Journal of Medical Microbiology (2011), **60**, 1200–1205 **DOI** 10.1099/jmm.0.029801-0

Characterization and antimicrobial susceptibility of *Clostridium difficile* strains isolated from adult patients with diarrhoea hospitalized in two university hospitals in Poland, 2004–2006

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This study analysed 330 *Clostridium difficile* strains isolated from patients with *C. difficile* infection who were hospitalized in two university hospitals (H1 and H2) in Warsaw, Poland, over the period 2004–2006. Strains were investigated for the presence of *tcdA* (A), *tcdB* (B) and binary toxin (CDT) genes, and antimicrobial susceptibility was determined against nine agents. Among the 330 *C. difficile* isolates, 150 (45.4 %) were classified as $A^+B^+CDT^-$, 18 (5.5 %) as $A^+B^+CDT^+$, 144 (43.6 %) as $A^-B^+CDT^-$ and 18 (5.5 %) as $A^-B^-CDT^-$. The predominant PCR ribotype in hospitals H1 and H2 was type 017 and accounted for 48.3 and 40.0 %, respectively. Only one PCR ribotype 027 strain was found. The rates of resistance to erythromycin and clindamycin in hospitals H1 and H2 were 53.6 and 53.6 %, and 48.6 and 47.5 %, respectively, whereas resistance rates to the newer fluoroquinolones gatifloxacin and moxifloxacin were 38.5 and 38.5 % (H1) and 38.4 and 40.1 % (H2). Erythromycin resistance was frequently associated with resistance to clindamycin and newer fluoroquinolones in strains belonging to type 017. No metronidazole- and vancomycin-resistant isolates were found, although two *C. difficile* isolates had elevated MIC values of metronidazole (MIC range 1.0–1.5 mg l^{-1}) and 15 strains revealed elevated MIC values for vancomycin (MIC range 1.5–2.0 mg l^{-1}). In conclusion, an increase in non-027 CDT-producing *C. difficile* strains was observed in Poland, but *C. difficile* PCR ribotype 017 remains a major circulating type.

Received 4 January 2011
Accepted 14 March 2011

INTRODUCTION

Clostridium difficile is a major cause of antibiotic-associated diarrhoea in hospitalized patients (Freeman *et al.*, 2010). Toxigenic isolates usually produce two toxins: toxin A (A) and toxin B (B). A^-B^+ strains do not produce detectable amounts of toxin A due to a deletion in the repeating sequence of the *tcdA* gene encoding the toxin (Kato *et al.*, 1998). Some *C. difficile* strains produce a third additional toxin, called *C. difficile* binary toxin (CDT), which can enhance the attachment of *C. difficile* to intestinal epithelial cells (Schwan *et al.*, 2009). Since 2003, outbreaks of *C. difficile* infection (CDI) due to an emerging strain of PCR ribotype 027 possessing CDT and resistant to erythromycin and/or clindamycin and newer fluoroquinolones have been reported in North America and Europe (Loo *et al.*, 2005; Kuijper *et al.*, 2008; Clements *et al.*, 2010). This strain has a point mutation in *tcdC*, a putative negative regulator of toxins A and B (Spigaglia & Mastrantonio, 2002; McDonald *et al.*, 2005).

Abbreviations: CDI, *Clostridium difficile* infection; CDT, binary toxin; CLSI, Clinical and Laboratory Standards Institute.

The most commonly used drugs for the treatment of CDI are metronidazole and vancomycin. However, some *C. diffficile* isolates have elevated MICs for metronidazole (MIC 32 mg l^{-1}) and vancomycin (MIC 16 mg l^{-1}) (Peláez *et al.*, 2002), although the clinical significance is not clear yet.

The aim of this study was to characterize 330 *C. difficile* strains isolated from adult patients with diarrhoea hospitalized in two university hospitals over the period 2004–2006. Additionally, MICs of nine different antimicrobial agents were determined.

METHODS

Hospitals. Two university-associated hospitals with regional and national reference functions for specialized care participated in this surveillance study. The hospitals are located in Warsaw, Poland: the Infant Jesus Teaching Hospital, assigned as H1 (n=675 beds; 12 clinics), and the Public Hospital of the Medical University of Warsaw, assigned as H2 (n=1177 beds; 16 clinics). Only faeces samples were included from patients with diarrhoea from whom the physician requested a diagnostic test for CDI. Diagnosis of CDI was based on a positive stool ELISA result using the *C. difficile* TOX A/B II kit (TechLab) for detection of toxin A and/or toxin B and on the isolation of toxigenic *C. difficile* strains over the period 2004–2006.

Micro-organisms. A total of 330 clinical *C. difficile* strains isolated from patients hospitalized between 2004 and 2006 in hospitals H1 (n=153) and H2 (n=177) were available for detailed characterization. In hospital H1, isolates were obtained from the following wards: general surgery (n=39), internal medicine (n=35), transplantation (n=33), orthopaedics (n=12), intensive care (n=10), urology (n=9), dermatology (n=6), gynaecology (n=1) and several other wards (n=8). In hospital H2, isolates were obtained from the following wards: haematology (n=55), gastrointestinal surgery (n=30), neurology (n=16), nephrology (n=14), vascular surgery (n=10), general surgery (n=7), neurosurgery (n=7), internal medicine (n=5), haematological intensive care (n=5), cardiac surgery (n=4), endocrinology (n=4), pulmonology (n=4), dialysis (n=2), thoracic surgery (n=2), neurological intensive care (n=1), cardiology (n=1) and several other wards (n=10). All isolates were stored at −70 °C and were sent to the Department of Medical Microbiology (hospital H1) for further characterization and determination of susceptibility to nine antimicrobial agents. Isolation of *C. difficile* was performed on selective Columbia agar supplemented with cycloserine/cefoxitin and amphotericin B (CLO medium; bioMérieux). The plates were incubated in an anaerobic chamber for 48 h at 37 °C. Isolates were identified as *C. difficile* by the characteristic morphology of the colonies and horse-like odour, green–yellow fluorescence under UV light (365 nm), Gram staining and an API 20A biochemical test (bioMérieux). Three reference strains were included in this study as controls: toxigenic *C. difficile* VPI 10463 (A^+B^+), non-toxigenic *C. difficile* NIHBRRIGS 8050 (A^-B^-) and *C. difficile* GAI 95 601 (A^-B^+) (from H. Kato, Institute of Anaerobic Bacteriology, Gifu University School of Medicine, Gifu, Japan). CDT-producing control strains were provided by Jon Brazier (Anaerobe Reference Laboratory, Cardiff, UK) and Ed Kuijper (Leiden University Medical Center, Leiden, The Netherlands) and consisted of R8637 (PCR ribotype 019), R5989 (PCR ribotype 023), R10456 (PCR ribotype 056) and strains from PCR ribotypes 045, 078 and 027.

Determination of toxin genes. PCRs to detect the *tcdA* and *tcdB* genes and deletions in the *tcdA* gene were conducted as described previously (Pituch *et al.*, 2006). Primers described by Stubbs *et al.* (2000) were used for amplification of the CDT genes *cdtA* and *cdtB*, as described previously (Pituch *et al.*, 2006). Amplification and sequencing of the *tcdC* gene was also performed to investigate the presence of deletions in this gene.

PCR ribotyping. *C. difficile* CDT gene-positive (n=18) and $A^-B^+CDT^-$ isolates (n=144) were typed by PCR ribotyping as described by Stubbs *et al.* (1999). Banding patterns were compared with those of the library of PCR ribotypes at the Anaerobe Reference Laboratory, Cardiff, UK.

Antimicrobial drug susceptibility testing. MICs of a panel of seven antimicrobial drugs were determined against the 330 *C. difficile* isolates using Etest strips (AB Biodisk) with exponential gradients of antimicrobial concentrations of 0.016–256.0 mg l^{-1}: erythromycin, clindamycin, metronidazole, vancomycin, ciprofloxacin, gatifloxacin and moxifloxacin. In addition, 100 randomly selected *C. difficile* isolates from hospital H1 and 164 from hospital H2 were tested against imipenem (0.002–32.0 mg l^{-1}), and 100 isolates from H1 and 133 from H2 were tested against tetracycline (0.002–32.0 mg l^{-1}). Cultures were adjusted to an OD_{950} of 1 (using a bioMérieux ATB1550 densitometer) on the McFarland scale, and streaked and grown to confluency on the surface of *Brucella* agar plates. Plastic strips with the antibiotics were applied and the plates were incubated anaerobically at 37 °C for 48 h. According to the Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI, 2007), antibiotic resistance was defined as follows: MIC $\geqslant$8.0 mg l^{-1} for clindamycin and erythromycin, MIC $\geqslant$32.0 mg l^{-1} for metronidazole, MIC $\geqslant$32 mg l^{-1} for vancomycin, MIC $\geqslant$4 mg l^{-1} for ciprofloxacin, gatifloxacin and moxifloxacin, and MIC $\geqslant$16 mg l^{-1} for imipenem and tetracycline. Quality-control strains (*Bacteroides fragilis* NCTC 11295, *Bacteroides thetaiotaomicron* ATCC 29741, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923) were always included. A 688 bp fragment of the *ermB* gene for erythromycin resistance was amplified using specific primer pairs 2980 (5′-AATAAGTAAACAGGTAACGTT-3′) and 2981 (5′-GCTCCTTGGAAGCTGTCAGTAG-3′) (Johnson *et al.*, 1999). PCR cycling conditions comprised 30 cycles of 60 s at 95 °C, 120 s at 55 °C and 180 s at 72 °C.

RESULTS

Characterization of *C. difficile* strains

Between 2004 and 2006, we characterized 330 *C. difficile* strains collected in two hospitals in Warsaw. Overall, 150 isolates (45.4 %) were classified as $A^+B^+CDT^-$, 144 (43.6 %) were $A^-B^+CDT^-$ (Table 1) and 18 (5.5 %) were $A^+B^+CDT^+$. Of 312 *C. difficile* isolates designated $A^+B^+CDT^-$, $A^-B^+CDT^-$ or $A^+B^+CDT^+$, PCR amplification with primer pairs YT28/YT29 and YT17/YT18 (Pituch *et al.*, 2006) generated products of 630 and 399 bp for the *tcdA* and *tcdB* genes, respectively. PCR to detect deletion of repeat sequences in the *tcdA* gene with the NK9/NKV011 (Pituch *et al.*, 2006) primer pair for the 144 $A^-B^+CDT^-$ strains generated a 700 bp product similar to that obtained for the Japanese GAI 95 601 *C. difficile* strain and for the prevalent group of A^-B^+ strains of PCR ribotype 017. The remaining 18 *C. difficile* isolates (5.5 %) were $A^-B^-CDT^-$. The distribution of *C. difficile* toxigenicity profiles was comparable for hospitals H1 and H2 (Table 1).

Table 1. Summary of toxigenicity profiles of 330 *C. difficile* strains isolated from patients with diarrhoea in two university hospitals (H1 and H2) in Warsaw between 2004 and 2006

Toxigenicity profile	H1 (n=153)	H2 (n=177)
$A^+B^+CDT^-$	62 (40.5 %)	88 (49.7 %)
$A^+B^+CDT^+$	8 (5.3 %)	10 (5.6 %)
$A^-B^+CDT^-$	74 (48.3 %)	70 (39.6 %)
$A^-B^-CDT^-$	9 (5.9 %)	9 (5.1 %)

PCR ribotyping of the 18 CDT^+ strains found that only one strain showed the same pattern as the control 027 strains. The remaining CDT^+ strains showed patterns similar to ribotype 023 (n=16) and ribotype 045 (n=1). All 144 isolates producing toxin B only ($A^-B^+CDT^-$) recovered from hospitals H1 and H2 belonged to ribotype 017.

Antimicrobial susceptibility

Overall, the *C. difficile* isolates showed some resistance to seven of the nine antimicrobial agents tested (Tables 2 and 3). All 330 *C. difficile* strains were susceptible to metronidazole (MIC range 0.016–1.5 mg l^{-1}) and vancomycin (MIC range 0.023–2.0 mg l^{-1}), according to CLSI breakpoints. Two strains had elevated MIC values for metronidazole (1.0 and 1.5 mg l^{-1}) and 15 strains had elevated MIC values for vancomycin (1.5–2.0 mg l^{-1}). Of the two isolates with elevated MICs for metronidazole, one isolate originated from the dermatology ward (H1) and one from the haematology ward (H2). Six of the *C. difficile* isolates with elevated MICs for vancomycin originated from patients hospitalized in H1 in surgery (n=3), dermatology (n=2) and cardiology intensive care (n=1), whilst the remaining nine isolates were obtained from patients hospitalized in H2 in nephrology (n=2), vascular surgery (n=2), internal medicine (n=1), haematology (n=1), gastrointestinal surgery (n=1), neurosurgery (n=1) and general surgery (n=1). In total, 163 (49.4 %) of the 330 *C. difficile* isolates were cross-resistant to erythromycin and clindamycin. Of these, 52.3 % (n=80) were from H1 and 46.9 % (n=83) were from H2. Among the $A^-B^+CDT^-$ *C. difficile* strains isolated in H1 and H2, 68 (91.9 %) and 63 strains (90.0 %), respectively, were cross-resistant to erythromycin and clindamycin and harboured the *ermB* gene. Among the 330 *C. difficile* strains, 324 (98.2 %) revealed resistance to ciprofloxacin. Resistance to both gatifloxacin and moxifloxacin was found in 134 (40.6 %) and 134 (40.6 %) isolates from H1 and H2. Among strains isolated in H1 and H2, 72.0 and 74.4 % were resistant to imipenem, respectively, and ~23 % (76 isolates) were resistant to tetracycline in both hospitals. Resistance to imipenem (MICs $\geqslant$16 mg l^{-1}) was observed among all the CDT^+ *C. difficile* strains belonging to ribotypes 023, 027 and 045. Of the $A^-B^+CDT^-$ *C. difficile* strains (PCR ribotype 017), 86.8 % were resistant to imipenem. Only one *cdtA*/*cdtB*-positive isolate belonging to PCR ribotype 027 (in H2) was found; this strain was resistant to erythromycin

Table 2. Distribution of MICs of antimicrobials against *C. difficile* strains isolated from patients in hospitals H1 and H2 in 2004–2006

Antimicrobial agent*	MIC (mg l^{-1})																					
	0.016	0.023	0.032	0.047	0.064	0.094	0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	3	4	6	8	12	24	32	48	256
***C. difficile* strains from H1 and H2 (n=330)**																								
EM				1	2		2	4	11	11	37	30	39	18	6				1		1			167
CM					2	2	2	2	4	3	6	7	25	52	44		11	2	3					165
CI						1					1		1		2	1						324		
MX		1	1		1	1	1			3	16	33	69	53	16	1	4	3	1			126		
GA		1				1		2		1	17	19	76	65	11	3	2		2			130		
MZ	47	21	40	34	45	46	42	28	17	4	2	2	1	1										
VA		2		1		2	3	9	10	38	107	82	61	14	1									
***C. difficile* strains from H1 (n=100; IP and TC) and from H2 (n=164, IP; n=133, TC)**																								
IP				2						7				10	11	12	9	15	4	9		185		
TC	6	3	9	12	36	34	26	2			7				1	2	4	5	10	42	22	12		

*EM, Erythromycin; CM, clindamycin; CI, ciprofloxacin; MX, moxifloxacin; GA, gatifloxacin; MZ, metronidazole; VA, vancomycin; IP, imipenem; TC, tetracycline.

Table 3. Summary of MICs (mg l^{-1}) of antimicrobial agents against 330 *C. difficile* strains isolated from adult patients with diarrhoea in two university hospitals (H1 and H2) between 2004 and 2006

Antimicrobial agent*	MIC range		MIC_{50}		MIC_{90}		Resistant strains (%)	
	H1 (n=153)	H2 (n=177)	H1	H2	H1	H2	H1	H2
EM	0.125–256.0	0.125–256.0	256.0	2.0	256.0	256.0	53.6	48.6
CM	0.047–256.0	0.047–256.0	256.0	4.0	256.0	256.0	53.6	47.5
CI	0.5–32.0	0.5–32.0	32.0	32.0	32.0	32.0	98.0	98.3
MX	0.094–32.0	0.094–32.0	1.5	1.5	32.0	32.0	38.5	38.4
GA	0.094–32.0	0.094–32.0	1.5	1.5	32.0	32.0	38.5	40.1
MZ	0.016–1.5	0.016–1.5	0.064	0.064	0.19	0.19	0	0
VA	0.023–1.0	0.023–2.0	0.5	0.75	1.0	1.0	0	0
IP	0.38–32.0†	0.047–32.0‡	32.0	32.0	32.0	32.0	72	74.4
TC	0.016–64.0†	0.016–64.0‡	6.0	0.125	32.0	24.0	23	22.6

*EM, Erythromycin; CM, clindamycin; CI, ciprofloxacin; MX, moxifloxacin; GA, gatifloxacin; MZ, metronidazole; VA, vancomycin; IP, imipenem; TC, tetracycline.

†The total number of isolates analysed for IP and TC resistance was 100.

‡The total number of isolates analysed for IP resistance was 164 and for TC resistance was 133.

and clindamycin and also to moxifloxacin and gatifloxacin. Multidrug resistance to erythromycin, clindamycin, ciprofloxacin, gatifloxacin and moxifloxacin was detected in 34.6 and 33.8 % of *C. difficile* strains in H1 and H2, respectively.

DISCUSSION

In a previous study conducted between 2002 and 2003 in Poland, we analysed 79 *C. difficile* strains isolated over a 2-year period from 785 adult patients with antibiotic-associated diarrhoea hospitalized in one university hospital in Warsaw (Pituch *et al.*, 2006). Among the strains investigated in that study, 44.3 % were $A^+B^+CDT^-$, 45.5 % were $A^-B^+CDT^-$, 1.3 % were $A^+B^+CDT^+$ and 8.9 % were $A^-B^-CDT^-$ (Pituch *et al.*, 2006). During the 2-year study period, one outbreak of CDI cases caused by a *C. difficile* PCR ribotype 017 strain occurred among 12 patients at the internal unit. The findings of the present study confirm the high prevalence of $A^-B^+CDT^-$ isolates in some hospitals in Warsaw and emphasize the significance of A^- strains (Pituch *et al.*, 2007). Outbreaks due to PCR ribotype 017 were also observed in the present survey at an internal unit (between January 2005 and May 2005 involving seven patients) and at a surgery unit (between February 2006 and March 2006 involving seven patients). Most A^-B^+ *C. difficile* isolates belong to ribotype 017, which is found more frequently in Asia than in some other continents (Shin *et al.*, 2008; Huang *et al.*, 2009). However, outbreaks of PCR ribotype 017 have also been reported from other continents and PCR ribotype 017 has a tendency to persist for a long time in hospitals (Arvand *et al.*, 2009; Goorhuis *et al.*, 2009).

In the current study, an increase of $A^+B^+CDT^+$ isolates was observed from 1.3 % in 2003 to 5.5 % in 2006. The PCR ribotypes involved were 023 (n=16) and 045 (n=1). *C. difficile* PCR ribotype 023 is not frequently found and was present in only 3 % of all isolates characterized in a recent pan-European survey (Bauer *et al.*, 2011). A similar trend of an increase in CDT^+ *C. difficile* strains has been found in Hungary (Terhes *et al.*, 2009) where an increase in $A^+B^+CDT^+$ isolates from 2.5 % in 2002–2003 to 6.7 % in 2006–2007 was observed. Barbut *et al.* (2007) found a higher prevalence of CDT^+ *C. difficile* strains in a European surveillance in 2005. The prevalence of $A^-B^+CDT^-$ *C. difficile* strains in Europe was 6.2 % in the same study.

Epidemiological surveys suggest a spread of *C. difficile* PCR ribotype 027 across North America and Europe (Kuijper *et al.*, 2008). However, a recently completed pan-European surveillance study revealed that the prevalence of PCR ribotype 027 was only 5 % and was restricted mainly to the UK (Bauer *et al.*, 2011). In Poland, we found a 0.3 % prevalence of ribotype 027. Barbut *et al.* (2007) observed a low prevalence of PCR ribotype 027 of 6.2 % in 2005 in a European survey.

The antibiotic susceptibility of 330 *C. difficile* strains was tested against nine antibiotics. All strains were susceptible to metronidazole and vancomycin (according to CLSI breakpoints). In a recent published study performed in the UK, 24.4 % of *C. difficile* ribotype 001 isolates had reduced susceptibility to metronidazole of $\geqslant$6 mg l^{-1}, measured by a spiral-gradient end-point screening method (Baines *et al.*, 2008), although the MIC values remained below the CLSI breakpoint. Other reports from the UK also mention higher MIC values for metronidazole of 1–2 mg l^{-1} (Burns *et al.*, 2007; Brazier *et al.*, 2008). Clinical breakpoints of metronidazole have not yet been determined, although it is likely that the currently applied CLSI breakpoint of 32 mg l^{-1} is too high. Our observation of elevated MIC values for

metronidazole could have implications in the clinical setting due to the poor penetration of metronidazole into the colon, and this needs further study.

We observed a high level of cross-resistance to erythromycin and clindamycin (MIC $\geqslant$256 mg l^{-1}) in the present study. Resistance against clindamycin and erythromycin among Polish $A^-B^+CDT^-$ (PCR ribotype 017) *C. difficile* strains was very high (91 %) but not among $A^+B^+CDT^-$ and $A^-B^-CDT^-$ strains (7 and 2.1 %, respectively), which confirmed our previous observations (Pituch *et al.*, 2006, 2007). Ilchmann *et al.* (2010), in a study performed in Germany, documented a significant increase from 13.0 to 54.8 % of erythromycin- and clindamycin-resistant *C. difficile* strains belonging to PCR ribotype 001 (Ilchmann *et al.*, 2010). Rates of resistance to erythromycin, clindamycin and moxifloxacin among strains isolated in 2006–2008 in Hungary were 25, 27.5 and 25 %, respectively (Terhes *et al.*, 2009).

In our study, resistance to newer fluoroquinolones was found in 38.9 % of the isolates. Resistance to newer fluoroquinolones has been described not only in the hypervirulent strain 027 but also in other emerging PCR ribotypes circulating in hospital settings (Pituch *et al.*, 2007; Spigaglia *et al.*, 2010). In a European prospective study conducted in 2005, strains resistant to moxifloxacin represented 37.5 % of all *C. difficile* clinical isolates and the majority of the fluoroquinolone-resistant isolates belonged to PCR ribotype 126 or 018 (Barbut *et al.*, 2007). In our study, combined resistance to erythromycin, clindamycin, gatifloxacin and moxifloxacin was associated with isolates belonging to PCR ribotype 017. Thus, this multiresistant PCR ribotype 017 still dominates in our hospitals.

ACKNOWLEDGEMENTS

We thank Dr Jon S. Brazier for kindly providing control strains and for his cooperation.

REFERENCES

Arvand, M., Hauri, A. M., Zaiss, N. H., Witte, W. & Bettge-Weller, G. (2009). *Clostridium difficile* ribotypes 001, 017, and 027 are associated with lethal *C. difficile* infection in Hesse, Germany. *Euro Surveill* **14**, pii-19403.

Baines, S. D., O'Connor, R., Freeman, J., Fawley, W. N., Harmanus, C., Mastrantonio, P., Kuijper, E. J. & Wilcox, M. H. (2008). Emergence of reduced susceptibility to metronidazole in *Clostridium difficile*. *J Antimicrob Chemother* **62**, 1046–1052.

Barbut, F., Mastrantonio, P., Delmée, M., Brazier, J., Kuijper, E. & Poxton, I. on behalf of the European Study Group on Clostridium difficile (ESGCD) (2007). Prospective study of *Clostridium difficile* infections in Europe with phenotypic and genotypic characterisation of the isolates. *Clin Microbiol Infect* **13**, 1048–1057.

Bauer, M. P., Notermans, D. W., van Benthem, B. H. B., Brazier, J. S., Wilcox, M. H., Rupnik, M., Monnet, D. L., van Dissel, J. T. & Kuijper, E. J. for the ECDIS Study Group (2011). *Clostridium difficile* infection in Europe: a hospital-based survey. *Lancet* **377**, 63–73.

Brazier, J. S., Raybould, R., Patel, B., Duckworth, G., Pearson, A., Charlett, A., Duerden, B. I. & the HPA Regional Microbiology Network (2008). Distribution and antimicrobial susceptibility patterns of *Clostridium difficile* PCR ribotypes in English hospitals, 2007–08. *Euro Surveill* **13**, pii:19000.

Burns, P., Wooton, M., Hall, V., Brazier, J. S. & Howe, R. (2007). Antimicrobial susceptibility of epidemic and nonepidemic strains of *Clostridium difficile*. In *Abstracts of the 47th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy*, Chicago, IL, 17–20 September. Abstract C2-2046. Washington, DC: American Society for Microbiology.

Clements, A. C., Magalhães, R. J., Tatem, A. J., Paterson, D. L. & Riley, T. V. (2010). *Clostridium difficile* PCR ribotype 027: assessing the risks of further worldwide spread. *Lancet Infect Dis* **10**, 395–404.

CLSI (2007). *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria*, 7th edn. Approved Standard. M11-A7. Wayne, PA: Clinical and Laboratory Standards Institute.

Freeman, J., Bauer, M. P., Baines, S. D., Corver, J., Fawley, W. N., Goorhuis, B., Kuijper, E. J. & Wilcox, M. H. (2010). The changing epidemiology of *Clostridium difficile* infections. *Clin Microbiol Rev* **23**, 529–549.

Goorhuis, A., Legaria, M. C., van den Berg, R. J., Harmanus, C., Klaassen, C. H., Brazier, J. S., Lumelsky, G. & Kuijper, E. J. (2009). Application of multiple-locus variable-number tandem-repeat analysis to determine clonal spread of toxin A-negative *Clostridium difficile* in a general hospital in Buenos Aires, Argentina. *Clin Microbiol Infect* **15**, 1080–1086.

Huang, H., Fang, H., Weintraub, A. & Nord, C. E. (2009). Distinct ribotypes and rates of antimicrobial drug resistance in *Clostridium difficile* from Shanghai and Stockholm. *Clin Microbiol Infect* **15**, 1170–1173.

Ilchmann, C., Zaiss, N. H., Speicher, A., Christner, M., Ackermann, G. & Rohde, H. (2010). Comparison of resistance against erythromycin and moxifloxacin, presence of binary toxin gene and PCR ribotypes in *Clostridium difficile* isolates from 1990 and 2008. *Eur J Clin Microbiol Infect Dis* **29**, 1571–1573.

Johnson, S., Samore, M. H., Farrow, K. A., Killgore, G. E., Tenover, F. C., Lyras, D., Rood, J. I., DeGirolami, P., Baltch, A. L. & other authors (1999). Epidemics of diarrhea caused by a clindamycin-resistant strain of *Clostridium difficile* in four hospitals. *N Engl J Med* **341**, 1645–1651.

Kato, H., Kato, N., Watanabe, K., Iwai, N., Nakamura, H., Yamamoto, T., Suzuki, K., Kim, S.-M., Chong, Y. & Wasito, E. B. (1998). Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. *J Clin Microbiol* **36**, 2178–2182.

Kuijper, E. J., Barbut, F., Brazier, J. S., Kleinkauf, N., Eckmanns, T., Lambert, M. L., Drudy, D., Fitzpatrick, F., Wiuff, C. & other authors (2008). Update of *Clostridium difficile* infection due to PCR ribotype 027 in Europe, 2008. *Euro Surveill* **13**, pii:18942.

Loo, V. G., Poirier, L., Miller, M. A., Oughton, M., Libman, M. D., Michaud, S., Bourgault, A. M., Nguyen, T., Frenette, C. & other authors (2005). A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. *N Engl J Med* **353**, 2442–2449.

McDonald, L. C., Killgore, G. E., Thompson, A., Owens, R. C., Jr, Kazakova, S. V., Sambol, S. P., Johnson, S. & Gerding, D. N. (2005). An epidemic, toxin gene-variant strain of *Clostridium difficile*. *N Engl J Med* **353**, 2433–2441.

Peláez, T., Alcalá, L., Alonso, R., Rodríguez-Créixems, M., García-Lechuz, J. M. & Bouza, E. (2002). Reassessment of *Clostridium difficile* susceptibility to metronidazole and vancomycin. *Antimicrob Agents Chemother* **46**, 1647–1650.

Pituch, H., Brazier, J. S., Obuch-Woszczatyński, P., Wultańska, D., Meisel-Mikołajczyk, F. & Łuczak, M. (2006). Prevalence and association of PCR ribotypes of *Clostridium difficile* isolated from symptomatic patients from Warsaw with macrolide-lincosamide-streptogramin B (MLS_B) type resistance. *J Med Microbiol* **55**, 207–213.

Pituch, H., van Leeuwen, W., Maquelin, K., Wultańska, D., Obuch-Woszczatyński, P., Nurzyńska, G., Kato, H., Reijans, M., Meisel-Mikołajczyk, F. & other authors (2007). Toxin profiles and resistances to macrolides and newer fluoroquinolones as epidemicity determinants of clinical isolates of *Clostridium difficile* from Warsaw, Poland. *J Clin Microbiol* **45**, 1607–1610.

Schwan, C., Stecher, B., Tzivelekidis, T., van Ham, M., Rohde, M., Hardt, W. D., Wehland, J. & Aktories, K. (2009). *Clostridium difficile* toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria. *PLoS Pathog* **5**, e1000626.

Shin, B.-M., Kuak, E. Y., Yoo, H. M., Kim, E. C., Lee, K., Kang, J.-O., Whang, D. H. & Shin, J.-H. (2008). Multicentre study of the prevalence of toxigenic *Clostridium difficile* in Korea: results of a retrospective study 2000–2005. *J Med Microbiol* **57**, 697–701.

Spigaglia, P. & Mastrantonio, P. (2002). Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. *J Clin Microbiol* **40**, 3470–3475.

Spigaglia, P., Barbanti, F., Dionisi, A. M. & Mastrantonio, P. (2010). *Clostridium difficile* isolates resistant to fluoroquinolones in Italy: emergence of PCR ribotype 018. *J Clin Microbiol* **48**, 2892–2896.

Stubbs, S. L., Brazier, J. S., O'Neill, G. L. & Duerden, B. I. (1999). PCR targeted to the 16S–23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. *J Clin Microbiol* **37**, 461–463.

Stubbs, S. L., Rupnik, M., Gibert, M., Brazier, J., Duerden, B. & Popoff, M. (2000). Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. *FEMS Microbiol Lett* **186**, 307–312.

Terhes, G., Urbán, E., Sóki, J., Szikra, L., Konkoly-Thege, M., Vollain, M. & Nagy, E. (2009). Assessment of changes in the epidemiology of *Clostridium difficile* isolated from diarrheal patients in Hungary. *Anaerobe* **15**, 237–240.