCR tubes (Amersham). PCR was carried out in a PTC 200 thermocycler (MJ Research).

**PCR-ribotyping.** This method was performed according to Bidet et al. (1999). The primer sequences (168 primers) were 5'-GTGCC-GGCTCAGTACCCCTC-3' and 5'-CCCTCGACCCCTTAAGTGGAGT- GACC-3'. Initial denaturation was carried out for one cycle of 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 57°C and 1 min at 72°C, and a final extension for 7 min at 72°C. Amplified products were analysed by electrophoresis on a 2% agarose gel (Eurobio) for 2 h at 85 V.

**PCR-RFLP.** The variability of the flIC gene encoding flagellin was studied by PCR-RFLP. The primer sequences were 5'-ATGA-GAGTAAATACAAATGTAAGTGC-3' and 5'-CTATCCTAAATATGGTAAAACTCC-3'. Initial denaturation was carried out at 95°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by a final extension at 72°C for 10 min. Amplified products were digested with the restriction enzymes HindIII, Dral, Rsal and HinfI. The digested amplified products were analysed by electrophoresis in a 2% (w/v) agarose gel with a 100 bp ladder (Eurobio) as the molecular size marker (Tasteyre et al., 2000).

The variable domain of the slpA gene encoding the S layer was amplified. The primer sequences were 5'-ATGATAAAGAAAAAAY- WTAAGCAATRGC-3' and 5'-CTATCCTATCTTCTCGCCATAC-3'. Initial denaturation was carried out at 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 45°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 10 min. Amplified products were digested with the restriction enzymes PvuII, HindIII, Dral and Rsal. The digested amplified products were analysed by electrophoresis in a 2% (w/v) agarose gel with a 100 bp ladder (Eurobio) as the molecular size marker (Karjalainen et al., 2002).

**RESULTS AND DISCUSSION**

Among the 28 isolates studied, 19 belonged to serogroup A, three to serogroup K, two to serogroup G, two to serogroup H, one to the non-toxigenic serogroup D and one isolate was non-typeable. Among the serogroup A isolates, 16 were toxigenic (toxin A and B positive) and all were toxino-type 0. These results are in accordance with other studies that reported the high frequency of toxinotype 0 among clinical isolates (Geric et al., 2004). No isolate belonged to the hypotoxigenic toxinotype III recently described in North America (Loo et al., 2005).

All the serogroup A isolates were tested by PCR-ribotyping and yielded seven different PCR-ribotypes. The most common PCR-ribotype 1 contained 10 isolates. PCR-ribotype 2 was identified for three isolates, PCR-ribotype 3 for two isolates and the PCR-ribotypes 4–7 for one isolate each. All isolates were typeable and all PCR-ribotype 1 isolates were toxigenic. The non-toxigenic isolates of this serogroup belonged to the PCR-ribotypes 3 and 5 (Table 1). PCR-ribotyping displayed good typeability and good discriminatory power, as suggested by numerous studies using PCR-ribotyping (in association with other typing methods) to study outbreaks due to *Clostridium difficile* (Fawley et al., 2005; Warny et al., 2005).

For RFLP analysis of the flIC gene, the amplified product (~1000 bp) was digested with four endonucleases. For each endonuclease, two patterns were identified (Fig. 1). The combination of these patterns allowed us to classify the isolates into two profiles, I and VII, according to Tasteyre et al. (2000). Eighteen isolates gave profile VII as did the reference strain A1; only one isolate displayed profile I (Table 1). All isolates were typeable. Tasteyre et al. (2000) showed that flagellin gene PCR-RFLP analysis could constitute an additional typing method. In their study of 47 *Clostridium difficile* isolates, this method discriminated nine different RFLP groups (I–IX). In our study, we chose endonucleases that offered, according to these authors, the best discriminatory power among serogroup A isolates. However, compared to PCR-ribotyping, it appeared less discriminatory for serogroup A isolates.

For the slpA gene, the amplified product (~1000 bp) was digested with four endonucleases. Four patterns were identified with the endonucleases HindIII, PvuII and Dral and only three with Rsal (Fig. 2). The combination of these patterns allowed us to distinguish four different profiles, 1, 2, 3 and 4, among the isolates. However, nine isolates were not typeable under our PCR conditions (Table 1). It has already been reported that *Clostridium difficile* surface proteins display a high variability (Péchinié et al., 2005). McCoubrey et al. (2003) studied the variation in the molecular mass of the *Clostridium difficile* S-layer proteins and identified seven S types among 100 isolates. The *Clostridium difficile* S layer is composed of two proteins of variable size: a higher-molecular-mass protein of 48–56 kDa encoded by the conserved 3'-terminal part of the slpA gene and a lower-molecular-mass protein of 36–45 kDa encoded by the variable 5’-terminal part of the gene. The lower-molecular-mass S-layer protein (P36 protein) is located on the exterior surface of the bacteria and exhibits a high degree of variability. Karjalainen et al. (2002) suggested that genotyping based on this variable domain of the slpA gene could be useful for typing. They showed that the gene could be easily amplified from various strains of different serogroups and that this PCR-RFLP typing method associated with DNA sequencing of the slpA variable region could constitute an alternative method for determining *Clostridium difficile* serogroups. Since the slpA sequences were quite similar in serogroups A1 and A10, the 20
subserogroups of serogroup A may not be completely specified by using this method. In our study, serogroup A isolates were classified into four groups and an additional group of non-typeable isolates. This suggests a high variability of the \( \text{slpA} \) gene in serogroup A. To enhance typeability of PCR-RFLP \( \text{slpA} \), it could be interesting to choose primers in more conserved regions. Kato et al. (2005) used nested PCR with two pairs of primers chosen in a conserved region of the \( \text{slpA} \) gene for \( \text{C. difficile} \) typing by sequencing the \( \text{slpA} \) gene. All the strains were typeable, and sequences could be compared among different laboratories from a database. However, as sequence analysis is not always available in clinical laboratories, we studied the discriminatory power and the typeability of PCR-RFLP on the \( \text{slpA} \)

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Ward</th>
<th>Toxinotype</th>
<th>PCR-ribotype</th>
<th>PCR-RFLP ( \text{fliC} )</th>
<th>PCR-RFLP ( \text{slpA}^* )</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>R</td>
<td>0</td>
<td>1</td>
<td>VII</td>
<td>1</td>
<td>I</td>
</tr>
<tr>
<td>4</td>
<td>H</td>
<td>0</td>
<td>1</td>
<td>VII</td>
<td>1</td>
<td>I</td>
</tr>
<tr>
<td>11</td>
<td>R</td>
<td>0</td>
<td>1</td>
<td>VII</td>
<td>1</td>
<td>I</td>
</tr>
<tr>
<td>33</td>
<td>R</td>
<td>0</td>
<td>1</td>
<td>VII</td>
<td>1</td>
<td>I</td>
</tr>
<tr>
<td>42</td>
<td>H</td>
<td>0</td>
<td>1</td>
<td>VII</td>
<td>1</td>
<td>I</td>
</tr>
<tr>
<td>48</td>
<td>H</td>
<td>0</td>
<td>1</td>
<td>VII</td>
<td>1</td>
<td>I</td>
</tr>
<tr>
<td>BO</td>
<td>G</td>
<td>0</td>
<td>1</td>
<td>VII</td>
<td>NT</td>
<td>II</td>
</tr>
<tr>
<td>1</td>
<td>R</td>
<td>0</td>
<td>1</td>
<td>VII</td>
<td>NT</td>
<td>II</td>
</tr>
<tr>
<td>6</td>
<td>H</td>
<td>0</td>
<td>1</td>
<td>VII</td>
<td>NT</td>
<td>II</td>
</tr>
<tr>
<td>36</td>
<td>H</td>
<td>0</td>
<td>1</td>
<td>VII</td>
<td>NT</td>
<td>II</td>
</tr>
<tr>
<td>14</td>
<td>H</td>
<td>0</td>
<td>2</td>
<td>VII</td>
<td>NT</td>
<td>III</td>
</tr>
<tr>
<td>53</td>
<td>H</td>
<td>0</td>
<td>2</td>
<td>VII</td>
<td>NT</td>
<td>III</td>
</tr>
<tr>
<td>60</td>
<td>H</td>
<td>0</td>
<td>2</td>
<td>VII</td>
<td>NT</td>
<td>III</td>
</tr>
<tr>
<td>41</td>
<td>H</td>
<td>ToxA(^{-})/B(^{-})</td>
<td>3</td>
<td>VII</td>
<td>2</td>
<td>IV</td>
</tr>
<tr>
<td>43</td>
<td>H</td>
<td>ToxA(^{-})/B(^{-})</td>
<td>3</td>
<td>VII</td>
<td>2</td>
<td>IV</td>
</tr>
<tr>
<td>8</td>
<td>H</td>
<td>0</td>
<td>4</td>
<td>VII</td>
<td>3</td>
<td>V</td>
</tr>
<tr>
<td>13</td>
<td>R</td>
<td>ToxA(^{-})/B(^{-})</td>
<td>5</td>
<td>VII</td>
<td>NT</td>
<td>VI</td>
</tr>
<tr>
<td>44</td>
<td>G</td>
<td>0</td>
<td>6</td>
<td>VII</td>
<td>NT</td>
<td>VII</td>
</tr>
<tr>
<td>40</td>
<td>G</td>
<td>0</td>
<td>7</td>
<td>I</td>
<td>4</td>
<td>VIII</td>
</tr>
</tbody>
</table>

*NT, Non-typeable.

![Fig. 1. RFLP patterns of the PCR-amplified \( \text{fliC} \) gene: two patterns, a and b, were obtained with each enzyme. Lanes M, 100 bp ladder (Eurobio).](image)

Table 1. Epidemiological study of \( \text{Clostridium difficile} \) serogroup A isolates from elderly patients by different typing methods
gene, which is a more practicable method. The variability of
\(slpA\) and \(fliC\) genes has been confirmed by multilocus
sequence analysis, demonstrating that these genes under-
went recombination events under environmental selective
pressure (Lemée et al., 2005).

Considering all these typing methods, the 19 serogroup A
isolates were distributed among eight main groups (I–VIII),
demonstrating the circulation of different strains in the
hospital (Table 1). The toxigenic serogroup A isolates
belonged to groups I, II, III, IV, VII and VIII whereas the
non-toxigenic isolates belonged to two other groups, IV and
VI. Six isolates belonging to group I were found to be closely
related by PCR-ribotyping and PCR-RFLP \(slpA\). The PCR-
RFLP \(slpA\) analysis allowed for the subdivision of PCR-
ribotype 1 into two groups.

In conclusion, PCR-ribotyping combined with PCR-RFLP
analysis of the \(slpA\) gene appears to be more discriminatory
than PCR-ribotyping alone. These methods are suitable for
investigating \(C.\) difficile cross-infections and understanding
the epidemiology of this enteropathogen in geriatric units.
They showed the spread of three main clones among
toxigenic serogroup A isolates and the persistence of a group
I strain over 11 months in two wards of the hospital. This
may have been due to transmission from patient to patient
or from environment to patients. Spores may have played a
main role in cross-infection.

**REFERENCES**

Development of a new PCR-ribotyping method for \(Clostridium\)
difficile based on ribosomal RNA gene sequencing. \(FEMS\) \(Microbiol\)
Lett 175, 261–266.

\(J\) \(Hosp\) \(Infect\) 40, 1–15.

Brazier, J. S. (2001). Typing of \(Clostridium\) difficile. \(Clin\) \(Microbiol\)
Infect 7, 428–431.

Calabi, E., Ward, S., Wren, B., Paxton, T., Panico, M., Morris, H., Dell, A.,
Dougan, G. & Fairweather, N. (2001). Molecular characterization of
the surface layer proteins from \(Clostridium\) difficile. \(Mol\) \(Microbiol\) 40,
1187–1199.

\(Clostridium\) difficile strains by slide agglutination. \(J\) \(Clin\) \(Microbiol\) 21,
323–327.

Fawley, W. N., Parnell, P., Verity, P., Freeman, J. & Wilcox, M. H.
(2005). Molecular epidemiology of endemic \(Clostridium\) difficile
infection and the significance of subtypes of the United Kingdom
epidemic strain (PCR Ribotype 1). \(J\) \(Clin\) \(Microbiol\) 43, 2685–2696.


