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

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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
isolate 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 Delmé et al. (1985).

Toxinotyping. DNA was extracted from 1 ml of an overnight anaer-
obic culture (Trypticase Glucose Yeast broth) using the protocol
provided in the INSTAGENE kit (Bio-Rad). Genetic identification of
the 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
HinII or Accl 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 USE Spol Swab kit (Invitrek) as
per 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-RFLP. The variability of the flIC gene encoding flagellin was
determined by PCR-RFLP. The primer sequences were 5' GTGC-
GGCTGATCACCCTC-3' and 5'-CCTCTGACCCCTTATAAGGT-
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% agar-
ose gel (Eurobio) for 2 h at 85 V.

PCR-ribotyping. This method was performed as described by Bidet
et al. (1999). The primer sequences (16S primers) were 5'-ATGA-
GATTAAATAAATTGTAATGCG-3' and 5'-CTATCCTAATAATT-
GAAAACCTC-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).

RESULTS AND DISCUSSION

Among the 28 isolates studied, 19 belonged to serogroup A,
three to serogroup K, two to serogroup G, and 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 toxino-type 0 among clinical
isolates (Geric et al., 2004). No isolate belonged to the
hypertoxicinogenic toxino-type 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 showed 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 C. difficile (Fawley et al.,
2005; Warny et al., 2005).

For RFLP analysis of the flIC gene, the amplified product
(870 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 C. 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 DraI
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 C. difficile surface proteins
display a high variability (Péchiné et al., 2005). McCoubrey
et al. (2003) studied the variation in the molecular mass of
the C. difficile S-layer proteins and identified seven S types
among 100 isolates. The C. 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 C. 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 \textit{slpA} gene in serogroup A. To enhance typeability of PCR-RFLP \textit{slpA}, it could be interesting to choose primers in more conserved regions. Kato \textit{et al.} (2005) used nested PCR with two pairs of primers chosen in a conserved region of the \textit{slpA} gene for \textit{C. difficile} typing by sequencing the \textit{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 \textit{slpA}

<table>
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<th>Isolate no.</th>
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<th>PCR-RFLP slpA*</th>
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*NT, Non-typeable.

**Table 1.** Epidemiological study of \textit{Clostridium difficile} serogroup A isolates from elderly patients by different typing methods

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


I. Poilane and others


