Characterization of *Clostridium difficile* strains isolated in Italy from 2007 to 2011 and comparative analysis of the predominant PCR-ribotypes

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Introduction and Purpose

The European surveillance performed in 2008 indicated Clostridium difficile PCR-ribotypes 078/126 and 018 as two emerging virulent types. The first type includes hypervirulent strains that cause infections in both hospital settings and community and that are currently spread in different European countries; the second type is predominantly isolated in Italy where it is the major cause of severe infections and outbreaks occurred in the last years. While type 078/126 has variations in the locus of pathogenicity (PaLoc), in particular in the toxin genes, and it is considered a toxin variant type, strains 018 are non-toxin variant, as the majority of isolates causing infections in Europe. In this study, 131 Italian clinical isolates isolated from 2007 and 2011 were characterized and a selection of strains 018 and 078/126 were investigated for sporulation and adhesion to Caco-2 cells. Strains were also compared by in vitro competition assays.

Table 1. Characteristics of the 131 *C. difficile* clinical isolates analyzed in the study

PCR-ribotype	Year of isolation (n. of strains)	ERY MIC range[a]	ERY % of resistance	CLI MIC range	CLI % of resistance	MXF MIC range	MXF % of resistance	RIF MIC range	RIF % of resistance
018	2007 (8) 2008 (20) 2009 (23) 2010 (27) 2011 (13)	1 - ≥256	99%	3 - 16	53%	1 - ≥32	99%	≤0.002 - ≥32	85%
078/126	2008 (7) 2009 (6) 2010 (4) 2011 (1)	0.75 - ≥256	89%	3 - ≥256	50%	0.38 - 12	33%	≤0.002 - ≥32	
095	2008 (5) 2009 (1)	0.75 - ≥256	83%	6 - ≥256	83%	0.5 - ≥32	83%	≤0.002 - ≥32	83%
010	2010 (2)	≥256	100%	≥256	100%	0.75		≤0.002	
014	2008 (1) 2009 (1)	0.75 - ≥256	50%	6		0.75 - 8	50%	≤0.002 - ≥32	50%
106	2009 (2)	≥256	100%	6		≥32	100%	≤0.002	
001	2008 (1)	≥256	100%	≥256	100%	12	100%	≥32	100%
002	2009 (1)	2		4		2		2	
003	2008 (1)	2		6		1		≤0.002	
012	2008 (1)	≥256	100%	≥256	100%	0.5		≤0.002	
015	2008 (1)	2		4		0.5		≤0.002	
087	2007 (1)	1.5		2		0.5		≤0.002	
116	2011 (1)	0.75		1.5		0.75		≤0.002	
131	2008 (1)	2		6		1		≤0.002	
137	2008 (1)	0.75		4		1		≤0.002	
212	2008 (1)	0.75		1.5		0.75		≤0.002	
Total	131	0.75 - ≥256	90%	1.5 - ≥256	50%	0.38 - ≥32	80%	≤0.002 - ≥32	65%

[a]: MIC values are reported in mg/L

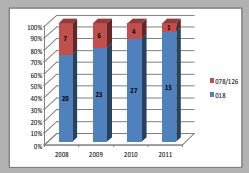

Fig 1. Ratio between strains 018 and 078/126 from 2008 to 2011

Table 2. Multi-resistant strains identified in the study

PCR-ribotype	N. of strains analyzed	N. of multi-resistant strains (%)	N. of strains resistant to: ERY MXF RIF	ERY MXF CLI	ERY CLI RIF	ERY MXF CLI RIF
018	91	85 (93)	37	8		40
078/126	18	4 (22)		2	2	
095	6	5 (83)				5
014	2	1 (50)	1			
001	1	1 (100)				1

Results

Among the 131 strains analyzed, the predominant PCR-ribotypes were 018 (69%) and 078/126 (14%), followed by 14 other types (Table 1). In 2007 only 9 strains were analyzed and 8 belong to PCR-ribotype 018. 73% of the strains (96/131) were multi-resistant and the majority of them (88%) were 018 (Table 2). The ratio between 018 and 078/126 increased from 2008 to 2011 (Fig. 1). In general, the majority of multi-resistant strains (84/96) were resistant to ERY, MXF and RIF. All strains were susceptible to VAN and MTZ.

Interestingly, two new SlpA variants were found in strains 018 (Table 3). These variants have 99% identity with that of a strain 018 recently analyzed by Dingle et al. (J Infect Dis, 2013, 207: 675) and 89% identity with the SlpA of strains 078/126.

Among the 20 selected strains belonging to PCR-ribotype 018 and 078/126, an *erm*B gene was detected only in those highly resistant to both ERY and CLI (MIC ≥ 256 mg/L) (Table 3). Resistance to MXF and RIF was associated to amino acid substitutions in GyrA (Thr82 → Ile) and in RpoB (Ser498 → Thr and/or Arg505 → Lys), respectively. Sporulation was strain-dependent but, in general, more abundant for strains 018 after 48h (Fig. 2). A significantly higher adhesion to CaCo-2 cells was observed for strains 018 (Fig. 3). Furthermore, growth competition assays indicated that strains 018 rapidly overcome strains 078/126 with a decrease in fitness for the latter (Table 4).

Table 3. Characteristics of 20 selected *C. difficile* strains belonging to PCR-ribotype 018 and 078/126

Strains	Year	PCR-ribotype	Resistance to	ermB	Sustitution in GyrA	Sustitution in RpoB	SlpA sequence
C253 (control strain)	1987	012	ERY CLI RIF	+	Thr82→Ile	Arg505→Lys	identical to AAZ05975 (strain HPA R13550)
3411	2006	018	ERY CLI MXF	+	Thr82→Ile		new type 1
IT0601	2006	018	ERY MXF	-	Thr82→Ile		new type 1
IT0707	2007	018	ERY CLI MXF	-	Thr82→Ile		new type 2
IT0805	2008	018	ERY CLI MXF RIF	-	Thr82→Ile	Ser498→Thr / Arg505→Lys	n. d.
IT0839	2008	018	ERY MXF RIF	-	Thr82→Ile	Ser498→Thr / Arg505→Lys	new type 1
IT0902	2009	018	ERY MXF RIF	-	Thr82→Ile	Arg505→Lys	new type 1
IT0926	2009	018	ERY MXF RIF	-	Thr82→Ile	Ser498→Thr / Arg505→Lys	new type 1
IT1006	2010	018	ERY CLI MXF RIF	-	Thr82→Ile	Ser498→Thr / Arg505→Lys	new type 1
IT1024	2010	018	ERY MXF RIF	-	Thr82→Ile	Arg505→Lys	new type 1
IT1118	2011	018	ERY CLI MXF RIF	-	Thr82→Ile	Arg505→Lys	new type 1
CD5	1998	078/126	ERY	-			identical to AAZ05994 (strain HPA R13540)
2353	2006	078/126	ERY MXF	-	Thr82→Ile		identical to AAZ05994 (strain HPA R13540)
IT0820	2008	078/126	ERY CLI RIF	+		Arg505→Lys	identical to AAZ05994 (strain HPA R13540)
IT0834	2008	078/126	ERY CLI MXF	+	Thr82→Ile		identical to AAZ05994 (strain HPA R13540)
IT0844	2008	078/126	ERY CLI MXF	-	Thr82→Ile		identical to AAZ05994 (strain HPA R13540)
IT0918	2009	078/126	ERY MXF	-	Thr82→Ile		identical to AAZ05994 (strain HPA R13540)
IT0925	2009	078/126	ERY	-			identical to AAZ05994 (strain HPA R13540)
IT1004	2010	078/126	ERY MXF	-	Thr82→Ile		identical to AAZ05994 (strain HPA R13540)
IT1008	2010	078/126	ERY MXF	-	Thr82→Ile		identical to AAZ05994 (strain HPA R13540)
IT1106	2011	078/126	ERY	-			identical to AAZ05994 (strain HPA R13540)

Fig. 2. Results of the *in vitro* sporulation assays performed on the selected strains 018 and 078/126. **A:** results obtained for each strain **B:** results expressed as average values

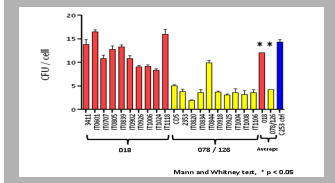

Fig. 3. Results of the *in vitro* adhesion assays performed on the selected strains 018 and 078/126

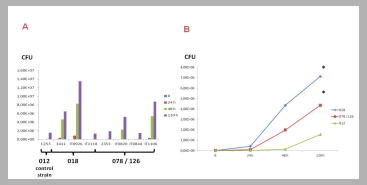

Table 4. Results of the *in vitro* growth competition assays performed on the selected strains 018 and 078/126

Strains		Ratio A / B[b]			s[b]	Relative fitness per generation[c]	P[d]
018	vs 078/126	1	2	3			
3411	2353	1.18	1.44	2.31	0.026	1.026 ± 0.009	<0.0001
3411	IT0844	1.11	1.36	2.08	0.021	1.021 ± 0.007	<0.0001
IT1118	IT1106	1.19	1.28	2.40	0.024	1.024 ± 0.009	<0.0001
IT0926	IT0820	1.17	1.25	2.00	0.021	1.021 ± 0.005	<0.0001

[a] Values represent the $\log_{10}$(cfu) ratios at the end of each transfer (ca. 8 generations).
[b] Selection coefficient.
[c] Fitness relative to strains PR 018.
[d] Statistical significance of difference in fitness relative to strains PR 018.

Methods

Bacterial strains. 131 *C. difficile* clinical isolates selected from the Istituto Superiore di Sanità (ISS) *C. difficile* national collection were included in this study. These strains were isolated from sporadic cases and outbreaks between 2007 and 2011 and were sent to ISS by different Italian hospitals for typing and/or molecular analysis. *C. difficile* C253, isolated in Italy (1987) and belonging to PCR-ribotype 012, were used as control strain.

Susceptibility test. Susceptibility to erythromycin (ERY), clindamycin (CLI), rifampicin (RIF), moxifloxacin (MXF), vancomycin (VAN) and metronidazole (MTZ) was determined by the E-test (AB Biodisk). The breakpoints used were 8 mg/L for ERY, CLI and MXF, and 32 mg/L for MTZ, in accordance with the CLSI interpretative categories approved for anaerobic bacteria (CLSI - Seventh Edition: Approved standard M11-A7. CLSI, Wayne, PA, USA, 2007.). The breakpoints for RIF and VAN were 4 and 16 mg/L, respectively, in accordance with the CLSI interpretive categories approved for Staphylococcus aureus (CLSI - Eighteenth Informational Supplement M100-S18. CLSI, Wayne, PA, USA, 2008.), since no interpretative values were provided for anaerobes.

SlpA characterization. The gene 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 analyzed 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.

Spore production and *in vitro* adhesion assay. Spore production was evaluated as already described (PLoSONE 6(9): e24894. doi:10.1371/journal.pone.0024894). Briefly, *C. difficile* isolates were cultured in BHIS broth in anaerobic conditions at 35°C for 5 days. 500 µl of cultures were removed at 0, 24, 48 and 120h from the anaerobic chamber and heated at 60°C for 25 min to kill vegetative forms. Samples were then plated onto BHIS plates supplemented with 0.1% bile salt taurocholate to induce germination and enhance recovery of spores. Statistical analysis was performed using Mann and Whitney test with GraphPad Prism software. A p value < 0.05 was considered significant.

In vitro adhesion was performed on Caco-2 cells at 15 days (post confluent monolayers), after a treatment with 0.1 mM EGTA to disrupt intercellular junctions. Results were expressed as number of adherent bacteria per cell. Statistical analyses were performed using Mann and Whitney test with GraphPad Prism software. A p value < 0.05 was considered significant.

Growth competition assays. Growth competition assays were performed as already described (PNAS, 2010,107:16964). Briefly, selected strains 018 and 078/126 were grown separately, mixed in a 1:1 ratio, and used to inoculate 25 ml of BHI broth. One hundred microliters were transferred to fresh 25-mL broth every 24 hours over three cycles. Aliquots were plated at the end of every cycle with and without the antibiotic of selection used. The selection coefficient s was calculated as $s=\ln(CI)/[t \times \ln(2)]$, where CI is the competition index (calculated as the cfu ratio of the two strains used for each experiment at time t1, t2 and t3 divided by the same ratio at time t0) and t is the number of generations. Fitness of strains 078/126 was set to 1 and the relative fitness of type 018 was determined as 1 + s. Statistical analysis was performed using GraphPad software.

Conclusions

- **Among 131 Italian *C. difficile* strains isolated between 2007 and 2011, 16 different PCR-ribotypes were identified. Types 018 (69%) and 078/126 (14%) were the predominant. Interestingly, the ratio between 018 and 078/126 increased from 2008 to 2011.**
- **73% of the strains analyzed were multi-resistant and the majority of them were resistant to ERY, RIF and MXF. 88% of the multi-resistant strains belonged to PCR-ribotype 018.**
- **Comparative *in vitro* assays showed a significant higher production of spores and adhesion to Caco-2 cells for strains 018. Interestingly, two new SlpA variants were found in strains 018. These variants could contribute to the higher adhesion of these strains to the cells.**
- **Growth competition assays between strains 018 and 078/126 also demonstrated that the first becomes predominant in a short time. This peculiar behavior could allow a more rapid colonization of the intestine by strains 018 if maintained *in vivo*.**
- **All these characteristics may have played a role in the enhancement of virulence and in facilitating the spread of type 018 towards type 078/126 in Italian hospitals, even if further studies will be necessary to in depth investigate this emerging type.**