Prevalence and association of PCR ribotypes of *Clostridium difficile* isolated from symptomatic patients from Warsaw with macrolide-lincosamide-streptogramin B (MLS_B) type resistance

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Isolates (79 in total) of *Clostridium difficile* obtained over a 2 year period from 785 patients suspected of having *C. difficile*-associated diarrhoea (CDAD) and being hospitalized in the University Hospital in Warsaw were characterized by toxigenicity profile and PCR ribotyping. Furthermore, their susceptibility to clindamycin and erythromycin was determined. Among the 79 *C. difficile* isolates, 35 were classified as A⁺B⁺, 1 as A⁺B⁺CDT⁺, 36 as A[−]B⁺ and 7 as A[−]B[−]. A total of 21 different PCR ribotypes was detected. Two main A⁺B⁺ strains circulated in our hospital: ribotype 014 and ribotype 046. Unexpectedly, the predominant PCR ribotype was type 017, a known A[−]B⁺ strain, and this accounted for about 45.5% of all isolates cultured from patients with CDAD. Isolates belonging to PCR ribotype 017 were found in cases from epidemics of antibiotic-associated diarrhoea in the internal and surgery units. High-level resistance (MIC ≥ 256 mg l^{−1}) to clindamycin and erythromycin was found in 39 (49%) of the *C. difficile* isolates. Interestingly, 34 (94%) of macrolide-lincosamide-streptogramin B (MLS_B) type resistance strains did not produce toxin A, but produced toxin B and were A[−]B⁺ ribotype 017. Thirty-seven of the high-level resistance strains harboured the erythromycin-resistance methylase gene (*ermB*). *C. difficile* isolates (2/29) that had high-level clindamycin and erythromycin resistance, and belonged to PCR ribotype 046, were *ermB* negative. These investigations revealed that the predominant *C. difficile* strain isolated from symptomatic patients hospitalized in University Hospital in Warsaw was MLS_B-positive clindamycin/erythromycin-resistant PCR ribotype 017.

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INTRODUCTION

Clostridium difficile is the main aetiological agent of antibiotic-associated diarrhoea, antibiotic-associated colitis and pseudomembranous colitis. The pathogenicity of this bacterium is determined by the production of two major toxins: enterotoxin A (TcdA) (A) and cytotoxin (TcdB) (B) (Borriello, 1998). However, *C. difficile* strains producing only toxin B (A[−]B⁺) can be isolated from cases of *C. difficile*-associated diarrhoea (CDAD) throughout the world (Brazier *et al.*, 1999; Pituch *et al.*, 2001, 2003; Kuijper *et al.*, 2001; Alfa *et al.*, 2000; Barbut *et al.*, 2002; Johnson *et al.*, 2003).

The application of different typing methods has revealed that *C. difficile* is a heterogeneous species. Workers at the

Anaerobe Reference Laboratory (ARL) in Cardiff developed a modification of the PCR-ribotyping method, based on the polymorphism in the 16S–23S rDNA intergenic region spacer, for the routine typing of *C. difficile* (O'Neill *et al.*, 1996). Every bacterial strain contains several rRNA operons, and there is a strain-dependent variation in the size and number of the 16S–23S intergenic spacer regions. Macrolide-lincosamide-streptogramin B (MLS_B)-resistant *C. difficile* strains have been demonstrated to be the cause of epidemics of CDAD in different countries (Johnson *et al.*, 1999; Kuijper *et al.*, 2001; Pituch *et al.*, 2003). High-level resistance to clindamycin and erythromycin in *C. difficile* is encoded by the *ermB* gene (the erythromycin ribosomal methylase gene B), and this determinant is located on a conjugative transposon called Tn5398 (Wust & Hardegger, 1993; Mullany *et al.*, 1995; Farrow *et al.*, 2001). The *ermB* MLS_B-resistance determinant from *C. difficile* 630 contains two copies of an *erm* (B) gene (Farrow *et al.*, 2000). Spigaglia

Abbreviations: CDAD, *Clostridium difficile*-associated diarrhoea; CPE, cytopathic effect; MLS_B, macrolide-lincosamide-streptogramin B.

& Mastrantonio (2004) described five phenotypic classes (EC-a to EC-e), with characteristic susceptibility/resistance patterns to erythromycin and clindamycin, among the *C. difficile* strains.

The aim of this study was to determine the distribution of PCR ribotypes of *C. difficile* circulating in the University Hospital in Warsaw, Poland and to establish the relationship with MLS_B-type resistance.

METHODS

C. difficile isolates. A total of 785 liquid stool samples was collected from patients for whom clinicians specifically requested investigations for *C. difficile* toxin, between January 2002 and December 2003. The samples originated from patients hospitalized in different wards of University Hospital in Warsaw. From these faecal samples, 79 *C. difficile* isolates were obtained from different wards: transplant ($n=36$), surgery ($n=16$), internal medicine ($n=16$), orthopaedics ($n=6$), intensive care ($n=2$), dermatology ($n=1$), gynaecology ($n=1$), urology ($n=1$). Three reference strains were included in this study as controls, namely, toxigenic *C. difficile* VPI 10463 (A⁺B⁺), a non-toxigenic *C. difficile* NIHBRIGS 8050 (A⁻B⁻) and A⁻B⁺ *C. difficile* GAI 95 601, isolated by H. Kato (Institute of Anaerobic Bacteriology, Gifu University School of Medicine, Gifu, Japan), were used as an internal control for detecting repeating sequences in the *tcdA* gene.

Culture and identification of *C. difficile* isolates. Isolation of *C. difficile* was performed on selective Columbia Agar supplemented with cycloserine-cefoxitin and amphotericin B (CCCA medium; bioMérieux), as described previously (Pituch *et al.*, 2001). Plates were incubated in an anaerobic chamber (Forma Scientific) at 37 °C for 4 days. The isolates were identified as *C. difficile* by the characteristic morphology of the colonies and horse odour, green-yellow fluorescence under UV light (365 nm), Gram staining and the API 20A biochemical test (bioMérieux).

Toxin detection. A single colony was transferred into brain heart infusion broth (BHI) (Difco) and grown for 48 h. Supernatants were collected by centrifugation at 3000 g for 15 min. TcdA was detected by an immunochromatography assay using anti-toxin A antibody labelled with latex: the *C. difficile* toxin A test (Oxoid). Additionally, the immunoenzymic assay *C. difficile* TOX A/B test (TechLab) was used for detection of either TcdA or TcdB toxins, or both. The procedures were carried out according to the manufacturer's instructions. TcdB was detected by a cytotoxicity assay on the McCoy cell line. Tenfold serial dilutions of culture filtrate were added in duplicate to McCoy cells and incubated for 24 h. *C. difficile* strain VPI 10463 was used as a positive control. The cytopathic effect (CPE) was observed by inverse microscopy. If this CPE could be neutralized by polyclonal antiserum to *C. difficile* (*C. difficile* TOX-B Test; TechLab), the test was considered positive.

PCR assays for detection of *tcdA* and *tcdB* genes. Crude template DNA was prepared using genomic DNA PREP-PLUS (A & A Biotechnology) according to the manufacturer's instructions. A 630 bp fragment of the *tcdA* gene and 399 bp fragment of *tcdB* gene were amplified using specific primer pairs YT28-YT29 and YT17-YT18, respectively, as described previously (Pituch *et al.*, 2003). The cycling conditions for both PCRs were: one predenaturation cycle at 94 °C for 45 s, and 55 °C for 30 s and 70 °C for 45 s, for 35 cycles, as described previously. Deletion in repeating regions of the *tcdA* gene was detected with the NK9-NKV011 primer pairs, as described previously (Kato *et al.*, 1999). The PCR cycling conditions were 95 °C for 20 s, 60 °C for 2 min for 40 cycles.

PCR assay for detection of binary-toxin genes (*cdtA* and *cdtB*). Primers described by Stubbs *et al.* (1999) were used for amplification of the binary-toxin genes *cdtA* and *cdtB*, as described previously (Pituch *et al.*, 2005).

PCR ribotyping. All isolates were typed by the PCR-ribotyping method described by O'Neill *et al.* (1996) and Stubbs *et al.* (1999). The oligonucleotide primers used were P3 (5'-CTG GGG TGA AGT CGT AAC AAG G-3') and P4 (5'-GCG CCC TTT GTA GCT TGA CC-3'). Banding patterns were compared with those of the library of PCR ribotypes at the ARL, Cardiff.

Determination of antibiotic susceptibility and *ermB* gene PCR. MICs of clindamycin and erythromycin were determined by E-test (AB Biodisc) according to the manufacturer's instructions. Cultures were adjusted to an OD₉₅₀ 1 (using a bioMérieux ATB 1550) on the McFarland scale and were streaked to confluence on the surface of Brucella agar plates. Plastic strips with antibiotics were applied and the plates were incubated anaerobically at 37 °C for 48 h. The MIC was measured at the intercept of the inhibition ellipses. According to NCCLS (National Committee for Clinical Laboratory Standards) recommendations, resistance was defined as follows: ≥ 8 mg clindamycin l⁻¹ and ≥ 8 mg erythromycin l⁻¹. A 688 bp fragment of *ermB* was amplified using specific primer pairs 2980 (5'-AAT AAG TAA ACA GGT AAC GTT-3') and 2981 (5'-GCT CCT TGG AAG C TG TCA GTA G-3') (Johnson *et al.*, 1999). The PCR cycling conditions included 30 cycles of 60 s at 95 °C, 120 s at 55 °C and 180 s at 72 °C.

RESULTS

Toxicogenicity of clinical isolates

During the study period, a total of 785 stool samples was investigated for *C. difficile*. All of these faecal samples were from symptomatic adults hospitalized in different units of the University Hospital in Warsaw. The overall incidence of toxin-positive *C. difficile*-positive symptomatic patients among those with suspected CDAD was 66 % (526), with individual incidence in the units as follows: transplant 64 % (156), surgery 23 % (22) and internal medicine 57 % (72). From toxin-positive patients, 79 *C. difficile* isolates were obtained: 36 from transplant patients, 16 from patients hospitalized in the internal medicine unit, 16 from patients hospitalized in the surgery unit, 6 from orthopaedic patients, 2 from patients hospitalized in the intensive care unit, 1 from a urology patient, 1 from a gynaecology patient and 1 from a patient hospitalized in the dermatology unit. These 79 *C. difficile* isolates were analysed for TcdA and/or TcdB toxins. Among these, 36 were A⁺B⁺, as demonstrated by the *C. difficile* toxin A test, the TOX A/B test and the TcdB cytotoxicity testing on McCoy cells. Thirty-six isolates were A⁻B⁺, because TcdA could not be detected using the commercial latex test for TcdA, but a CPE on the McCoy cell line was observed. The TOX A/B tests gave positive results for all 36 A⁻B⁺ strains. The remaining seven *C. difficile* isolates were A⁻B⁻ because all tests gave negative results. For the 72 isolates designated A⁺B⁺ or A⁻B⁺, PCR amplification with primer pairs YT28-YT29 and YT17-YT18 generated products of 630 bp and 399 bp for the *tcdA* and *tcdB* genes, respectively. For the seven A⁻B⁻ isolates, PCR with the above primer pairs did not generate a product. PCR

for detecting repeating sequences in toxin A with NK9-NKV011 primer pairs for 36 A^-B^+ strains generated a 700 bp product similar to that obtained for the Japanese GAI 95 601 *C. difficile* strain, and similar to that obtained for prevalent group of A^-B^+ strains (serogroup F, toxinotype VIII, ribotype 017). The presence of the *cdtA* and *cdtB* genes was tested by PCR for the same set of strains. Both *cdtA* and *cdtB* genes were identified in A^+B^+ *C. difficile* isolate no. 2145.

PCR ribotyping

By PCR ribotyping, we distinguished 21 different types among 79 *C. difficile* isolates as shown in Table 1. Of the 35 *C. difficile* A^+B^+ isolates 15 could be classified into visually distinct ribotypes. One distinct $A^+B^+CDT^+$ isolate belonged to ribotype 033. All isolates producing only toxin B (A^-B^+) belonged to ribotype 017. Seven non-toxicogenic (A^-B^-) isolates were classified into four ribotypes: three isolates belonged to ribotype 010, two to ribotype 128, one to ribotype 031 and one to ribotype 114. Interestingly, among *C. difficile* strains isolated from patients hospitalized in the internal unit, 4 A^+B^+ isolates belonged to 3 ribotypes, ribotype 023 (1), ribotype 046 (1) and ribotype 070 (2), but 12 A^-B^+ strains belonged to ribotype 017. Among strains isolated in the surgery unit the 4 A^+B^+ isolates belonged to 3 ribotypes, ribotype 046 (1), ribotype 090 (1), ribotype 094 (2), but 11 A^-B^+ strains belonged to ribotype 017. One non-toxicogenic (A^-B^-) strain belonged to ribotype 010.

Antimicrobial susceptibility

The results of the investigation of antimicrobial susceptibility of *C. difficile* strains belonging to the four toxigenicity groups A^+B^+ , $A^+B^+CDT^+$, A^-B^+ and A^-B^- , showed a MIC range from 0.023 mg l⁻¹ to 256 mg l⁻¹ for both erythromycin and clindamycin.

Three phenotypic classes of patterns of susceptibility/resistance to erythromycin/clindamycin were identified in all strains studied (Table 2). In our laboratory, the EC-a class was characterized by susceptibility to both erythromycin (MIC range 0.023–1.5 mg l⁻¹) and clindamycin (MIC range 0.023–3.0 mg l⁻¹), the EC-b class by high level resistance to both erythromycin and clindamycin (MIC $\geq$ 256 mg l⁻¹), and the new class designated by us as EC-f by decreased susceptibility to clindamycin (MIC 4 mg l⁻¹) and susceptibility to erythromycin (MIC range 0.5–0.75 mg l⁻¹). The phenotypic EC-a class was composed of 36 *C. difficile* isolates, the EC-b class of 39 isolates and the EC-f class of 4 isolates.

Among 35 toxigenic *C. difficile* isolates (A^+B^+) 3 classes of patterns of susceptibility/resistance to erythromycin and clindamycin were observed: 28 isolates were class EC-a, 4 isolates (belonging to ribotypes 012 and 046) were class EC-b, 3 isolates were class EC-f. One $A^+B^+CDT^+$ strain (ribotype 033) was class EC-a. Among 36 A^-B^+ isolates

belonging to ribotype 017, 3 classes of susceptibility/resistance to erythromycin and clindamycin were observed; 1 isolate was class EC-a, 34 were class EC-b and 1 was class EC-f. Among seven non-toxicogenic (A^-B^-) strains two classes of patterns of susceptibility/resistance to erythromycin and clindamycin were observed. Six non-toxicogenic isolates were EC-a and one was EC-b.

Of the *C. difficile* isolates of class EC-a, all were *ermB* negative. Out of the 37 strains showing the EC-b phenotype, 35 harboured the *ermB* gene, but 2 strains did not. All four *C. difficile* strains of class EC-f were *ermB* gene negative.

DISCUSSION

The present study describes a comparative analysis of *C. difficile* strains isolated from symptomatic patients hospitalized in the University Hospital in Warsaw between 2002 and 2003. Our investigations focused on toxigenicity studies, comparative analysis of ribotypes isolated in Poland with the UK ARL PCR-ribotype library, and MLS_B-type resistance. We analysed 79 *C. difficile* strains isolated over a 2 year period from a large group of patients with antibiotic-associated diarrhoea. Among the strains investigated in this study 44.3% (35/79) were A^+B^+ , 1.3% (1/79) were $A^+B^+CDT^+$, 45.5% (36/79) were A^-B^+ and 8.9% (7/79) were A^-B^- . Among 33 strains isolated in the period 1999–2001 from patients with CDAD in our hospital, 45% were A^+B^+ but 55% were A^-B^+ . The findings of the present study confirmed the high prevalence of A^-B^+ isolates in our institution, as was described previously (for 1999–2001) (Pituch *et al.*, 2003).

The data generated from the present study showed that all 79 *C. difficile* strains isolated from patients with antibiotic-associated diarrhoea were typable by the PCR-ribotyping method. Interestingly, the 35 *C. difficile*-positive patients harboured 15 highly diverse PCR ribotypes of A^+B^+ strains.

Two distinct toxigenic (A^+B^+) clones were found to circulate our hospital (PCR ribotypes 014 and 046). Rotimi *et al.* (2003) described three different clones belonging to PCR ribotypes 097, 078 and 039, among isolates from patients in hospitals in Kuwait. The most predominant ribotype in a Hungarian survey was PCR ribotype 087 (A^+B^+), which accounted for 39% of all isolates (Urban *et al.*, 2001). In Warsaw, we did not observe these ribotypes that were present in Hungary and Kuwait. Among clinical strains isolated in the University Hospital in Warsaw only one isolate was found that belonged to ribotype 001, which is dominant in the United Kingdom (Stubbs *et al.*, 1999). The most predominant PCR ribotype in the University Hospital in Warsaw (between 2002 and 2003), from a survey of 79 isolates, was ribotype 017 (A^-B^+), which accounted for 45.5% of all isolates. During the 24 month period, an outbreak of CDAD cases caused by *C. difficile* strains ribotype 017 occurred among 12 patients at the internal unit versus 4 patients with CDAD caused by A^+B^+ *C. difficile*

Table 1. Distribution of PCR ribotypes, clindamycin resistance and toxigenicity among *C. difficile* strains isolated in 2002–2003, from patients with CDAD in different wards at University Hospital in Warsaw

Polish reference no.	Year	Month	Unit	Toxin test*			<i>cdtA/cdtB</i> †	PCR ribotype	<i>ermB</i> †	C/E MIC (mg l ⁻¹) ‡
				Toxin A†	Toxin A/B†	Toxin B				
1128	2003	III	Transplant	+	+	10 ⁻³	-/-	001	-	0.75/0.5
4053	2002	XI	Surgery	-	-	-	-/-	010	-	1.0/0.75
1587	2002	V	Transplant	-	-	-	-/-	010	-	0.5/0.75
3863	2002	XI	Transplant	-	-	-	-/-	010	+	256/256
147	2003	I	Transplant	+	+	10 ⁻³	-/-	011	-	3.0/0.5
499	2003	II	Orthopaedics	+	+	10 ⁻⁶	-/-	012	+	256/256
2357	2002	VII	Transplant	+	+	10 ⁻³	-/-	012	+	256/256
953	2003	III	Transplant	+	+	10 ⁻³	-/-	012	-	2.0/0.75
806	2002	III	Intensive care	+	+	10 ⁻²	-/-	014	-	0.75/1.0
3791	2002	XI	Orthopaedics	+	+	10 ⁻⁵	-/-	014	-	0.75/0.19
3771	2002	XI	Orthopaedics	+	+	10 ⁻³	-/-	014	-	1.0/1.0
857	2002	III	Transplant	+	+	10 ⁻¹	-/-	014	-	1.0/0.75
4014	2002	XI	Transplant	+	+	10 ⁻²	-/-	014	-	0.38/0.75
4198	2002	XII	Transplant	+	+	10 ⁻²	-/-	014	-	0.75/0.5
284	2003	I	Transplant	+	+	10 ⁻²	-/-	014	-	0.125/0.19
1172	2002	IV	Internal	-	+	10 ⁻³	-/-	017	+	256/256
1694	2002	V	Internal	-	+	10 ⁻¹	-/-	017	+	256/256
2811	2002	VIII	Internal	-	+	10 ⁻²	-/-	017	+	256/256
3415	2002	X	Internal	-	+	10 ⁻²	-/-	017	+	256/256
4185	2002	XII	Internal	-	+	10 ⁻⁵	-/-	017	+	256/256
657	2003	II	Internal	-	+	10 ⁻¹	-/-	017	+	256/256
1201	2003	III	Internal	-	+	10 ⁻⁴	-/-	017	+	256/256
1388	2003	IV	Internal	-	+	10 ⁻⁴	-/-	017	-	0.75/1.5
1909	2003	V	Internal	-	+	10 ⁻³	-/-	017	+	256/256
1910	2003	V	Internal	-	+	10 ⁻³	-/-	017	+	256/256
2194	2003	VI	Internal	-	+	10 ⁻²	-/-	017	+	256/256
2260	2003	VI	Internal	-	+	10 ⁻³	-/-	017	+	256/256
4270	2002	XII	Orthopaedics	-	+	10 ⁻⁴	-/-	017	+	256/256
2113	2003	V	Orthopaedics	-	+	10 ⁻¹	-/-	017	+	256/256
1702	2002	V	Surgery	-	+	10 ⁻³	-/-	017	+	256/256
1718	2002	V	Surgery	-	+	10 ⁻²	-/-	017	+	256/256
2836	2002	IX	Surgery	-	+	10 ⁻²	-/-	017	+	256/256
4124	2002	XII	Surgery	-	+	10 ⁻⁴	-/-	017	+	256/256
4409	2002	XII	Surgery	-	+	10 ⁻³	-/-	017	+	256/256
983	2003	III	Surgery	-	+	10 ⁻⁵	-/-	017	+	256/256
1005	2003	III	Surgery	-	+	10 ⁻²	-/-	017	+	256/256
1193	2003	III	Surgery	-	+	10 ⁻²	-/-	017	+	256/256
1788	2003	V	Surgery	-	+	10 ⁻⁶	-/-	017	+	256/256
2121	2003	V	Surgery	-	+	10 ⁻³	-/-	017	+	256/256
2146	2003	VI	Surgery	-	+	10 ⁻²	-/-	017	+	256/256
422	2002	II	Transplant	-	+	10 ⁻²	-/-	017	+	256/256
1929	2002	VI	Transplant	-	+	10 ⁻²	-/-	017	+	256/256
4110	2002	XII	Transplant	-	+	10 ⁻⁴	-/-	017	+	256/256
1093	2003	III	Transplant	-	+	10 ⁻²	-/-	017	+	256/256
1479	2003	IV	Transplant	-	+	10 ⁻²	-/-	017	+	256/256
2044	2003	V	Transplant	-	+	10 ⁻⁴	-/-	017	+	256/256
2175	2003	VI	Transplant	-	+	10 ⁻³	-/-	017	-	4.0/0.75
2314	2003	VI	Transplant	-	+	10 ⁻¹	-/-	017	+	256/256
2370	2003	VI	Transplant	-	+	10 ⁻⁴	-/-	017	+	256/256
4408	2003	XII	Transplant	-	+	10 ⁻²	-/-	017	+	256/256

Table 1. cont.

Polish reference no.	Year	Month	Unit	Toxin test*			<i>cdtA/cdtB</i> †	PCR ribotype	<i>ermB</i> †	C/E MIC (mg l ⁻¹) ‡
				Toxin A†	Toxin A/B†	Toxin B				
3707	2003	X	Transplant	—	+	10 ⁻³	—/—	017	+	256/256
1428	2003	IV	Transplant	+	+	10 ⁻³	—/—	018	—	4·0/0·5
3197	2003	IX	Transplant	+	+	10 ⁻²	—/—	018	—	1·5/0·5
886	2002	III	Internal	+	+	10 ⁻⁸	—/—	023	—	0·064/0·023
3059	2002	IX	Transplant	+	+	10 ⁻²	—/—	023	—	0·38/0·19
3058	2002	IX	Transplant	+	+	10 ⁻⁵	—/—	023	—	0·19/0·125
2064	2003	V	Transplant	+	+	10 ⁻¹	—/—	025	—	4·0/0·75
517	2002	II	Dermatology	+	+	10 ⁻²	—/—	029	—	0·75/0·75
1979	2003	V	Intensive care	—	—	—	—/—	031	—	0·38/0·38
2145	2003	VI	Gynaecology	+	+	10 ⁻⁷	+/+	033	—	0·023/0·25
4430	2003	XII	Internal	+	+	10 ⁻¹	—/—	046	—	256/256
2063	2003	V	Surgery	+	+	10 ⁻²	—/—	046	—	4·0/0·5
3558	2002	X	Transplant	+	+	10 ⁻²	—/—	046	—	2·0/0·75
3573	2002	X	Transplant	+	+	10 ⁻⁴	—/—	046	—	0·064/1·0
4396	2002	XII	Transplant	+	+	10 ⁻²	—/—	046	—	256/256
1200	2003	III	Internal	+	+	10 ⁻²	—/—	070	—	1·5/0·094
4290	2003	XI	Internal	+	+	10 ⁻¹	—/—	070	—	1·5/0·75
2150	2003	VI	Orthopaedics	+	+	10 ⁻³	—/—	072	—	2·0/0·75
514	2002	II	Urology	+	+	10 ⁻⁴	—/—	077	—	1·0/0·047
1417	2003	IV	Transplant	+	+	10 ⁻²	—/—	081	—	0·5/0·38
2220	2003	VI	Transplant	+	+	10 ⁻²	—/—	081	—	0·38/0·19
1376	2002	V	Surgery	+	+	10 ⁻²	—/—	090	—	1·0/0·25
800	2002	III	Transplant	+	+	10 ⁻³	—/—	090	—	0·38/0·5
3118	2002	IX	Transplant	+	+	10 ⁻⁵	—/—	090	—	0·38/0·75
1362	2003	IV	Surgery	+	+	10 ⁻²	—/—	094	—	2·0/1·0
1557	2003	IV	Surgery	+	+	10 ⁻³	—/—	094	—	3·0/0·75
760	2003	II	Transplant	—	—	—	—/—	114	—	0·5/0·25
4001	2002	XI	Transplant	—	—	—	—/—	128	—	0·75/0·38
4242	2002	XII	Transplant	—	—	—	—/—	128	—	0·32/0·124

*The toxin tests were as follows: *C. difficile* toxin A test (Oxoid) for detection of toxin A, *C. difficile* TOX A/B test (TechLab) for detection of toxin A and toxin B, and a cytotoxic test on the McCoy cell line for detection of toxin B. Numbers for toxin B indicate the last dilution of supernatant that demonstrated a CPE.

†+, Toxin or gene present; —, toxin or gene absent.

‡C, Clindamycin; E, erythromycin.

strains (ribotypes 023, 046 and 070), and among 16 patients at the surgery unit versus 4 patients with CDAD caused by toxigenic strains (ribotypes 046, 090, 094). *C. difficile* A⁻B⁺ strains isolated between 1999 and 2001 belonged to the Polish ribotype designated ribotype A (Pituch *et al.*, 2003). One *C. difficile* binary positive strain (no. 2145) belonged to ribotype 033. In our earlier study, the same isolate belonged to Polish ribotype D (Pituch *et al.*, 2005).

In our hospital we observed MLS_B-type resistant variant toxin *C. difficile* strains that were responsible for many cases of CDAD. In our earlier study we found MLS_B resistance among all analysed *C. difficile* A⁻B⁺ strains and we concluded that *C. difficile* strains harbouring the *ermB* gene are significantly associated with CDAD (Pituch *et al.*, 2001,

2003). Van den Berg *et al.* (2004) analysed 39 *C. difficile* strains that did not produce toxin A but produced toxin B (A⁻B⁺), originating from Canada, the United States, Poland, the United Kingdom, France, Japan and The Netherlands. All Polish A⁻B⁺ isolates, designated earlier as Polish ribotype A, belonged to ribotype 017. Clindamycin resistance encoded by the *ermB* gene was found in 33 of the 39 *C. difficile* strains. They concluded that clindamycin-resistant *C. difficile* A⁻B⁺ strains of PCR ribotype 017 have a clonal worldwide spread. MLS_B-type resistance is frequently found in *C. difficile* strains that are resistant to other antibiotics (Ackermann *et al.*, 2003; Barbut *et al.*, 2002). It is interesting because Delmee & Avesani (1988) did not find resistance to clindamycin in toxin variant *C. difficile* strains (A⁻B⁺).

Table 2. Summary of phenotypic classes identified in 79 Polish *C. difficile* strains analysed for erythromycin and clindamycin susceptibility (Spigaglia & Mastrantonio, 2004).

Phenotypic class/no. of strains	Detection of <i>ermB</i> gene	Erythromycin MIC (mg l ⁻¹)	Clindamycin MIC (mg l ⁻¹)
EC-a/36	—	0.023–1.5	0.023–3.0
EC-b ₁ /37	+	≥256	≥256
EC-b ₂ /2	—	≥256	≥256
EC-f/4	—	0.5–0.75	4.0

+ or —, *ermB* gene present or absent, respectively.

Spigaglia & Mastrantonio (2004) performed a comparative analysis of *C. difficile* strains belonging to distinct genetic lineages, focusing on PaLoc (pathogenicity locus) analysis and antibiotic resistance. In this survey, five classes of patterns of susceptibility/resistance (EC-a to EC-e) to erythromycin and clindamycin were identified. Most of the recent isolates belonged to EC-d and EC-e classes, although erythromycin resistant *in vitro*, they did not harbour the *ermB* gene, and two strains of the EC-d class were resistant to clindamycin only after induction with a subinhibitory concentration of the antibiotic. In our study, the main phenotypic classes of patterns of susceptibility/resistance to erythromycin/clindamycin, for all Polish strains, were identified: EC-a, EC-b (described by Spigaglia & Mastrantonio, 2004) and a new class designated by us as EC-f. We did not observe classes EC-c, EC-d and EC-e in our collection. The analysis of the genetic background of the resistance to clindamycin and erythromycin using PCR, showed the presence of the erythromycin-resistance methylase gene (*ermB*) in 36 *C. difficile* strains belonging to the EC-b₁ phenotypic subclass. Interestingly, two MLS_B-resistant *C. difficile* strains belonging to PCR ribotype 046, from the EC-b₂ phenotypic subclass, were *ermB* negative. Resistance in those strains could possibly be due to mutations within the target site in the 23S rRNA, or a new mechanism of high resistance. Spigaglia & Mastrantonio (2004) showed that the EC-b phenotypic class in their Italian collection was always associated with the presence of an *ermB* gene, but Ackermann *et al.* (2003) have described *ermB*-negative *C. difficile* strains with high level resistance to clindamycin and/or erythromycin. Ackermann *et al.* (2003) suggest it is probable that other *erm* genes are responsible for this high-level resistance to clindamycin and erythromycin.

In streptococci, as well as in many other Gram-positive bacteria, target-site modification is a common resistance mechanism (Weisblum, 1995). Genes belonging to the *ermAM* (*ermB*) gene class, which was founded in *Streptococcus pyogenes*, are linked with MLS_B resistance. In addition, Seppala *et al.* (1998) found in *S. pyogenes* a gene other than *ermB* that mediated MLS_B resistance called *ermTR*.

In our collection of *C. difficile* strains, we identified phenotype EC-f by the decreased susceptibility to clindamycin (MIC 4 mg l⁻¹) and susceptibility to erythromycin (MIC

range 0.5–0.75 mg l⁻¹), and they were always *ermB*-gene negative. Spigaglia & Mastrantonio (2004) described that only *C. difficile* strains with susceptibility or decreased susceptibility to clindamycin were *ermB*-negative. Efflux-mediated resistance confers only a low-level resistance to antimicrobial agents. Interestingly, among *C. difficile* strains belonging to ribotype 017 we observed three type clindamycin/erythromycin resistance patterns: EC-a/*ermB* negative, EC-b₁/*ermB* positive, EC-f/*ermB* negative. MLS_B type resistance was always associated with the *ermB* gene. Clindamycin-resistant *C. difficile* strains were found to be responsible for a large outbreak of CDAD in four hospitals in the USA. High-level resistance to clindamycin (MIC ≥ 256 mg l⁻¹) was present in all 85 epidemic-strain isolates, but in only 7 out of 46 non-epidemic strain isolates. The epidemic strains were also highly resistant to erythromycin (MIC ≥ 256 mg l⁻¹) (Johnson *et al.*, 1999). In summary, resistance against clindamycin and erythromycin among Polish A⁻B⁺ (ribotype 017) *C. difficile* strains was very frequent (94 %), but among A⁺B⁺ and A⁻B⁻ strains it was very rare (11 and 3 %, respectively). However, clindamycin resistance among *C. difficile* strain isolates is not new (Gerding & Johnson, 2001), but further work is needed to elucidate the association of MLS_B resistance with epidemic-spreading toxin variant *C. difficile* strains.

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