Characterization of *Clostridium difficile* 018: an epidemic PCR-ribotype recently emerged in Italy

P. Spigaglia, F. Barbanti, P. Mastrantonio*

Department of Infectious, Parasitic and Immuno-mediated Diseases, Istituto Superiore di Sanità, Rome, ITALY

Introduction and Purpose

PCR-ribotype 018 has recently emerged in the panorama of *Clostridium difficile* strains as a cause of severe infections and outbreaks.
Data from the European hospital-based survey of 2008, performed in 34 countries, showed that this type was the fourth most frequent PCR-ribotype and that strains 018 were significantly associated with complicated infection in patients with an age of 65 years or older (The Lancet, 2011, 377: 63). In that survey, the majority of strains 018 was isolated in Italy, indicating a regional spread of this type.
In this study, we evaluated the frequency of isolation of strains 018 on a collection of 257 Italian clinical strains, isolated from 1985 to 2011, and analysed the characteristics of representative isolates 018 in comparison with two epidemic strains belonging to PCR-ribotype 012 and 126, the predominant types in our country before the emergence of PCR-ribotype 018.

Methods

***C. difficile* clinical isolates and control strains.** PCR-ribotype 018 frequency was evaluated on 257 *C. difficile* strains from the Istituto Superiore di Sanità (ISS) *C. difficile* national collection.
These strains were isolated from sporadic cases and outbreaks between 1985 and 2011 and were sent to ISS by different Italian hospitals for typing and/or molecular analysis.
C. difficile 630, a PCR-ribotype 012 strain, was used as control in the different assays.

Phenotypic and genotypic analysis
A selection of strain 018 was analysed in the different assays, together with two isolates, one PCR-ribotype 012 and one 126, both from severe outbreaks and representative of the two types predominant before the emergence of PCR-ribotype 018.

Antibiotic susceptibility and mechanisms of resistance. MICs to moxifloxacin (MX), erythromycin (EM) and clindamycin (CM) were determined using the E-test (AB Biodisk, Solna, Sweden) on Brucella agar plates containing vitamin K1 (0.5 mg/L), hemin (5 mg/L) and 5% defibrinated sheep red blood cells, according to the manufacturer's instructions. The breakpoint used for all antibiotics tested in the study was 8 mg/L, in accordance with the guidelines established by the Clinical and Laboratory Standards Institute (CLSI, 2007, M11-A7).
erm(B) genes were detected by the amplification of an internal fragment using the primer pair E5/E6, as previously described (JMM, 2004, 53: 1129). Mutations in the *gyr*A and *gyr*B genes in *C. difficile* strains resistant to fluoroquinolones were detected as previously described (AAC, 2009, 53: 2463). Briefly, the quinolone-resistance determining region (QRDR) of both *gyr*A and *gyr*B genes was amplified using two different couples of primers, sequenced and analysed for mutations conferring resistance to moxifloxacin and others fluoroquinolones.
Sequencing and phylogenetic analysis. The gene encoding for the toxin negative regulator TcdC and that encoding for the S-layer proteins precursor (SlpA) were amplified as already described (JCM, 2002, 40: 3470). PCR products were purified using the NucleoSpin Extract kit (Macherey-Nagel) and sequenced by the Big Dye Terminator v.1.1 Cycle Sequencing kit (Applied Biosystems) and an Applied Biosystems 3730 DNA Analyser. Sequences were analysed and compared by the SeqMan and MegAlign in DNAStar Lasergene® v8.0 software (DNASTAR Inc., Madison, WI).
Comparisons of SlpA amino acid sequences were accomplished using the European Bioinformatics Institute ClustalW server and the output was used for the construction of the phylogenetic tree by TreeView 1.6.6.
Adhesion and cytotoxicity assays. In vitro adhesion was performed on Caco-2 cells. Monolayers were used at 3 (non confluent monolayers) and 15 days (post confluent monolayers) after seeding. 0.1 mM EGTA was added in the media to disrupt intercellular junctions. Overnight cultures of different *C. difficile* strains were pelleted, washed and added to each tissue-culture plate well. After 1.5 h of incubation in anaerobic conditions the non adherent bacteria were removed by washing with PBS and the bound bacteria were detached by adding saponine 1%. Serial dilutions were plated and cfu were counted after 48 h of incubation. Statistical analyses were performed using Mann and Whitney test with GraphPad Prism software. A p value< 0.05 was considered significant.
As far as cytotoxicity is concerned, overnight cultures of the different *C. difficile* strains were pelleted and the supernatants were filtered, diluted and added to confluent CaCo-2 cell monolayers. The cells were examined after 6 and 24 h of incubation at 37 °C in a 5% CO2 atmosphere. The result of the cytotoxicity assay was considered positive if cell rounding and detachment were observed.

Results

PCR-ribotyping. Typing results are shown in Table1. In general, from 1985 to 2000 the predominant type was 012 (44.1%), during the period 2001-2006 the majority of strains were 126 (44.0%), whereas strains 018 (70.3%) were the most frequently isolated from 2007 to 2011.
Strain 018 was first detected in 2006, when four (4.7%) were isolated (data not shown).

Table 1. Predominant PCR-ribotypes in the different time periods

Period	N. of strains isolated	Predominant PCR-ribotypes (%)
1985 - 2000	95	012 (44.1) - 014/020 (25.5) - 078 (8.8)
2001 - 2006	86	126 (44.0) - 078 (11.6) - 014/020 (8.3)
2007 - 2011	138	018 (70.3) - 078 (10.1) - 095 (4.3)

SlpA and TcdC analysis. Strains 018 had a new SlpA variant, showing 89% of identity with that of PCR-ribotype 126/078 (Fig. 1), whereas the SlpA found in strain 126 and 012 was identical to that already observed in strains belonging to the same PCR-ribotype (data not shown).

No amino acid substitutions were found in the toxin negative regulator TcdC of strains 018, as already observed in other strains of this type.
This analysis performed on the TcdC of strain 126 showed that this strain had a new variant with an identity of 96% to that of strain 81A330 (GenBank accession number ABI29910). Strain 012 was analysed in a previous study (Infect. Immun, 2001, 69: 3442) and its sequence deposited in GenBank (accession number AJ291709).

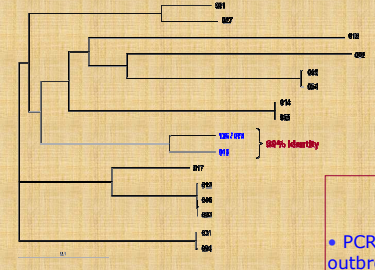

Fig. 1. Phylogenetic tree based on the alignment of the SlpA sequence of *C. difficile* 018 with those of PCR-ribotypes 001, 002, 005, 010, 012, 014, 017, 027, 031, 046, 054, 066, 126/078, 092 and 094 (GenBank accession n. AAZ05957, AAZ05964, AAZ05968, AAZ05974, AAZ05975, AAZ05984, AAZ05988, CBE06198, AAZ05989, AAZ05980, AAZ05972, AAZ05986, AAZ05994, AAZ05982, AAZ05991, respectively). The phylogram was generated using TreeView 1.6.6. The branch lengths are scaled in proportion to the extent of the change per position, as indicated by the scale bar.

Table 2. Antibiotic susceptibility of 37 selected clinical isolates PCR-ribotype 018.

Period (n. of strains isolated)	N. of strains analysed	MICs range (mg/L)			erm(B) (n. of strains)	Amino acid substitution in GyrA
		EM	CM	MX		
1985 - 2000 (0)	0	-	-	-	-	-
2001 - 2006 (4)	4	1 - ≥256	1 - ≥256	16 - ≥32	positive (1) negative (3)	Thr82 to Ile
2007 - 2011 (97)	33	1- ≥256	1 - ≥256	1 - ≥32	negative (33)	Thr82 to Ile

Antibiotics susceptibility and mechanisms of resistance. A selection of 37 isolates PCR-ribotype 018, representative of different outbreaks were analysed for antibiotic susceptibility. MIC ranges for the 37 strains analysed were 1 - ≥256 mg/L for EM, 3 - ≥256 mg/L for CM and 1 - ≥32 mg/L for MX (Table 2). Twelve isolates were multi-resistant, since they were resistant to the 3 classes tested, whereas only one strain was susceptible to all these antibiotics.
Twenty three strains were resistant to both EM and MX and one isolate only to MX. Only one strain, highly resistant to EM and CM (MIC ≥256 mg/L for both antibiotics), was *erm*(B)-positive (Table 2). The amino acid substitution Thr82 to Ile in GyrA was observed in all strains resistant to MX.
The representative epidemic strain 012 was resistant to both EM and CM and positive for *erm*(B), whereas the representative epidemic strain 126 was resistant to EM and MX, *erm*(B)-positive and negative for the amino acid substitution Thr82 to Ile (data not shown).

Adhesion to CaCo2 cells and citotoxicity. As showed in Fig. 2, strains 018 showed a number of adherent bacteria per cell significantly higher compared to the other strains in all assays performed at 3 days. This significant difference was still observed in comparison with strain 126 and the control strain *C. difficile* 630 but not with the epidemic strain 012, that it is known to have high levels of adhesiveness (FEMS Immunol Med Microbiol, 2002, 32: 211), in the assays performed at 15 days. Cytotoxicity assays indicated an early activity of strains 018, visible as cell damage at 6 hours, compared to the other types (data not shown).

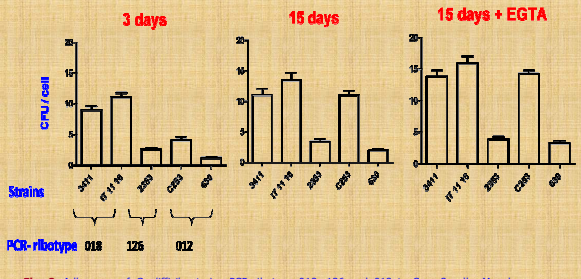

Fig. 2. Adherence of *C. difficile* strains PCR-ribotype 018, 126 and 012 to Caco-2 cells. Monolayers were infected after 3 days or 15 days (untreated or pretreated with EGTA) in anaerobic condition. The adhesion index is given as the average number of adhering bacteria per cell ± the standard deviation from four different assays

Conclusions

- PCR-ribotype 018 is the most frequent type isolated in Italy since 2007 and it is the cause of severe CDI and outbreaks.
- In this study, all strains 018, except one, resulted resistant to MX and EM. Twelve strains (32%) were multi-resistant showing resistance also to CM.
- A new SlpA variant was identified in strains 018. This variant has 89% of identity with that of type 126/078. Since SlpA is involved in adhesion, it is probably implicated in the high level of adhesiveness of strains PCR-ribotype 018.
- Preliminary results also indicate an early cytotoxic activity by the representative strains 018 analysed in this study.
- All these characteristics may have played a role in the enhancement of virulence and in facilitating the spread of PCR-ribotype 018 in the last years.
- Further studies will be undertaken to better investigate the peculiar characteristics of this emerging type.

patrizia.spigaglia@iss.it