Predominance of *Clostridium difficile* 027 during a five-year period in Bolzano, Northern Italy

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SUMMARY

Toxigenic *Clostridium difficile* is responsible for antibiotic-associated diarrhoea and other diseases. The increasing frequency and severity is attributed to highly-virulent ribotypes such as 027. The aim of the study was to collect epidemiological and molecular data for *C. difficile* isolates during 2009-13 in the Central Hospital of Bolzano, Northern Italy. Stool samples from inpatients of the Bolzano Central Hospital were screened for toxins A and B, and *C. difficile* was cultured and tested for antibiotic susceptibility. PCRs were performed for genes of toxin A, toxin B, binary toxin and ribotyping. During the period 2009-13 from 320 patients (9% of patients tested) at least one stool sample proved positive for *C. difficile* toxins, and incidences for all hospital inpatients per 10,000 patient days (per 1,000 admissions) varied between 2.2 (1.5) and 4.3 (3.0). Out of 138 isolates (43% of total isolates were studied), 24 different ribotypes were identified. Isolates with ribotype 027 were predominant (38%), followed by 018 (13%) and 607 (10%). Whereas for ribotype 018 a significant decrease was seen during the five-year period, ribotype 027 increased significantly from 0% in 2009 to 64% in 2012, decreasing then to 10% in 2013. Isolates were sensitive to metronidazole and vancomycin, whereas isolates of the three major ribotypes were resistant to moxifloxacin. Our data indicates a significant change in *C. difficile* incidence rates and ribotype frequencies during the five-year period in the Central Hospital in Bolzano.

Keywords: *Clostridium difficile*, ribotype 027, ribotype 018, ribotype 607.

INTRODUCTION

Toxigenic *Clostridium difficile* is recognized as the primary pathogen of antibiotic-associated clinical manifestations, especially in geriatric patients, with diseases ranging from diarrhoea to fulminant and sometimes fatal pseudomembranous colitis, bowel perforation or toxic megacolon [1-3]. Important virulence factors of *C. difficile* are toxins A and B responsible for intestinal fluid secretion, mucosal injury and inflammation [4]. The observed increase in frequency and severity of *C. difficile* infection (CDI) during the last years is generally attributed to the emergence of *C. difficile* ribotype 027, both in North America and in Europe [5-8]. Higher levels of toxins, expression

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of binary toxin and other virulence factors in this strain are associated with a more severe course of infection, higher risk of relapse and mortality [9, 10]; ribotype 078 has molecular characteristics like ribotype 027 (both contain the binary toxin gene *cdt* and frameshift deletions in the gene *tcdC*), but CDI due to type 078 is more frequently community associated [11].
The purpose of this study was to provide data on epidemiological surveillance, ribotyping and antibiotic susceptibility for *C. difficile* stool isolates from inpatients of the Bolzano Central Hospital, Northern Italy, during the five-year period 2009-13.

PATIENTS AND METHODS

The study population included inpatients >2 years of age in the Central Hospital in Bolzano, an 800-bed tertiary care reference hospital in the Province of Bolzano, Northern Italy, with a catchment area of 220,000 people and centralized trauma and neurosurgery services for about half a million inhabitants as well as an active bone marrow transplantation program. Stool samples were sent to the Hospital Laboratory of Microbiology and Virology from inpatients with diarrhea, defined as passage of 3 or more unformed stools, according to Bristol stool chart types 6-7, in 24 consecutive hours, following the guidelines of the Society of Healthcare Epidemiology of America (SHEA) and Infectious Diseases Society of America (IDSA) [2, 12]. During the study period 2009-13, all stool samples were screened for *C. difficile* toxins A and B by immunoassay (Vidas® *C. difficile* A&B, bioMérieux, Marcy l'Etoile, France). *C. difficile* toxin positive samples were cultured on cefoxitin, cycloserine, fructose agar (bioMérieux, Marcy l'Etoile, France) at 37°C for 48 h under anaerobic conditions for isolates coming from the Departments of Internal Medicine, Urology and Geriatrics in 2009-10, extended to stool samples from all hospital units after 2010. Presumptive *C. difficile* isolates were identified morphologically, subcultured on Columbia blood agar (bioMérieux), identified by matrix-assisted laser desorption/ionization - time of flight - mass spectrometry (MALDI-TOF Vitek-MS; bioMérieux) and stored at -80°C using the Microbank microbial preservation system (Pro-Labs Diagnostics, Ontario, Canada). The routine testing algorithm for *C. difficile* has been modified in 2016 (after the study period); the new algorithm is based on a glutamate dehydrogenase (GDH) stool screening test (VIDAS *C. difficile* GDH, bioMérieux) and TECHLAB C. DIFF QUIK CHEK COMPLETE® (Alere), combined in positive samples with a molecular assay (Xpert® *C. difficile*, Cepheid) detecting the genes TCDB (coding for toxin B), CDT (coding for binary toxin) and the deletion TCDCΔ117 (markers for presumptive identification of ribotype 027).
Ribotyping was performed as described elsewhere [13]. In short, after DNA extraction, a fluorescence marked primer was used for PCR amplification. PCR products were mixed with an internal size standard and analyzed using an automated capillary gel electrophoresis system (ABI3130, Thermo Fisher). PCR-Ribotypes were identified using AGES-WEBRIBO (webribo.ages.at).
In vitro susceptibility testing was performed as described previously [14]. Briefly, agar-diffusion testing was performed on Brucella agar plates supplemented with hemin (5 µg/ml), vitamin K1 (1 µg/ml), and lysed sheep blood (5% v/v) using the epsilometer test (Etest®) (AB-Biodisk, Solna, Schweden) and the respective European Committee on Antimicrobial Susceptibility Testing (EUCAST) minimal inhibitory concentration (MIC) breakpoints for metronidazole and vancomycin, Clinical and Laboratory Standards Institute (CLSI) MIC breakpoints for clindamycin and moxifloxacin, and the suggested MIC breakpoints for rifampicin and an in-house disc (40 µg) diffusion test for rifaximin, as described by Huhulescu et al. [14].
Statistical analysis of data was performed using the software MedCalc version 13.1.2.0 (MedCalc Software, bvba). For trend analysis, the Chi-squared test for trends was used, the level of significance was set as $p<0.01$.
The study was approved by the Ethics Committee of the Bolzano Central Hospital and procedures followed were in accordance with the ethical standards of the Ethical Principles for Medical Research Involving Human Subjects [15].

RESULTS

During the period from January 2009 to December 2013 screening tests for *C. difficile* toxins A/B

from patients hospitalized in the Bolzano Central Hospital were requested for stool samples from a total of 3,400 patients >2 years of age, their mean age was 69 years (range 3 to 101 years) and 48% of these were females. For 320 patients (9% of tested patients; 90% hospitalized for >2 days) at least one stool sample resulted positive for *C. difficile* toxins, with a fluctuating monthly trend during the five-year study period (Figure 1); 4.2% of toxin positive patients were aged >2-20 years, 2.2% were aged >20-40 years, 13.2% were aged >40-60 years, 40.0% were aged >60-80 years and 40.4% were aged >80 years. Mean hospital wide incidence rates of *C. difficile* toxin A/B positive inpatients per 10,000 patient-days (per 1,000 admissions) varied between 1.8 (1.2) and 4.3 (3.0); 70% of patients were from Internal Medicine, Urology, Geriatrics, Pneumology or Nephrology/Hemodialysis units. Incidence rates ranging from 4.0-5.0/10,000 patient days, remaining constant over the five-year period, were found for the Geriatrics unit, whereas highly variable (1.1-29.6/10,000 patient days) and fluctuating incidence rates were observed in the Internal Medicine, Urology, Pneumology and Nephrology/Hemodialysis units (Table 1). During the years 2014-15, following the five-year study period, we found hospital wide incidence rates of *C. difficile* toxin A/B positive inpatients per 10,000 patient days (per 1,000 admissions) of 2.9 (1.7) in 2014 and 2.6 (1.8) in 2015. First non-replicate *C. difficile* isolates from 138 (43% of total first isolates) toxin A/B positive stool samples from inpatients in the Bolzano Central Hospital were PCR-ribotyped (Table 2); further 9 isolates from long-term care facilities in the Bolzano health district and 6 isolates from a neighboring hospital (Bressanone) were also typed. In total, 24 different ribotypes were identified. In the Bolzano Central Hospital isolates with ribotype 027 were predominant (38%), followed by 018 (13%), 607 (10%) and 014/020 (8%). Ribotype 027 increased significantly (p=0.003) during the peri-

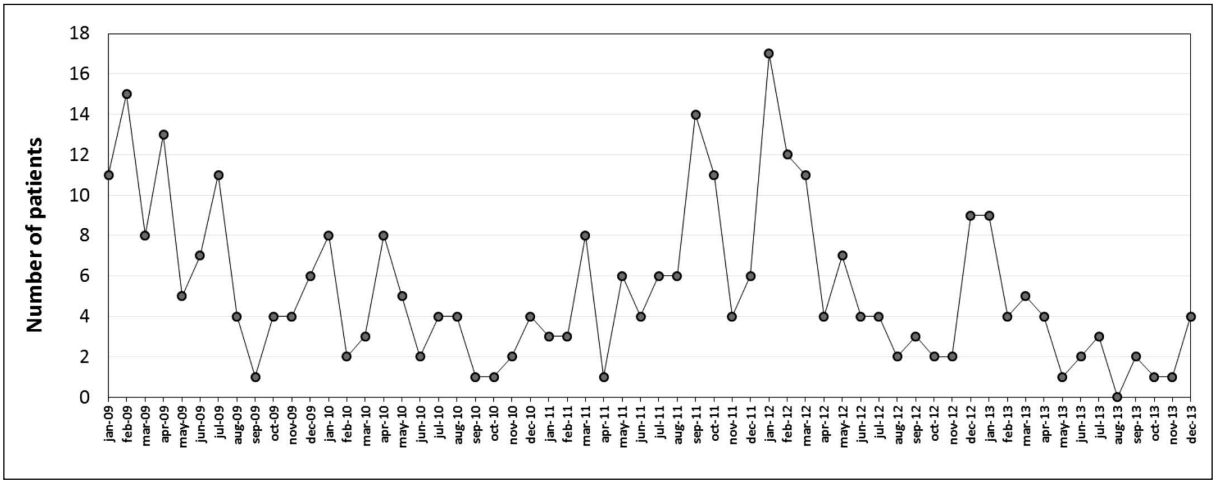

Figure 1 - Monthly trend of first *C. difficile* toxin A/B positive hospital inpatients.

Table 1 - Prevalence and incidence of *C. difficile* toxin A/B positive patients during 2009-13.

	Year	*All inpatients*	*Internal Medicine*	*Urology*	*Geriatrics*	*Pneumology*	*Nephrology/ Hemodialysis*
Cases per 10,000 patient-days (Cases per 1,000 admissions)	2009	4.3 (3.0)	4.8 (3.6)	32.1 (15.1)	4.0 (3.3)	14.0 (16.8)	16.6 (16.5)
	2010	2.2 (1.5)	2.5 (1.9)	2.6 (1.1)	4.0 (3.0)	2.5 (3.0)	27.4 (29.6)
	2011	3.6 (2.5)	9.3 (6.9)	5.7 (2.3)	4.0 (3.5)	2.5 (2.6)	7.6 (8.3)
	2012	3.8 (2.6)	8.5 (6.7)	15.2 (6.4)	5.0 (4.5)	8.9 (9.5)	2.5 (2.6)
	2013	1.8 (1.2)	3.0 (2.3)	2.6 (1.1)	4.4 (4.0)	1.2 (1.3)	10.0 (10.4)

Table 2 - *Clostridium difficile* ribotypes during 2009-13.

	027	*018*	*607*	*014/020*	*029*	*017*	*126*	*628*	*078*	*012*	*070*	*241*	*081*	*033*	*001*	*005*	*043*	*056*	*076/1*	*440*	*AI-8/0**	*AI-75**	*AI-78**	*AI-84**	*All isolates*
2009		10		1		1			1		1	1		1					1						17
2010	7	6	1	1					1		1	1								1			1	1	21
2011	14		5	3				3		1															26
2012	29	1	5	2	5		2								1										45
2013	3	2	4	5		3	2		1	2			2			1	1	1			1	1			29
All isolates	53	19	15	12	5	4	4	3	3	3	2	2	2	1	1	1	1	1	1	1	1	1	1	1	138

*Austrian nomenclature.

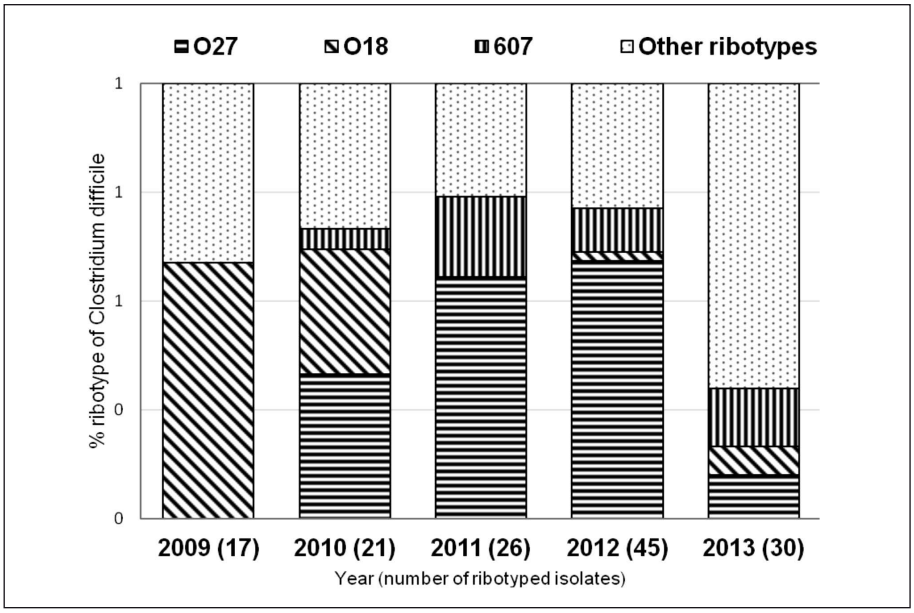

Figure 2 - Distribution of *C. difficile* ribotypes per year.

od 2009-12 (2009: 0%; 2010: 33%; 2011: 53%; 2012: 64%), decreasing then again (p<0.001) to 10% in 2013 (Figure 2); 51% of patients with ribotype 027 were females (vs 52% of patients with non-027 ribotypes). Ribotype 027 was isolated mainly from patients of the Internal Medicine (44%), followed by Geriatrics (10%), Urology (10%), Pneumology (8%), Infectious Diseases (7%), Nephrology (5%) and the Intensive Care unit (5%), with low isolate numbers coming from patients of other units. The mean age was 80 years (range 47-101 years) for hospitalized patients with CDI by ribotype 027 and 75 years (range 3-99 years) for patients with infections by non-027 ribotypes. On the other hand, during the five-year period a significant decrease (p<0.001) was registered for ribotype 018, highly prevalent in 2009 (2009: 58%; 2010: 28%; 2011: 0%; 2012: 2%; 2013: 6%). Five out of 6 ribotype 017 isolates were PCR-positive for toxin B and negative for toxin A, all other isolates were PCR-positive for both toxin genes. All ribotype 027, 078, 126 and 033 isolates were PCR-positive for binary toxin. Moreover, 2/9 *C. difficile* isolated during the same five-year period from long-term care facility residents in the Bolzano health district and 5/6 isolates from a hospital

serving a neighboring health district (Bressanone) had ribotype 027. On the other hand, in 2016, by direct molecular testing of 41 GDH and toxin B gene positive stools from patients of the Bolzano Central Hospital, no sample positive for *cdt* and deletion *tcdC*Δ117 (markers for ribotype 027) has been found.
Antimicrobial susceptibility testing was done for all isolates from 2011-13 and all were susceptible to metronidazole and vancomycin. All isolates belonging to ribotypes 027, 607, 017, 018, 126 and the single isolate with ribotype AI-8/0 were resistant to moxifloxacin, whereas 5/6 ribotype 014/020 isolates and all belonging to other ribotypes were susceptible to moxifloxacin. The 10 isolates with ribotype 607 were also tested for rifampicin and rifaximin, all but one resulting resistant to both antibiotics.

DISCUSSION

The present study describes the incidence of CDI from hospitalized patients and the distribution of ribotypes from first stool isolates during 2009-13 in the Bolzano Central Hospital. In a previous study in the same hospital, considering data collected during 2007 from the Internal Medicine unit, 16.9% of patients with nosocomial diarrhoea were detected to be positive, with an overall CDI incidence rate of 6.2 cases/10,000 patient days and 5 cases/1,000 patient admissions [16]. In the present study, similar incidence rates of 2.5-9.3 cases/10,000 patient days and 1.9 to 6.7 cases/1,000 patient admissions were found for the period 2009-13 in the same unit, with highest incidence rates in 2011-12. In four Internal Medicine wards of another hospital in Northern Italy higher incidence rates of 23.3/10,000 patient days or 25.6/1,000 hospitalizations were found in 2008-09 [17]. Another Italian study in five hospitals in Rome reported increasing incidence of CDI episodes from 0.3 in 2006 to 2.3 per 10,000 patient days in 2011; 80% of episodes occurred in medical wards [18]. A study over four months during 2013-14 in 40 Italian Internal Medicine wards, enrolling 10,780 patients, found an overall CDI incidence rate of 5.3/10,000 patient-days [19]. In a teaching hospital in Liguria the annual incidence rate of CDI/10,000 patient days significantly increased from 0.5 in 2010 to 3.0 in 2014 [20].
A European study in 2014-15, including 60 hospitals in 3 European countries (Italy, France, UK), found a mean annual CDI rate per hospital of 2.5/10,000 patient days, with the highest rate present in Italy (4.7 cases/10,000 patient days) [21]. A network of 106 laboratories in 34 European countries in 2008 found a mean incidence rate of 4.1 cases per 10,000 patient days (range 0.0-36.3) [22], whereas a European study involving 482 hospitals across 20 countries in 2012-13 reported a mean incidence rate of 7.0 cases/10,000 patient days (range 0.7-28.7) [23]. In the last study in Italy, CDI rates of 9.5/10,000 and 8.3/10,000 patient days were reported in 2012 and 2013, respectively [24]. Thus, incidence rates of CDI (identified by *C. difficile* toxin positivity) found in our study are in the mean range of Italian and other European hospitals.
Fifty-three out of 138 typed isolates (38%), collected during 2009-13 from patients hospitalized in the Bolzano Central Hospital, had ribotype 027; moreover, 7 isolates collected from long-term care facilities in the Bolzano health district or from a hospital serving a neighboring health district had also ribotype 027. On the other hand, a previous study, testing 44 toxin A/B positive *C. difficile* isolates collected during 2007 in the Bolzano health district (Internal Medicine, Geriatrics, Nephrology/Hemodialysis, other hospital wards, long-term-care facilities or outpatients), did not find ribotype 027 and in 2016 out of 41 GDH and toxin B gene positive stool samples no ribotype 027 isolate was identified [25]. Previously, in two hospitals in Northern Italy, authors found *C. difficile* ribotype 027 in 8 CDI cases and ribotype 078 in 26 cases of generally younger patients [26]. Other authors found ribotype 027 in stool samples collected in 2011-12 from 41% (10/24) of patients with severe CDI admitted to 7 hospitals in Rome, Italy [27]. Another multicentre study in 2014 in six tertiary care hospitals in Rome found that, out of 563 patients, 47.9% had CDI caused by ribotype 027, showing statistically significant association with residence in nursing homes [28]. A European study in 2012-13, typing 1,196 *C. difficile* isolates from 482 hospitals among 19 countries, identified 027 as the most common ribotype (19%), with 88% of occurrences recorded in only 4 countries (Germany, Hungary, Poland and Romania); 3% of the isolates belonged to ribotype 078 [7,23]. Ribotypes 078 and 033, found in our strain collection (3

and 1 isolates, respectively), have been previously associated with domestic animals such as pigs and calves [22, 29, 30].
In the present study 42% (16/38) of isolates from 2009-10 had ribotype 018, highly prevalent in 2009 (58%) but rarely found in 2011-13. This ribotype has been found to be prevalent in Italy and to frequently express fluoroquinolone resistance; the three ribotype 018 isolates collected in our hospital in 2011-13 (strains isolated in 2009-10 were not tested) were also resistant to moxifloxacin [31, 32].
The third most frequent ribotype, found in 11% (15/129) of our isolates, was 607. Typing of 103 Italian *C. difficile* isolates from 2012-13 identified 31 ribotypes and the predominant were 607 (27%) and 018 (12%); PCR-ribotypes 027 and 078 represented 8% and 4% of the strains, respectively [33]. A European study in 2012-13 commonly identified ribotypes 018 and 607 in Italy (22% and 17% prevalence, respectively), whereas these ribotypes were rarely isolated in other countries [7]. In a 2008 survey, including 389 toxigenic *C. difficile* isolates from 34 European countries, the 4 most frequent PCR-ribotypes were 014/020 (16%), 001 (10%), 078 (8%) and 018 (6%; because of high prevalence in 3 Italian hospitals), whereas 027 (5%) was the sixth most frequent ribotype [22]. In a European study in 2012-13, out of 1,196 isolates, the following 4 most prevalent ribotypes were identified: 027 (19%), 001/072 (11%), 014/020 (10%), 140 (4%) [7]. The 4 most frequent ribotypes in the two European studies include three (027, 018 and 014/020) out of the 4 most frequent ribotypes in our strain collection.
Antimicrobial susceptibility of our *C. difficile* isolates to metronidazole and vancomycin confirms data from a European study, finding rare resistance to metronidazole (0.1%) and vancomycin (0.8%) [34]. Resistance to moxifloxacin of prevalent ribotypes in our hospital during 2009-12 (027, 607, 018, 126) has been described by other authors [32, 34].
The higher prevalence of CDI in 2009 (4.3/10,000 patient days) compared to following years could be explained by healthcare associated transmission of the epidemic Italian ribotype 018 [31-32], replaced in following years by 027. Compared with 2011-12 in 2013 we noticed decreasing CDI incidence rates (1.8/10,000 in 2013 vs 3.6 and 3.8/10,000 patient days in 2011 and 2012, respectively), decreasing prevalence of ribotype 027 (10% in 2013 vs 53% and 64% in 2011 and 2012, respectively) and increasing ribotype diversity (14 ribotypes in 2013 vs 5 and 6 ribotypes in 2011 and 2012, respectively). Similarly, a European study found that ribotype diversity decreased as the prevalence of ribotype 027 increased and this might be explained by more healthcare associated transmission of dominant ribotypes [7]. Incidence rates 2014-15 in our hospital were 2.9 and 2.6 cases/10,000 patient days in 2014 and 2015, respectively, and no ribotype 027 isolate has been identified out of 41 *C. difficile* toxin B positive stools tested in 2016.
Low level but highly variable and fluctuating incidence rates in different wards in the Bolzano Central Hospital probably indicates low-level endemic presence of *C. difficile* in the hospital and local epidemics in particular wards.
To conclude, the high prevalence of pathogenic *C. difficile* ribotypes in the hospital environment in 2010-12 and their rapid decrease in 2013 confirms the important role of epidemiological surveillance by molecular typing. Moreover, because of suboptimal laboratory diagnostic methods such as only toxin A/B testing, stool samples positive for CDI may not be diagnosed, and the possible overestimation of the 027 frequency by immunoenzymatic methods could bias the relative frequency of different ribotypes (7,35). Therefore, in 2016 we have optimized our testing algorithm for *C. difficile* infection by introduction of molecular methods with real-time PCR identification of toxin genes and presumptive hypervirulent strains, together with a GDH assay, combined with a toxin A/B test and a culture method for *C. difficile*, allowing conservation of isolates for further genotyping studies.

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Conflict of interest. The authors have no conflicts of interest to disclose.

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