Characterization of clinical *Clostridium difficile* isolates by PCR ribotyping and detection of toxin genes in Austria, 2006–2007

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In order to assess the lethality of *Clostridium difficile*-associated disease (CDAD) and the PCR ribotypes prevalent in Austria, the Austrian Agency for Health and Food Safety requested isolates of *C. difficile* from patients in a structured but arbitrary sampling scheme. In the allocated period from February 2006 to January 2007, local hospital laboratories within each of the nine provinces were asked to submit *C. difficile* isolates from at least ten cases of CDAD. Confirmation of species identification, toxin detection, susceptibility testing against four antimicrobial agents and typing using a PCR ribotyping method were performed at the reference laboratory. In total, 149 isolates of putative *C. difficile* were submitted, from which 142 were included for study. Antimicrobial susceptibility patterns revealed resistance to clindamycin in 57% and high-level resistance to moxifloxacin in 38% of isolates tested. CDAD manifested as diarrhoea (including eight cases of bloody diarrhoea) in 126 cases (88.7%), as pseudomembranous colitis in 15 cases (10.6%) and as toxic megacolon in one case. Twelve of the 142 patients died within 30 days of specimen collection (8.45% lethality). A lethal outcome occurred in 2/15 cases (13.3%) when pseudomembranous colitis was present and in 10/126 cases (7.9%) in the absence of pseudomembranous colitis or toxic megacolon. Among the 142 isolates from 25 health-care facilities, 41 PCR ribotype patterns were found. The most frequent ribotypes were AI-5 (including six lethal cases out of 26 patients), 014 (two out of 24) and 053 (one out of 24). The typing patterns demonstrated the occurrence of clusters in hospitals.

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INTRODUCTION

Clostridium difficile is a sporogenic anaerobic bacterium that can cause intestinal disease. Soil and the intestinal tracts of wild and domestic animals and humans are the main reservoirs. In 1977, infection with *C. difficile* was recognized as a cause of pseudomembranous colitis and of *C. difficile*-associated diarrhoea (Bartlett & Gorbach, 1977). It is the most frequently identified cause of hospital-acquired diarrhoea. The disease is mediated by the production of toxins. *C. difficile* has a pathogenicity locus comprising genes encoding enterotoxin A (*tcdA*) and cytotoxin B (*tcdB*). Genes for the binary toxin are located outside the pathogenicity locus, but the role of this toxin is still unclear (Kuijper *et al.*, 2006). A hypervirulent epidemic strain of *C. difficile*, PCR ribotype 027, causes a more severe disease, more frequent recurrences and is associated with a higher mortality (Kuijper *et al.*, 2006). *C. difficile* colonizes the human intestinal tract in 3% of healthy adults and up to 80% of healthy newborns and

infants (Bartlett, 2002; Viscidi *et al.*, 1981). Stool carriage reaches 16–35% among hospitalized patients; the risk of colonization is increased with duration of hospital stay and exposure to antibiotics (Aslam *et al.*, 2005). The case fatality rate of *C. difficile*-associated disease (CDAD) ranges from 6 to 30% when pseudomembranous colitis is present (Aslam *et al.*, 2005; Bartlett, 2002). *C. difficile* can also be found in food (Rupnik, 2007). *C. difficile* was isolated from 12 (20%) of 60 retail ground meat samples purchased over a 10-month period in 2005 in Canada; 11 isolates were toxigenic (Rodriguez-Palacios *et al.*, 2007). *C. difficile* is also associated with enteric disease in animals, including horses, dogs and pigs (Baverud, 2002; Rodriguez-Palacios *et al.*, 2006). Recent reports indicating that human and animal isolates are often indistinguishable and that PCR ribotype 027 has been isolated from a dog have created concerns regarding potential public health implications (Arroyo *et al.*, 2005; Lefebvre *et al.*, 2006; Rodriguez-Palacios *et al.*, 2006). Various authors have postulated that CDAD is increasing rapidly (Pituch *et al.*, 2006; Kuijper *et al.*, 2006; McDonald *et al.*, 2006; Vonberg *et al.*, 2007). To address this emerging threat, we conducted a

Abbreviation: CDAD, *Clostridium difficile*-associated disease.

prospective study to characterize the *C. difficile* strains prevalent in Austrian hospitals and to analyse the lethality associated with the PCR ribotypes found.

METHODS

Specimen collection and case selection procedure. Hospital microbiology laboratories from all nine provinces of Austria were asked to submit isolates of toxin-positive *C. difficile* from at least ten cases of CDAD to the reference laboratory of the Austrian Agency for Health and Food Safety (Vienna) for subtyping and characterization of their toxins. A case of CDAD was defined as a patient with diarrhoea or toxic megacolon and toxin detection in stool samples. Stool specimens were spread on *C. difficile* agar (a selective cycloserine/cefoxitin agar; bioMérieux) and incubated at $35 \pm 2^\circ\text{C}$ in an anaerobic atmosphere (85% nitrogen, 10% hydrogen, 5% carbon dioxide) for 48 h. Suspected *C. difficile* colonies (based on morphological criteria, Gram-stain results and odour) were confirmed by testing for the common antigen of *C. difficile* using a latex slide agglutination test (*C. difficile* Agglutination Test kit; Oxoid). From February 2006 to January 2007, a total of 149 isolates of *C. difficile* originating from 25 health-care facilities were sent by 15 laboratories, with 142 cases fulfilling the case definition. After surface transport to the reference laboratory, isolates were recultured using *C. difficile* agar and Columbia blood agar plates (bioMérieux) in an anaerobic atmosphere as above for 48 h at 37°C . Species identity was confirmed using a commercial biochemical identification system (RapID ANA; bioMérieux). The demonstration of toxin production was carried out as a 'second-look' assay with strains isolated on cycloserine/cefoxitin/fructose agar medium using a Toxin A+B test (Meridian Bioscience). Strains were stored at -80°C in cryobank tubes (Mast Diagnostics) until further testing.

Typing. PCR ribotyping was performed with primers 16S (5'-GTGCGGCTGGATCACCTCCT-3') and 23S (5'-CCCTGCACCC-TTAATAACTTGACC-3') as described by Bidet *et al.* (2000). DNA extraction from cultures was carried out using a MagnaPure Compact (Roche) according to the manufacturer's recommendations to a final volume of 100 μl . HotStar Taq master mix (25 μl ; Qiagen) was used with 1 μl each primer (10 pmol μl^{-1}), 18 μl water and 5 μl DNA. Amplification was carried out in a FlexCycler (Analytik Jena) with a 15 min initial enzyme activation at 95°C , followed by 35 cycles of 1 min at 95°C , 1 min at 57°C and 1 min at 72°C , and a 5 min final elongation step at 72°C . The amplified products were separated by electrophoresis on 1.5% agarose (Bio-Rad) for 3 h at 100 V using a 100–1000 bp ladder (Fermentas) as size standard. Control strains of *C. difficile* ribotypes 001, 014, 017 and 027, and strain VPI for toxin typing, were kindly provided by Ed J. Kuijper (University Medical Center, Leiden, the Netherlands). Ribotype 053 was verified for an Austrian isolate accepted for ribotyping by Jon Brazier (University Hospital of Wales, Cardiff, UK). Isolates with PCR ribotype patterns different from those of the available reference strains (001, 014, 017, 027 and 053) were labelled with consecutive numbers and the prefix AI.

Toxin typing was performed as described by van den Berg *et al.* (2004). Briefly, for detection of toxin A, region *tcdA* was tested using primers NKV011 (5'-TTTGTATCCTATAGAATCTAAGTCTAGTAAC-3') and NK9 (5'-CCACCAGCTGCAGCCATA-3') as described elsewhere (van den Berg *et al.*, 2004). Toxin A-positive strains produced a 2535 bp amplicon, whilst toxin A-negative strains had deletions of 1700–1800 bp.

For the detection of toxin B, region *tcdB* was tested using primers NK104 (5'-GTGTAGCAATGAAAGTCCAAGTTTACGC-3') and

NK105 (5'-CACTTAGCTCTTTGATTGCTGCACCT-3') as described previously (Kato *et al.*, 1991, 1999); amplicons with a size of 204 bp were produced for *tcdB*-positive strains. Detection of the amplification products was carried out on a 1.5% agarose gel (Bio-Rad) for 60–90 min at 100 V.

Analysis of polymorphisms in the negative regulator of toxin production (*tcdC*) was performed as described by Spigaglia & Mastrantonio (2002) using primers *tcdC*fw (5'-CATATCCTTTCT-TCTCCTCTTC-3') and *tcdC*rev (5'-AATTGTCTGATGCTGAACC-3'). Amplification was carried out in a FlexCycler with a 15 min initial enzyme activation at 95°C , followed by 35 cycles of 30 s at 95°C , 30 s at 50°C and 30 s at 72°C , with a 10 min final elongation step at 72°C . Control strain ribotype 027, with an 18 bp deletion, and strain VPI (without this 18 bp deletion) were used as internal controls on every test run for *tcdC*.

The presence of binary toxin was tested by detection of the binary toxin genes *cdtA* and *cdtB* using primers and conditions as described by Stubbs *et al.* (2000). Forward primer *cdtA*fw (5'-TGAACCTG-GAAAAGGTGATG-3') and reverse primer *cdtA*rev (5'-AGGATTA-TTACTGGACCATTG-3') were used for *cdtA*, and forward primer *cdtB*fw (5'-CTTAATGCAAGTAAATACTGAG-3') and reverse primer *cdtB*rev (5'-AACGGATCTCTTGCTTCAGTC-3') were used for *cdtB*, with HotStarTaq master mix (Qiagen) instead of the standard Taq DNA polymerase. For amplicon detection, a 1.5% agarose gel was run for 60 min at 100 V.

PCR ribotype profile analysis and virulence gene profile analysis were carried out using Bionumerics software (version 4.6; Applied Maths).

Antimicrobial susceptibility testing. Isolates were tested against metronidazole, moxifloxacin, vancomycin and clindamycin using E-test strips (AB Biodisk). A suspension of *C. difficile* equivalent to 1 McFarland turbidity standard was spread on *Brucella* agar supplemented with haemin and vitamin K1 (BD Diagnostic Systems) and incubated in an anaerobic atmosphere. The MIC was read after 24 h (except for clindamycin, which was read after 48 h) by the intersection of the inhibition ellipse on the scale. The interpretation of MIC results was based on the recommendations given by the guidelines of the Clinical and Laboratory Standards Institute (CLSI) for metronidazole, clindamycin and moxifloxacin (CLSI, 2007). The breakpoints for metronidazole were $\leq 8 \mu\text{g ml}^{-1}$ (susceptible), $16 \mu\text{g ml}^{-1}$ (intermediate) and $\geq 32 \mu\text{g ml}^{-1}$ (resistant), and for clindamycin were $\leq 2 \mu\text{g ml}^{-1}$ (susceptible) and $\geq 8 \mu\text{g ml}^{-1}$ (resistant). Moxifloxacin resistance was determined using a breakpoint of $\leq 2 \mu\text{g ml}^{-1}$ (susceptible) and $\geq 8 \mu\text{g ml}^{-1}$ (resistant); high-level moxifloxacin resistance was determined using a breakpoint of $\geq 32 \mu\text{g ml}^{-1}$. For vancomycin, we used breakpoints of $\leq 2 \mu\text{g ml}^{-1}$ (susceptible), $4\text{--}16 \mu\text{g ml}^{-1}$ (intermediate) and $\geq 32 \mu\text{g ml}^{-1}$ (resistant), as no standard has been defined by the CLSI.

Case characteristics. Data on clinical manifestations of infection and lethality within 30 days of specimen collection were obtained by reviewing patients' medical charts. Medical records were reviewed by the respective hospital and by M. H. and R. G. No attempt was made to differentiate between direct and indirect causes of CDAD-associated lethality. A case of CDAD was defined as a patient with diarrhoea or toxic megacolon and *C. difficile* toxin detection in stool samples. A case of community-acquired CDAD was defined as a case of CDAD with clinical onset outside the hospital or within 72 h after hospital admission with a negative history of hospitalization in the previous 12 weeks. Data for the 12-week history of cases were obtained by interviewing the patient and reviewing hospital admission register data for the previous 12 weeks.

RESULTS

Table 1 shows characteristics of the 142 cases of CDAD by PCR ribotyping. Of the 142 patients, 56 were male (39.4%). The median age was 64.5 years (range 2–95 years). At least seven cases were cases of community-acquired CDAD, including the one from which *C. difficile* ribotype 027 was isolated.

CDAD manifested as diarrhoea (including eight cases of bloody diarrhoea) in 126 patients (88.7%), as pseudo-membranous colitis in 15 (10.6%) and as toxic megacolon in one. Twelve of the 142 patients died within 30 days after specimen collection (8.45% case fatality). The deceased patients ranged in age from 70 to 93 (mean, 81 years; median, 80 years) and six were male (median age for males, 83 years; median age for females, 80 years). A lethal outcome occurred in 2/15 cases (13.3%) with pseudo-membranous colitis and in 10/126 (7.9%) cases with diarrhoea only.

Antimicrobial susceptibility patterns revealed resistance to clindamycin in 81/142 isolates (57.0%). One isolate (PCR ribotype AI-18) was also resistant to metronidazole, whilst one isolate (PCR ribotype AI-29) showed resistance to metronidazole only. Both isolates reverted to MICs of 0.5 µg ml⁻¹ on storage. Fifty-four of the 142 isolates demonstrated high-level resistance (≥ 32 µg ml⁻¹) to moxifloxacin (38.0%), whilst 56 were resistant (≥ 8 µg ml⁻¹; 39.4%). All isolates were susceptible to vancomycin.

Fig. 1 shows the various patterns of PCR ribotypes detected among the 142 isolates. A total of 41 different patterns were found. The patterns found in lethal cases were: AI-5, six lethal cases out of 26 patients; 014, two out of 24 patients; 053, one out of 24 patients; AI-1, one out of three patients; AI-30, one out of one patient; and AI-32, one out of one patient (Table 1).

For all but two isolates, *in vitro* production of enterotoxin A and cytotoxin B was detected: one isolate of ribotype 017 and one of AI-51 were negative for enterotoxin A.

Binary toxin genes encoding binary toxin were found in 10/142 isolates (7.0%): once for ribotype 027, in three cases of ribotype AI-1, in three cases of AI-10, once for ribotype AI-10-1, once for ribotype AI-27 and once for ribotype AI-30. None of the ten binary toxin-positive cases in our study was community-acquired.

C. difficile ribotype 027 was found in a British tourist with pseudomembranous colitis. This isolate showed an 18 bp deletion in *tcdC*.

The subtyping patterns demonstrated the occurrence of clusters of cases of CDAD in hospitals. The largest cluster affected a tertiary teaching hospital where ribotype 053 was obtained from 10/21 cases. The same health-care facility was affected by a second cluster of four cases of ribotype AI-5, with an unusually high case fatality rate (4/4). Another cluster with 7/18 cases showing ribotype pattern AI-5 affected a non-teaching hospital.

DISCUSSION

C. difficile has received much attention in recent years (Rupnik, 2007). Although the principal risk factors remain prolonged hospitalization, an age of ≥ 65 years and antibiotic therapy, some recent changes in the epidemiology of CDAD have been observed. Overall, the incidence and severity of the disease seem to be increasing (Rodriguez-Palacios *et al.*, 2007), and reports of severe cases with a community onset are becoming more numerous (Rupnik, 2007). Furthermore, some of the community-acquired cases have occurred in a low-risk population (i.e. younger than 65 years of age, or without previous antibiotic therapy or previous hospitalization) (CDC, 2005).

The toxinogenic profiles of the Austrian isolates studied here were in accordance with findings by other authors (van den Berg *et al.*, 2004; Viscidi *et al.*, 1981). All but two of the 142 Austrian isolates tested expressed enterotoxin A plus cytotoxin B: the exceptions belonged to ribotypes 017 and AI-51. Ribotype 017 usually shows a deletion of 1700–1800 bp in the region *tcdA* (Kato *et al.*, 1999). The role of binary toxin in CDAD, present in 7% of our Austrian isolates, needs further elucidation. In early studies, the prevalence of binary toxin-positive strains among human isolates was between 1.6 and 10% in non-outbreak situations (Rupnik *et al.*, 2003). In some countries, the proportion of binary toxin-positive strains in the human population has gradually increased. An Italian study of isolates from three different periods (pre-1990, 1991–1999 and 2000–2001) revealed that the proportions of binary toxin-positive strains in the three periods were 0, 24 and 45%, respectively (Spigaglia & Mastrantonio, 2004). In contrast to the claim that binary toxin-producing strains seem to be associated with community-acquired *C. difficile* infections of such severity that hospitalization is required, only one of the ten binary toxin-positive cases in our study was community-acquired (Rupnik *et al.*, 2003; Terhes *et al.*, 2004).

The resistance profiles of the isolates were also comparable to those described in the literature: high-level resistance to moxifloxacin was documented for 38% of isolates tested. Increased use of newer quinolones is considered a major factor contributing to the increase in CDAD (Kuijper *et al.*, 2006). Two isolates that initially tested resistant to metronidazole showed full *in vitro* susceptibility when retested some weeks later. The loss of metronidazole resistance in *C. difficile* seems to be a common phenomenon *in vitro* (J. Brazier, personal communication).

It has been postulated by van den Berg *et al.* (2005) that the coexistence of multiple PCR ribotype strains of *C. difficile* in faecal samples limits the value of PCR ribotyping for studying the epidemiology of CDAD. Of 23 patients with a first episode of CDAD studied by van den Berg *et al.* (2005), two (8.7%) harboured two different types, with no differences in toxinogenicity or clindamycin resistance, within one faecal sample. In our limited experience, PCR

Table 1. Characteristics of 142 CDAD cases that occurred in Austria from February 2006 to January 2007 by PCR ribotyping

Patterns different from those of the available reference strains (001, 014, 017, 027 and 053) were labelled with consecutive numbers and the prefix AI. Results are shown as the number of cases.

Ribotype	Deaths	Pseudomembranous colitis	Clindamycin-resistant isolates	High-level moxifloxacin-resistant isolates	Binary toxin gene-positive	Toxin A-positive/ toxin B-positive	Toxin A-negative/ toxin B-positive	Total
AI-5	6	2	24	22	0	26	0	26
014	2	2	7	1	0	24	0	24
053	1		22	21	0	24	0	24
AI-6		2	9	3	0	9	0	9
AI-14			1		0	7	0	7
AI-23			1		0	4	0	4
AI-9			3	1	0	4	0	4
AI-1	1		1	1	3	3	0	3
AI-10			1	1	3	3	0	3
AI-2					0	3	0	3
AI-17			2		0	2	0	2
AI-19			1		0	2	0	2
AI-3			2		0	2	0	2
AI-50		1	2		0	2	0	2
AI-8					0	2	0	2
AI-30	1	1			1	1	0	1
AI-32	1				0	1	0	1
017			1	1	0	0	1	1
027		1		1	1	1	0	1
AI-10-1					1	1	0	1
AI-12					0	1	0	1
AI-52			1	1	0	1	0	1
AI-15					0	1	0	1
AI-16			1	1	0	1	0	1
AI-18			1		0	1	0	1
AI-20					0	1	0	1
AI-21					0	1	0	1
AI-22					0	1	0	1
AI-24					0	1	0	1
AI-25					0	1	0	1
AI-26					0	1	0	1
AI-27					1	1	0	1
AI-29					0	1	0	1
AI-31					0	1	0	1
AI-35					0	1	0	1
AI-4		1	1		0	1	0	1
AI-45					0	1	0	1
AI-48					0	1	0	1
AI-51					0	0	1	1
AI-9-1					0	1	0	1
Total	12	10	81	54	10	140	2	142

ribotyping might be a valuable tool for recognizing related cases of CDAD in health-care facilities. Of the 142 Austrian isolates tested here, 22 were from one hospital experiencing a cluster of severe cases of CDAD (severe case defined as requiring intensive care or surgical intervention, or having a lethal outcome within 30 days of diagnosis; McDonald *et al.*, 2007). The cluster included 16 cases of severe CDAD and a further six cases that were related in time and place to these severe cases. Ten of the 22 isolates (45.5%) from cluster cases were ribotype 053. Clustering of certain

ribotypes in a health-care setting should prompt an in-depth epidemiological investigation of possible nosocomial outbreaks.

Recent outbreaks of CDAD in the USA, Canada and some European Union countries can be attributed to the emergence of the highly virulent *C. difficile* clone 027 (Kuijper *et al.*, 2007). However, only part of the recent increase in mortality and morbidity caused by *C. difficile* infections can be accounted for by this new, highly virulent

Fig. 1. PCR ribotype patterns of the 142 *C. difficile* isolates. Isolates with PCR ribotype patterns different from the available reference strains (001, 014, 017, 027 and 053) were labelled with consecutive numbers and the prefix AI.

type (Rupnik, 2007). Of the 142 Austrian isolates tested, only one showed ribotype 027; details on the British tourist with pseudomembranous colitis from whom the isolate was obtained have been reported elsewhere (Indra *et al.*, 2006). In Europe, this strain had been detected previously in France, Belgium, the UK and the Netherlands, and outside Europe in the USA and Canada. The Austrian case is considered to be the first documented community-acquired 027 infection in Europe. The patient had been repeatedly visiting her father in a hospital with a known 027 prevalence while herself taking antibiotics for bronchitis. Meanwhile, 027 has been reported in Ireland, Poland, Luxembourg, Denmark and Japan (Terhes *et al.*, 2004).

In our study, only 50/142 isolates (35.2%) could be allocated to a previously described PCR ribotype. The limited availability of reference strains severely hampers a comparison of the prevalence of various types across Europe and explains the lack of a standardized nomenclature for PCR ribotyping using the primers described by Bidet *et al.* (2000).

According to Terhes *et al.* (2006), PCR ribotypes 014 and 002 are the most common types in Hungary, and were found to account for 24.8 and 13.3% of 105 isolates tested, respectively. In Poland, ribotype 017 dominated among 79 *C. difficile* isolates tested, followed by ribotypes 014 and 046 (Pituch *et al.*, 2006).

Brazier *et al.* (2007) recently reported that type 106 is the most common strain currently prevalent in England; type 001 was by far the most common strain in the UK in the mid-1990s, when the British *C. difficile* typing service was set up. Type 106 appears to be a peculiarly British strain, as collaborative studies with several European countries and others further afield, including the USA and Canada, have not detected this strain (Pituch *et al.*, 2006). Whether or not one of the patterns described for our Austrian isolates by AI numbers matches type 106 cannot be answered because of the unavailability of an appropriate reference strain collection.

With the exception of ribotype AI-5, which had a case fatality rate of 23% (six lethal cases among 26 patients), ribotype patterns found in lethal cases were not significantly different from those found in survivors. It is possible that ribotype AI-5 might be identical to the British ribotype 001, a ribotype not known to be associated with increased lethality (J. Brazier, personal communication). According to the literature, the rate of fatality associated with CDAD ranges from 6 to 30% when pseudomembranous colitis is present, and is 'substantial' even in the absence of colitis (Aslam *et al.*, 2005; Bartlett, 2002). Overall, the case fatality was 8.45% among our study population, affecting only patients of at least 70 years of age. Whilst the case fatality rate is unknown for most countries, authors from northern France reported a 4% attributable mortality of CDAD and a Dutch group found 6.3% for the Netherlands (Kuijper *et al.*, 2007). It should be noted that the Austrian isolates were chosen arbitrarily and therefore this high case fatality rate may have been biased by a preference for choosing isolates from severe cases.

Whilst the community-acquired case of the British tourist suffering from 027 CDAD in an Austrian hospital could be traced back to a hospital in England, the discovery of the presence of *C. difficile* in food animals and in meat products strongly suggests that animal reservoirs and transmission via foods are possible sources of community-associated infection. *C. difficile*-associated infection may be a zoonotic and food-borne disease. Delaney (2007) studied the association between antimicrobial drugs and community-acquired CDAD and confirmed antimicrobial drugs as a risk factor for CDAD. Despite the high risk that appears to be associated with fluoroquinolone use,

only 7% of the case patients were exposed to a fluoroquinolone, and only 37% were exposed to any class of antimicrobial drug. Therefore, whilst good prescribing practices for antimicrobial drugs should continue to be encouraged, these drugs are unlikely to be the primary driving force of community-acquired CDAD infections in this population (Delaney, 2007). Recently, Rodriguez-Palacios *et al.* (2006) reported a surprisingly high degree of overlap in Canada between isolates from symptomatic and asymptomatic calves and recent human isolates.

In Austria, so far there are no data concerning the ribotypes of isolates from animal or food reservoirs. If animals are indeed a source of *C. difficile* infection, food could be one of the transmission routes from animals to humans. In Canada, approximately 20% of retail ground meat samples have been shown to harbour *C. difficile* (Rodriguez-Palacios *et al.*, 2007).

Whether *C. difficile*-associated infection could be a zoonotic and food-borne disease clearly needs further evaluation. The cases in our study were primarily health-care-acquired. Furthermore, the subtyping patterns found demonstrated the occurrence of clusters within hospitals. These findings indicate the need for increased awareness of this pathogen and a concerted effort to control CDAD by reducing unnecessary antimicrobial drug use and implementing currently recommended infection control measures. The study also highlights the need to develop a surveillance system for CDAD (McDonald *et al.*, 2007). Health authorities should be aware of the risk for CDAD and should make efforts to control the transmission of *C. difficile* and to prevent disease.

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REFERENCES

- Arroyo, L. G., Kruth, S. A., Willey, B. M., Staempfli, H. R., Low, D. E. & Weese, J. S. (2005). PCR ribotyping of *Clostridium difficile* isolates originating from human and animal sources. *J Med Microbiol* **54**, 163–166.
- Aslam, S., Hamill, R. J. & Musher, D. M. (2005). Treatment of *Clostridium difficile*-associated disease: old therapies and new strategies. *Lancet Infect Dis* **5**, 549–557.
- Bartlett, J. G. (2002). Antibiotic-associated diarrhea. *N Engl J Med* **346**, 334–339.
- Bartlett, J. G. & Gorbach, S. L. (1977). Pseudomembranous enterocolitis (antibiotic-related colitis). *Adv Intern Med* **22**, 455–476.
- Baverud, V. (2002). *Clostridium difficile* infections in animals with special reference to the horse. A review. *Vet Q* **24**, 203–219.
- Bidet, P., Lalande, V., Salauze, B., Burghoffer, B., Avesani, V., Delmee, M., Rossier, A., Barbut, F. & Petit, J. C. (2000). Comparison of PCR-ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for typing *Clostridium difficile*. *J Clin Microbiol* **38**, 2484–2487.
- Brazier, J., Patel, B. & Pearson, A. (2007). Distribution of *Clostridium difficile* PCR ribotype 027 in British hospitals. *Euro Surveill* **12**, E070426.2.
- CDC (2005). Severe *Clostridium difficile*-associated disease in populations previously at low risk – four states, 2005. *MMWR Morb Mortal Wkly Rep* **54**, 1201–1205.
- CLSI (2007). *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria*. Approved standard M11-A7, 7th edn. Wayne, PA: Clinical and Laboratory Standards Institute.
- Delaney, J. A. C. (2007). Antimicrobial drugs and community acquired *Clostridium difficile*-associated disease, UK. *Emerg Infect Dis* **13**, 761–763.
- Indra, A., Huhulescu, S., Hasenberger, P., Schmid, D., Alfery, C., Wuerzner, R., Fille, M., Gattringer, K., Kuijper, E. & Allerberger, F. (2006). First isolation of *Clostridium difficile* PCR ribotype 027 in Austria. *Euro Surveill* **11**, E060914.3.
- Kato, N., Ou, C. Y., Kato, H., Bartley, S. L., Brown, V. K., Dowell, V. R. J. & Ueno, K. (1991). Identification of toxigenic *Clostridium difficile* by the polymerase chain reaction. *J Clin Microbiol* **29**, 33–37.
- Kato, H., Kato, N., Katow, S., Maegawa, T., Nakamura, S. & Lyerly, D. M. (1999). Deletions in the repeating sequences of the toxin A gene of toxin A-negative, toxin B-positive *Clostridium difficile* strains. *FEMS Microbiol Lett* **175**, 197–203.
- Kuijper, E. J., Coignard, B. & Tull, P. (2006). Emergence of *Clostridium difficile*-associated disease in North America and Europe. *Clin Microbiol Infect* **12** (Suppl. 6), 2–18.
- Kuijper, E. J., Coignard, B., Brazier, J., Suetens, C., Drudy, D., Wiuff, C., Pituch, H., Reichert, P., Schneider, F. & other authors (2007). Update of *Clostridium difficile*-associated disease due to PCR ribotype 027 in Europe. *Euro Surveill* **12**, 163–166.
- Lefebvre, S. L., Arroyo, L. G. & Weese, J. S. (2006). Epidemic *Clostridium difficile* strain in hospital visitation dog. *Emerg Infect Dis* **12**, 1036–1037.
- McDonald, L. C., Owings, M. & Jernigan, D. B. (2006). *Clostridium difficile* infection in patients discharged from US short-stay hospitals, 1996–2003. *Emerg Infect Dis* **12**, 409–415.
- McDonald, L. C., Coignard, B., Dubberke, E., Song, X., Horan, T. & Kuttu, P. K. (2007). Recommendations for surveillance of *Clostridium difficile*-associated disease. *Infect Control Hosp Epidemiol* **28**, 140–145.
- Pituch, H., Brazier, J. S., Obuch-Woszczatynski, 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 (MLSB) type resistance. *J Med Microbiol* **55**, 207–213.
- Rodriguez-Palacios, A., Staempfli, H. R., Duffield, T., Peregrine, A. S., Trotz-Williams, L. A., Arroyo, L. G., Brazier, J. S. & Weese, J. S. (2006). *Clostridium difficile* PCR ribotypes in calves, Canada. *Emerg Infect Dis* **12**, 1730–1736.
- Rodriguez-Palacios, A., Staempfli, H. R., Duffield, T. & Weese, J. S. (2007). *Clostridium difficile* in retail ground meat, Canada. *Emerg Infect Dis* **13**, 485–487.
- Rupnik, M. (2007). Is *Clostridium difficile*-associated infection a potentially zoonotic and foodborne disease? *Clin Microbiol Infect* **13**, 457–459.
- Rupnik, M., Grabnar, M. & Geric, B. (2003). Binary toxin producing *Clostridium difficile* strains. *Anaerobe* **9**, 289–294.
- 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. & Mastrantonio, P. (2004).** Comparative analysis of *Clostridium difficile* clinical isolates belonging to different genetic lineages and time periods. *J Med Microbiol* **53**, 1129–1136.
- Stubbs, S., 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., Urban, E., Soki, J., Hamid, K. A. & Nagy, E. (2004).** Community-acquired *Clostridium difficile* diarrhea caused by binary toxin, toxin A, and toxin B gene-positive isolates in Hungary. *J Clin Microbiol* **42**, 4316–4318.
- Terhes, G., Brazier, J. S., Urban, E., Soki, J. & Nagy, E. (2006).** Distribution of *Clostridium difficile* PCR ribotypes in regions of Hungary. *J Med Microbiol* **55**, 279–282.
- van den Berg, R. J., Claas, E. C. J., Oyib, D. H., Klaassen, C. H. W., Dijkshoorn, L., Brazier, J. S. & Kuijper, E. J. (2004).** Characterization of toxin A-negative, toxin B-positive *Clostridium difficile* isolates from outbreaks in different countries by amplified fragment length polymorphism and PCR ribotyping. *J Clin Microbiol* **42**, 1035–1041.
- van den Berg, R. J., Ameen, H. A., Furusawa, T., Claas, E. C., van der Vorm, E. R. & Kuijper, E. J. (2005).** Coexistence of multiple PCR-ribotype strains of *Clostridium difficile* in faecal samples limits epidemiological studies. *J Med Microbiol* **54**, 173–179.
- Viscidi, R., Willey, S. & Bartlett, J. G. (1981).** Isolation rates and toxigenic potential of *Clostridium difficile* isolates from various patient populations. *Gastroenterology* **81**, 5–9.
- Vonberg, R. P., Schwab, F. & Gastmeier, P. (2007).** *Clostridium difficile* in discharged inpatients, Germany. *Emerg Infect Dis* **13**, 179–180.