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Comparative analysis of *Clostridium difficile* clinical isolates belonging to different genetic lineages and time periods

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Recent studies have shown that *Clostridium difficile* strains with variant toxins and those with resistance to macrolide–lincosamide–streptogramin B (MLSB) are increasingly causing severe disease and outbreaks in hospital settings. Here, the pathogenicity locus (PaLoc), the acquisition of binary toxin, and the genotypic and phenotypic characteristics of antibiotic resistance of 74 *C. difficile* clinical strains isolated from symptomatic patients in Italy during different time periods were studied. These strains were found to belong to two different lineages, and those isolated before 1991 were genetically unrelated to the more recent strains. The majority of recent *C. difficile* strains showed variations in toxin genes and in the toxin negative regulator (*tcdC*) and had the binary toxin. In 62 % of them, variations in *tcdC* and the presence of the binary toxin were associated. Five classes of susceptibility/resistance pattern (EC-a to -e) for erythromycin and clindamycin were identified in all strains studied. Most of the recent isolates belonged to EC-d and EC-e and, although erythromycin-resistant *in vitro*, did not harbour the commonly associated *ermB* determinant. Interestingly, two strains of the EC-d class were resistant to clindamycin only after induction with subinhibitory concentrations of the antibiotic. A decrease in tetracycline and chloramphenicol MIC values was also observed in the recently isolated strains, associated with less frequent detection of the *catD* and *tetM* genes. Two *tetM*-positive strains were resistant *in vitro* only after induction with subinhibitory concentrations of the antibiotic. The acquisition of the binary toxin, the possible increase in toxin production due to a mutated negative regulator and a decrease in the fitness cost as a result of lower levels of antibiotic resistance or other mechanisms may have led to the successful establishment of these new phenotypes, with potentially serious clinical implications.

INTRODUCTION

Recently, the involvement of *Clostridium difficile* strains with variant toxins, as well as strains with resistance to macrolide–lincosamide–streptogramin B (MLSB), in causing severe diseases and outbreaks has been demonstrated (Alfa *et al.*, 2000; Johnson *et al.*, 1999; Pituch *et al.*, 2003; Sambol *et al.*, 2000; Spigaglia & Mastrantonio, 2002).

C. difficile toxin A (TcdA, an enterotoxin) and toxin B (TcdB, a cytotoxin) are encoded by two genes, *tcdA* and *tcdB*, which, together with three accessory genes, *tcdE*, *tcdD* and *tcdC*, form a pathogenicity locus (PaLoc) (Braun *et al.*, 1996; Hammond & Johnson, 1995). TcdD and TcdC have been indicated as the positive and negative regulators of toxin A and B expression, respectively, whereas TcdE seems to have a holin function (Hammond *et al.*, 1997; Hundsberger *et al.*, 1997; Tan *et al.*, 2001). Much heterogeneity has been observed in the toxin A and B genes and currently 20 groups of variant *C. difficile* strains have been recognized and defined as toxinotypes I–XX (Rupnik *et al.*, 1997, 1998, 2003). Some studies have also focused on the production of a third toxin, binary toxin CDT, by several *C. difficile* isolates (Perelle *et al.*, 1997; Stubbs *et al.*, 2000). Binary toxin CDT is produced by the majority of strains with mutations in the *tcdA* and *tcdB* genes and its detection has been suggested as a method for the rapid identification of these variant strains (Rupnik *et al.*, 2001). Mutations have been also detected in PaLoc accessory genes. Three different alleles of *tcdC*, which encodes the negative toxin regulator, have been identified and it has been hypothesized that these variants could have some influence on the amounts of toxins A and B released (Soehn *et al.*, 1998; Spigaglia & Mastrantonio, 2002).

MLSB-resistant *C. difficile* strains have been demonstrated to be the cause of epidemics of diarrhoea in different hospitals (Johnson *et al.*, 1999; Pituch *et al.*, 2003). The *erm* genes, in particular the *ermB* class, are the main known cause of

Abbreviation: MLSB, macrolide–lincosamide–streptogramin B.

resistance to MLSB in *C. difficile* and *Clostridium perfringens* (Berryman & Rood, 1989; Farrow *et al.*, 2000; Roberts, 1995). In *C. difficile*, this determinant is located on a conjugative transposon called Tn*5398*. Recent studies have demonstrated much heterogeneity in the genetic arrangement of this element (Farrow *et al.*, 2001; Spigaglia & Mastrantonio, 2003). Furthermore, an association between different levels of resistance to erythromycin, different alleles and the number of *ermB* copies has been observed. Resistance to MLSB is frequently found in *C. difficile* strains that are also resistant to other antibiotics (Ackermann *et al.*, 2003; Barbut *et al.*, 1999; Delmee & Avesani, 1988; Roberts *et al.*, 1994), underlining the importance of monitoring their circulation.

In a previous study, we analysed *C. difficile* clinical isolates from different Italian hospitals by PCR ribotyping and PFGE (Spigaglia *et al.*, 2001). In the present study, the PaLoc genes, the presence of the binary toxin, and the genotypic and phenotypic characteristics of antibiotic resistance in several toxinogenic *C. difficile* clinical isolates were analysed with the aim of identifying characteristics differentiating older *C. difficile* strains from more recently isolated strains.

METHODS

Bacterial strains and PCR ribotyping. Seventy-four toxinogenic *C. difficile* strains from sporadic symptomatic cases or representative of outbreaks were examined in this study. These isolates, from diarrhoeic adults and children, were collected in five hospitals during the course of the study period and sent to our laboratory for further characterization. Twenty isolates were selected from 53 strains isolated in period I (before 1990), 34 from 90 strains in period II (1991–1999) and 20 from 56 strains isolated in period III (2000–2001). Thirty-five *C. difficile* isolates were selected from 100 strains collected from 1985 to 1999 and typed in a previous study (Spigaglia *et al.*, 2001), while 39 isolates were selected from 110 strains isolated between 1991 and 2001 and typed during this study. PCR ribotyping was performed on these strains as previously described (Spigaglia *et al.*, 2001). Briefly, 10 µl crude DNA was used as a template. Amplification was performed in a final volume of 100 µl with a reaction mixture containing buffer (10 mM Tris/HCl, 50 mM KCl and 1·5 mM $MgCl_2$), 200 mM each deoxynucleoside triphosphate, 100 pmol each primer and 2·5 U Takara *Ex Taq* (Takara Shuzo). The primers were complementary to conserved regions at the 3′ and 5′ ends of the 16S and 23S rRNA genes, respectively, as previously described by Kostman *et al.* (1992). DNA was amplified for 30 cycles, each consisting of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. After amplification, 10 µl of the product was electrophoresed on a 1·5 % agarose gel. The genomic DNA fingerprinting patterns produced by PCR ribotyping were analysed using Molecular Analyst Fingerprinting Plus Software, version 1.0 (Bio-Rad). A similarity analysis was performed using Dice's coefficient and clustering was performed using the unweighted pair group mean association.

C. difficile 630 (Wüst & Hardegger, 1983), C250 (Wren *et al.*, 1988) and F17 (Spigaglia & Mastrantonio, 2003) were used as control strains in multiplex PCRs to detect antibiotic-resistance determinants and in PCR-RFLP of *ermB* genes.

Multiplex PCR for *tcdA* and *tcdB* detection and PCR for the repetitive region of *tcdA*. Multiplex PCR for toxin A and B genes, amplifying fragments of 642 and 412 bp, respectively, were performed as previously described (Spigaglia & Mastrantonio, 2002). The amplification of a 3·1 kb fragment of the repetitive region of *tcdA* was performed by PCR with primers A3C and A4N, as reported by Rupnik *et al.* (1997), using 2 µl purified DNA as the template in each PCR. DNA was purified using the NUCLEOBOND Buffer Set III and NUCLEOBOND cartridges AXG 20.

Multiplex PCR for the PaLoc accessory genes (*tcdC*, *tcdD* and *tcdE*) and detection of binary toxin gene *cdtB*. Multiplex PCR for *tcdC*, *tcdD* and *tcdE* gene amplification were performed using the primer pairs PaL15/PaL16, PaL11/PaL12 and PaL13/PaL14, respectively (Table 1). Primers were designed based on the *C. difficile* PaLoc sequence (GenBank accession no. X92982). Five microlitres of crude DNA was used as the template, and was denatured for 5 min at 94 °C and amplified for 30 cycles consisting of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C. Ten microlitres of the PCR product was electrophoresed on a 1·5 % agarose gel. The expected sizes for PCR fragments were approximately 670 bp for *tcdC*, 470 bp for *tcdD* and 350 bp for *tcdE*. As suggested by M. Rupnik and others (www.uni-lj.si/∼bfbcdiff), binary toxin was detected by amplification of the *cdtB* gene encoding the binary-toxin-binding component. A PCR for an internal region of *cdtB* was performed as previously described (Stubbs *et al.*, 2000).

PCR to detect antibiotic-resistance determinants. Multiplex PCR for *tetM*, *ermB* and *catD* gene amplification were performed using the primer pairs TETMd/TETMr (Marchese *et al.*, 1998), E5/E6 and CL1/CL2 (Table 1), respectively. E5/E6 and CL1/CL2 primer pairs were designed from *ermB* (GenBank accession nos AF109075 and U18931) and *catD* (GenBank accession no. AF226276) sequences, respectively. Five microlitres of crude DNA were denatured for 5 min at 94 °C and amplified for 30 cycles consisting of 1 min at 94 °C, 1 min at 50 °C and 1·5 min at 72 °C. Ten microlitres of the PCR products were

Table 1. Primers used in the study

Primer	Sequence (5′⟶3′)	Gene	Product size (bp)
PaL11	CTCAGTAGATGATTTGCAAGAA	*tcdD*	473
PaL12	TTTTAAATGCTCTATTTTTAGCC		
PaL13	AGGAGGCGTTATGAATATGA	*tcdE*	358
PaL14	TGGTAATCCACATAAGCACA		
PaL15	TCTCTACAGCTATCCCTGGT	*tcdC*	673
PaL16	AAAAATGAGGGTAACGAATTT		
E5	CTCAAAACTTTTTAACGAGTG	*ermB*	711
E6	CCTCCCGTTAAATAATAGATA		
CL1	ATACAGCATGACCGTTAAAG	*catD*	500
CL2	ATGTGAAATCCGTCACATAC		

electrophoresed on a 1·5 % agarose gel. The expected sizes of the PCR fragments were approximately 1·0 kb for *tetM*, 0·6 kb for *ermB* and 0·5 kb for *catD*.

Specific primers for rRNA methylases, classes A, C, F and Q, and the *mefA* gene, encoding a membrane-bound efflux protein, have been reported by M. C. Roberts (http://faculty.washington.edu/marilynr/). PCRs were performed as described previously (Chung *et al.*, 1999; Luna *et al.*, 2000).

PCR-RFLP of *ermB* genes. Twenty microlitres of PCR products, obtained using the primers E5 and E6 and the same PCR conditions as for the multiplex PCR to detect antibiotic-resistance determinants, were digested with 40 U *Pvu*II restriction enzyme. This enzyme has a cutting site in the *C. difficile* 630 *ermB* sequence (SZ-type sequence) but no site in the *C. perfringens* CP592 *ermB* sequence (SP-type sequence). The digestion of *ermB* PCR products with a sequence similar to *C. difficile* 630 *ermB* would thus produce two fragments of approximately 589 and 122 bp, whereas *ermB* PCR products with a sequence similar to *C. perfringens* CP592 should remain undigested. The fragments were separated on a 2 % agarose gel and visualized by ethidium bromide staining.

Susceptibility tests and induction of resistance. MIC values for erythromycin, clindamycin, tetracycline and chloramphenicol were determined by the E-test (AB Biodisk), following the manufacturer's instructions. Wilkins–Chalgren agar plates (Unipath) were incubated anaerobically at 37 °C for 24 h for erythromycin, tetracycline and chloramphenicol and for 48 h for clindamycin. The cut-off values were $\geqslant$4 mg l^{-1} for erythromycin and clindamycin, $\geqslant$8 mg l^{-1} for tetracycline and $\geqslant$16 mg l^{-1} for chloramphenicol (National Committee for Clinical Laboratory Standards, 1993). Induction of resistance to clindamycin, tetracycline and chloramphenicol in *C. difficile* isolates was evaluated by pre-growth for 18 h on blood agar plates containing subinhibitory concentrations of each antibiotic. Erythromycin, at a subinhibitory concentration of 0·05 mg l^{-1}, was used to induce MLSB resistance as described previously (Giovanetti *et al.*, 1999). The subinhibitory concentration of 0·01 mg l^{-1} was used for both tetracycline and chloramphenicol (Doherty *et al.*, 2000; Wren *et al.*, 1988). MICs were then determined by the E-test as described above.

RESULTS

Detection of toxins A and B and PCR ribotyping results

All *C. difficile* isolates were confirmed as toxinogenic by multiplex PCR for toxin A and B gene detection (data not shown).

Comparison of all 74 strains included in this work showed that they clustered into two main lineages, previously described for some by Spigaglia *et al.* (2001). We have named these lineages 1 and 2. Five of the 13 PCR ribotypes (A, D, E, F and C) belonged to lineage 1 and eight (B, H, I, L, O, P, R and U) to lineage 2.

The main PCR ribotypes were A (21 strains), D (13 strains) and R (15 strains). The other PCR ribotypes each contained between one and seven *C. difficile* strains.

The majority (95 %) of *C. difficile* strains isolated in period I (before 1990) were PCR ribotypes of lineage 1, whereas the majority (90 %) of isolates from period III (2000–2001) were PCR ribotypes of lineage 2. Strains isolated in period II (1991–1999) were equally distributed in both clusters.

PCR ribotype A represented 52 % of the strains belonging to lineage 1 (21/40), whereas PCR ribotype R contained 44 % of those belonging to lineage 2 (15/34).

Analysis of PaLoc genes and detection of the binary toxin gene *cdtB*

A PCR specific for the toxin A repetitive region identified six isolates with noticeable deletions (Table 2). Five of them, four of lineage 2 (PCR ribotype R) and one of lineage 1 (PCR ribotype E), had a deletion of 0·8 kb and were very similar to toxinotype VII described by Rupnik *et al.* (1998, 2003), whereas one isolate of lineage 1, belonging to PCR ribotype E, had a deletion of 1·9 kb and could be recognized as a toxin A$^-$ toxin B$^+$ strain (toxinotype VIII).

None of the strains belonging to lineage 1 showed any variations in the *tcdC*, *tcdD* and *tcdE* accessory genes, whereas 15 *C. difficile* isolates of lineage 2 showed a different size for the PCR fragment obtained by amplification of the regulatory gene *tcdC*. Thirteen strains of PCR ribotype R had a *tcdC* gene type A, with a deletion of 39 bp, whereas the two strains of PCR ribotype L had a *tcdC* gene type B or C, with a deletion of 18 bp (Fig. 1 and Table 2).

No isolate of lineage 1 was positive for the binary toxin gene *cdtB*, whereas in lineage 2 all strains of PCR ribotype U and R were positive for this gene, suggesting the presence of minor variations in toxin A and B genes.

Detection of antibiotic-resistance genes and antimicrobial susceptibility

The results obtained by multiplex PCR for *ermB*, *tetM* and *catD* gene detection and MIC values for tetracycline and chloramphenicol are shown in Fig. 2 and Table 3, respectively, while MIC values for erythromycin and clindamycin are shown in Table 4.

Three PCR ribotypes of lineage 1 (A, D and E) and three of lineage 2 (B, L and R) contained all the antibiotic-resistant *C. difficile* strains.

Twenty-five of the 40 lineage 1 strains were found to have at least one resistance determinant. In particular, 19 isolates, belonging to PCR ribotype A, were positive for *ermB*, *tetM* and *catD* genes, four strains (two of PCR ribotype A, one of PCR ribotype D and one of PCR ribotype E) had both *ermB* and *tetM* genes and two isolates (one of PCR ribotype D and one of PCR ribotype E) had only an *ermB* gene. Of the 34 lineage 2 strains, one PCR ribotype R was positive for *ermB*, *tetM* and *catD* genes, one of the same PCR ribotype had both *ermB* and *tetM* genes, nine isolates (eight PCR ribotype R and one L) harboured only a *tetM* gene and two (one PCR ribotype B and one R) only an *ermB* gene.

catD genes have always been found with *ermB* and *tetM* genes.

Table 2. PaLoc analysis and detection of the binary toxin gene *cdtB* in 74 *C. difficile* isolates

The number of isolates is shown in parentheses. *tcdC*, *tcdD* and *tcdE* were detected in all isolates.

PCR ribotype	Number of isolates in each time period*			Size of *tcdA* repetitive region (kb)	Size of *tcdC* PCR fragment (bp)	Detection of binary toxin gene *cdtB*
	I	II	III			
Lineage 1 (40)						
A (21)	15	5	1	3·1 (21)	673 (21)	− (21)
D (13)	2	10	1	3·1 (13)	673 (13)	− (13)
E (2)	1	1		2·3 (1)–1·2 (1)	673 (2)	− (2)
F (3)	1	2		3·1 (3)	673 (3)	− (3)
C (1)	1			3·1 (1)	673 (1)	− (1)
Lineage 2 (34)						
B (1)		1		3·1 (1)	673 (1)	− (1)
H (1)	1			3·1 (1)	673 (1)	− (1)
I (2)		1	1	3·1 (2)	673 (2)	− (2)
L (2)		2		3·1 (2)	655 (2)	− (2)
O (7)		1	6	3·1 (7)	673 (7)	− (7)
P (4)		2	2	3·1 (4)	673 (4)	− (4)
R (15)		8	7	2·3 (4)–3·1 (11)	634 (13)	+ (15)
U (2)			2	3·1 (2)	673 (2)	+ (2)

*Period I, before 1990; period II, 1991–1999; period III, 2000–2001.

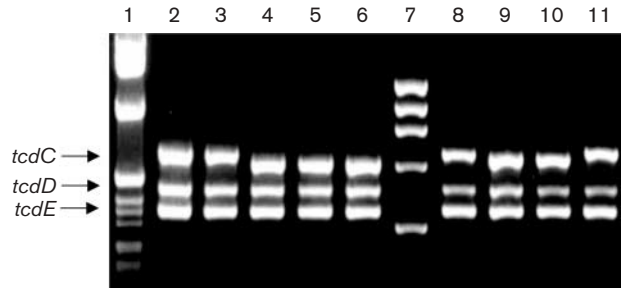

Fig. 1. Multiplex PCR for *tcdC*, *tcdD* and *tcdE* detection in *C. difficile* isolates. The expected size for the *tcdC* PCR fragment was approximately 670 bp. *tcdC* type A showed a deletion of 39 bp, whereas *tcdC* type B and type C both showed a deletion of 18 bp. Lanes: 1, DNA Molecular Weight Marker VIII (Roche); 2, 3, 8 and 11, isolates with a classic *tcdC*; 4–6, isolates with a *tcdC* gene type A; 7, DNA Molecular Weight Marker IX (Roche); 9 and 10, isolates with a *tcdC* gene type B or C.

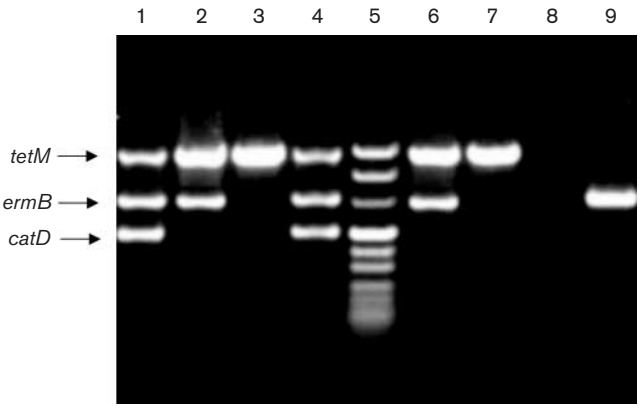

Fig. 2. Multiplex PCR for *tetM*, *ermB* and *catD* detection in *C. difficile* isolates. Lanes: 1 and 4, isolates positive for *tetM*, *ermB* and *catD*; 2 and 6, isolates positive for *tetM* and *ermB*; 3 and 7, isolates positive for *tetM*; 5, DNA Molecular Weight Marker IX (Roche); 9, isolate positive for *ermB*; 8, susceptible isolate.

C. difficile isolates belonging to lineage 1 showed MICs for erythromycin between 0·094 and 256 mg l^{-1} and for clindamycin between 0·047 and 256 mg l^{-1}.

Twenty-six isolates of lineage 1 were resistant to both antibiotics. Erythromycin and clindamycin resistance patterns could be distinguished in four classes designated EC-a, EC-b, EC-c and EC-d (Tables 3 and 4). EC-a was characterized by susceptibility to both erythromycin (MIC 0·032–2 mg l^{-1}) and clindamycin (MIC 0·047–3 mg l^{-1}), class EC-b by high levels of resistance to both erythromycin and clindamycin (MIC $\geqslant$ 256 mg l^{-1}), class EC-c by low levels of resistance to erythromycin (MIC 12–48 mg l^{-1}) and high resistance to clindamycin (MIC $\geqslant$ 256 mg l^{-1}) and class EC-d by a high level of resistance to erythromycin (MIC $\geqslant$ 256 mg l^{-1}) and susceptibility to clindamycin (MICs 1·5–3 mg l^{-1}). Sixty-five per cent (17/26) of the *C. difficile* isolates of lineage 1, resistant to both antibiotics, showed a class EC-c phenotype, 31 % (8/26) a class EC-b phenotype and 4 % (1/26) a class EC-d phenotype. This last strain was *ermB*-negative. *C. difficile* isolates belonging to lineage 2 showed an MIC for erythromycin between 0·032

Table 3. Genotypic and phenotypic analysis of antibiotic resistance in 74 *C. difficile* isolates

The number of isolates is shown in parentheses.

PCR ribotype	Detection of *ermB*, *tetM* and *catD*	*ermB* sequence type	Phenotypic class for ERY and CLI*	MIC (mg l^{-1})	
				TET*	CHL*
Lineage 1 (40)					
A (21)	*ermB* (21)/*tetM* (21)/*catD* (19)	SZ (21)	EC-b (4)/EC-c (17)	12–256 (21)	16–256 (19)
D (13)	*ermB* (2)/*tetM* (1)	SP (2)	EC-b (2)/EC-d (1)	0·016–0·064 (12)/32 (1)	0·75–4 (13)
E (2)	*ermB* (2)/*tetM* (1)	SP (2)	EC-b (2)	0·016 (1)/16 (1)	0·5–6 (2)
F (3)	–	–	EC-a (3)	0·023–0·032 (2)	0·75–1·5 (2)
C (1)	–	–	EC-a (1)	0·016 (1)	0·032 (1)
Lineage 2 (34)					
B (1)	*ermB* (1)	SP (1)	EC-b (1)	0·064 (1)	3 (1)
H (1)	–	–	EC-a (1)	0·016 (1)	0·032 (1)
I (2)	–	–	EC-a (2)	0·047–0·064 (2)	0·032–1·5 (2)
L (2)	*tetM* (1)	–	EC-a (2)	0·032–4 (1)	0·75–1·5 (2)
O (7)	–	–	EC-a (7)	0·016–0·94 (7)	1·0–4 (7)
P (4)	–	–	EC-a (4)	0·032–0·064 (4)	0·75–1 (4)
R (15)	*ermB* (3)/*tetM* (10)/*catD* (1)	SP (3)	EC-b (3)/EC-d (2)†/EC-e (3)	8–16 (6)/0·023–4 (9)‡	0·75 (1)
U (2)	–	–	EC-a (2)	0·064–0·125 (2)	0·032–3 (2)

*ERY, erythromycin; CLI, clindamycin; TET, tetracycline; CHL, chloramphenicol.

†These isolates showed inducible resistance to clindamycin.

‡Two of the four *tetM*-positive strains showed inducible resistance to tetracycline.

Table 4. Phenotypic classes identified in 35 *C. difficile* isolates analysed for erythromycin and clindamycin susceptibility

Phenotypic class	Detection of *ermB*	*ermB* sequence type	MIC (mg l^{-1})	
			Erythromycin	Clindamycin
EC-a	–	–	0·032–2	0·047–3
EC-b	+	SZ/SP	⩾256	⩾256
EC-c	+	SZ	12–48	⩾256
EC-d	–	–	⩾256	1·5–3
EC-e	–	–	⩾256	4–12

and 256 mg l^{-1} and for clindamycin between 0·047 and 256 mg l^{-1}. Nine strains were resistant to erythromycin and seven to clindamycin. Four strains had an *ermB* gene and showed a class EC-b phenotype and five were *ermB*-negative. Three of the latter showed a phenotype with a high level of resistance to erythromycin (MIC ⩾ 256 mg l^{-1}) and low to clindamycin (MIC 4–12 mg ml^{-1}), designated class EC-e, and two strains showed a class EC-d phenotype.

All *C. difficile* isolates were erythromycin-resistant but *ermB*-negative isolates were also PCR-negative for the *ermA*, *ermC*, *ermF*, *ermQ* and *mefA* genes.

C. difficile isolates of lineage 1 with a *tetM* gene were resistant to tetracycline with MICs ranging from 12 to 256 mg l^{-1} (MIC_{50} = 32 mg l^{-1}, MIC_{90} = 64 mg l^{-1}). Five of the 11 *C. difficile* isolates of lineage 2 that were *tetM*-positive were susceptible to this antibiotic, with MICs between 0·023 and 4 mg l^{-1} (MIC_{50} = 3 mg l^{-1}, MIC_{90} = 12 mg l^{-1}); one of these strains was also *ermB*-positive but erythromycin-susceptible. All isolates of lineage 1 that were *catD*-positive were resistant to chloramphenicol, whereas the only *catD*-positive isolate of lineage 2 was susceptible to chloramphenicol *in vitro*.

Induction of antibiotic resistance

All *C. difficile* strains in phenotypic classes EC-d and EC-e were analysed for the induction of resistance to clindamycin.

Two isolates of the EC-d class, belonging to PCR ribotype R, showed induced resistance *in vitro* with MICs of 4 and 12 mg l^{-1}, respectively.

All *tetM*-positive strains that were susceptible *in vitro* were analysed for induction of tetracycline resistance. Two strains, belonging to PCR ribotype R, showed induced resistance with MICs of 8 and 12 mg l^{-1}, respectively.

No *C. difficile* isolate was found that showed induced resistance towards both clindamycin and tetracycline.

The only *C. difficile* isolate with a *catD* gene that was susceptible to chloramphenicol *in vitro* (PCR ribotype R) did not show induced resistance.

ermB characterization

All *ermB* genes were analysed to define the nucleotide sequence type (Table 3 and Fig. 3). Twenty-one of the 25 isolates of lineage 1 (84 %) with an *ermB* gene showed an SZ-type sequence, similar to *C. difficile* 630, and the remaining four strains had an SP-type sequence, similar to *C. perfringens* CP592. All *ermB* genes detected in isolates of lineage 2 had an SP-type sequence. *C. difficile* isolates showing a class EC-b phenotype had an *ermB* SZ-type sequence, whereas the majority of isolates (67 %) showing a class EC-a phenotype had an *ermB* SP-type sequence.

DISCUSSION

In this study we performed a comparative analysis of *C. difficile* clinical isolates belonging to two distinct genetic lineages, focusing our investigations on analysis of PaLoc genes, detection of binary toxin and antibiotic-resistance patterns.

The results showed that the majority of strains of recent isolation grouped in lineage 2 and had unusual characteristics that differed from those of older isolates belonging to lineage 1.

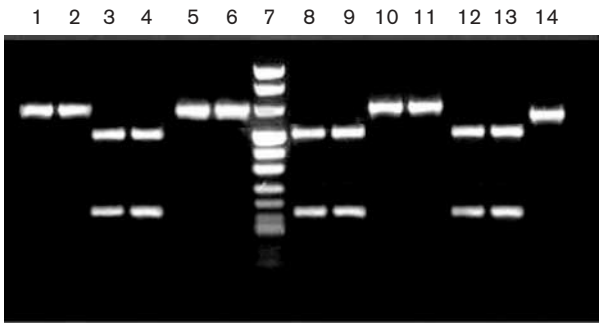

Fig. 3. PCR-RFLP of the *ermB* gene using *Pvu*II. Genes with the SP-type sequence remained undigested, whereas genes with the SZ-type sequence were digested into two fragments of approximately 450 and 200 bp. Lanes: 1, 2, 5, 6, 10, 11 and 14, *ermB* with SP-type sequence; 3, 4, 8, 9, 12 and 13, *ermB* with SZ-type sequence; 7, DNA Molecular Weight Marker VIII (Roche).

Various studies of *C. difficile* isolates with variant toxins have been published (Barbut *et al.*, 2002; Rupnik *et al.*, 2001, 2003), but there is no report of a comparison between *C. difficile* strains of older and more recent isolation. The results of this study seem to indicate an increase in *C. difficile* strains with variant PaLoc genes over time. The majority of these strains were identified as PCR ribotype R and represented 56 % of all isolates of lineage 2, whereas in lineage 1, they represented only 5 % and belonged to PCR ribotype E. Most of the recent strains with variant PaLoc genes (81 %) showed variations in toxin genes, as suggested by the presence of the binary toxin. Only six strains, four of lineage 2 and two of lineage 1, had major deletions in *tcdA*. Together with the acquisition of the binary toxin, mutations in the negative toxin regulator of toxins A and B (*tcdC*) differentiated the recent strains from the older ones. Seventy-one per cent of isolates with a variant PaLoc showed mutations in the *tcdC* gene; in particular, the allele *tcdC-A* was prevalent. *tcdC-A* encodes a truncated protein of only 61 amino acids that probably has an altered function, determining an increase in toxin production. It was interesting to note that 62 % of the isolates with a variant PaLoc showed both a mutated negative regulator of toxins A and B and the binary toxin.

Different characteristics were also found in the *C. difficile* isolates of the two lineages when resistance to MLSB, tetracycline and chloramphenicol was investigated. All multi-resistant strains belonged to lineage 1, in particular to PCR ribotype A, and represented 47 % of *C. difficile* isolates of that lineage. No multi-resistant strain was found in lineage 2. The only strain of PCR ribotype R that was positive for all the antibiotic-resistance determinants examined was resistant to erythromycin only *in vitro*. Barbut *et al.* (1999) described a similar trend in recently isolated strains, which were more susceptible to this panel of antibiotics than those isolated in the past, due to a major change in the serogroup distribution.

Five different classes were identified on the basis of erythromycin and clindamycin resistance patterns. Interestingly, all *C. difficile* isolates showing an EC-d and EC-e phenotype were *ermB*-negative, whereas the EC-b and EC-c phenotypes were always associated with the presence of an *ermB* gene. In particular, strains of the EC-c class were always associated with an allele showing an SZ-type sequence, whereas 67 % of those of the EC-b class were associated with an allele showing an SP-type sequence. As we observed in a previous study (Spigaglia & Matrantonio, 2003), these phenotypes were also associated with a different number of *ermB* copies: EC-c strains were associated with one copy of a gene with an SZ-type sequence, whereas EC-b strains had one or two copies of a gene with an SP-type sequence or two copies of a gene with an SZ-type sequence. In lineage 1, the predominant phenotypic class was EC-b, which contained 73 % of the strains resistant to erythromycin. In contrast, this phenotype was not found in lineage 2, where the predominant classes were

EC-d and EC-e containing 56 % of the strains resistant to erythromycin. Recent studies have described *ermB*-negative *C. difficile* strains with high levels of resistance to clindamycin and/or erythromycin (Ackermann *et al.*, 2003; Pituch *et al.*, 2003). In our study, only *C. difficile* isolates with susceptibility or low levels of resistance to clindamycin were *ermB*-negative. These strains were also PCR-negative for the *ermA*, *ermC*, *ermF*, *ermQ* and *mefA* genes. Induction of resistance to clindamycin indicated that two strains of class EC-d phenotype were inducibly resistant. All these data suggest that *erm* genes different from those tested, or different mechanisms, may be involved in MLSB resistance in strains with an EC-d or EC-e phenotype.

As far as tetracycline resistance was concerned, we observed a decrease in MIC_{50} and MIC_{90} values in strains belonging to lineage 2. Furthermore, an inducible resistance to tetracycline was observed in two of the four isolates susceptible *in vitro* to this antibiotic, but *tetM*-positive.

The detection of inducible resistance to antibiotics in recent *C. difficile* isolates could be clinically relevant and further analysis should be performed to characterize these strains further and monitor their circulation.

A decrease in the presence of the *catD* gene and in chloramphenicol MIC values was observed on comparison of isolates from lineage 1 and 2.

In conclusion, strains belonging to lineage 2, in contrast to strains belonging to lineage 1, showed the presence of binary toxin, variations in toxins A and B and in their negative regulator *tcdC*, and the absence of multi-drug resistance.

Taken together, these results provide evidence of a recent circulation of *C. difficile* strains with characteristics that could increase their pathogenic potential. It may be hypothesized that additional virulence factors, such as the binary toxin and a possible increase in toxins A and B production due to a mutated negative regulator, could enhance *C. difficile* virulence (Soehn *et al.*, 1998; Spigaglia & Mastrantonio, 2002). Moreover, a decrease in the biological fitness cost necessary to maintain constitutive expression and high levels of antibiotic resistance (Andersson & Levin, 1999; Bjorkman & Andersson, 2000; Johanesen *et al.*, 2001) could be relevant for bacterial survival and for its competitive performance. Further studies will be necessary to confirm these hypotheses, but the possible clinical implications following the spread of *C. difficile* strains with these characteristics demand the monitoring of the molecular features in these clinical isolates.

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