Original Article

Occurrence of *Clostridium difficile* infections due to PCR ribotype 027 in Bucharest, Romania

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Abstract

Introduction: Little is known about prevailing ribotypes of *Clostridium difficile* infection in Romania where CDI is not a mandatory notifiable disease.

Methodology: We studied 64 non-duplicate *C. difficile* isolates from patients hospitalised at the National Institute of Infectious Diseases, Bucharest, Romania between March 2011 and March 2012.

Results: Sixty-three of the 64 *C. difficile* isolates produced toxins A and B whereas 44 (69%) isolates produced a binary toxin. Ribotype 027 accounted for 43 (68%) of the 63 toxigenic strains. The remaining 20 isolates belonged to ribotypes 018 (n = 9), 012 (n = 3), and, with one isolate each, 014, 031, 081, 416, 433, 500, 507 and PR03035 (new ribotype). Information on hospital mortality was available for 62 of the 64 patients; among these 62 cases, 4 (6.4%) ended fatal. Recurrence was documented for 11 (18.3%) of the 60 patients for whom this information was available. Multilocus variable-number tandem repeat analysis of the 43 isolates of ribotype 027 yielded a unique cluster for the Romanian isolates when compared to Austrian or Italian isolates.

Conclusion: Our findings sustain the hypothesis of a recent emerged outbreak of *C. difficile* PCR ribotype 027 infections in the area of Bucharest.

Key words: *Clostridium difficile*; ribotype 027; Romania.

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Introduction

Clostridium difficile is the major identifiable cause of nosocomial and antibiotic-associated infectious diarrhea worldwide [1,2]. Clinical manifestations of *C. difficile* infection (CDI) range from asymptomatic carriage, to diarrhea, pseudomembranous colitis, toxic megacolon and death. In recent years, an increase in CDI has been reported worldwide, partly due to the spread of a specific clone, PCR ribotype 027 [3-5]. *Clostridium difficile* PCR ribotype 027 is primarily characterised by an increased production of toxins A and B, production of a binary toxin and resistance to newer fluoroquinolones such as moxifloxacin [3]. In Europe, PCR ribotype 027 was reported for the first time in 2005 in England and one year later in the Netherlands [4,6]. Subsequently, many European countries reported occurence of CDI cases caused by PCR ribotype 027 [7]. Prior to 2008 *C. difficile* 027 had been reported in 16 European countries. In 2008, a hospital-based European-wide study funded by the European Centre for Disease Prevention and Control (ECDC) found that the prevalence of PCR ribotype 027 was 5% in the 34 European countries included in the study and that ribotypes other than 027, including 014, 020, 001 and 078, were more prevalent [5].

Because CDI is not a mandatory notifiable disease the number of CDI cases in Romania is probably largely underestimated. Since 2011, the National Institute for Infectious Diseases Matei Bals (NIIDMB) in Bucharest (a 700-beds hospital, the largest hospital for infectious diseases in Romania) noticed an increase of admissions of patients with CDI; the average number of hospitalised cases rose from 1.7 cases per month in 2010, to 12 cases per month in 2011 and even 31 cases per month in the first quarter of 2012 [15]. While no case of CDI-related fatality was noticed in 2010, ten fatalities due to CDI were recorded in 2011 and within the first quarter of 2012 ten fatal outcomes were documented in patients with diagnosis CDI [15]. To our knowledge, there are currently very

few laboratories in Romania that perform toxin diagnosis by PCR and *C. difficile* detection by culture techniques and these laboratories are sometimes confronted with reagent supply shortage. The majority of CDI cases in Romania are diagnosed by physicians on the basis of clinical symptoms and medical history, without microbiological testing. To obtain information on microbiological characteristic of this disease in Romania, with a focus on ribotypes and toxin genes, we studied a collection of 64 isolates of *C. difficile* consecutively sampled in Bucharest at the NIIDMB, between March 2011 and March 2012.

Methodology

Patient population

During the twelve months period, from March 2011 to March 2012, 200 patients were diagnosed with CDI in NIIDMB in Bucharest. We used the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) definitions regarding diagnosis of defined CDI cases, severe cases, fatality rate, and recurrence [9]. However, due to reagent supply shortages for *Clostridium* cultures, for toxin identification or for detection of genes encoding for toxin A and toxin B, we also considered a new category of possible cases of CDI which included patients with clinical manifestations and epidemiological risk factors, combined with presence of *C. difficile* in stool samples and in the absence of evidences for another cause of diarrhea. The SHEA&IDSA case definitions for CDI surveillance were used for differentiation of community-associated versus healthcare-associated CDI [10]. We considered the diagnosis date, the date of the request for stool tests submitted to the department of microbiology in the NIIDMB. Written consent was obtained from each patient prior to obtain samples. All patient demographic data were anonymised**.**

Bacterial isolates

We prospectively collected *C. difficile* isolates originating from patients receiving medical care in NIIDMB, Bucharest, for CDI between 1 March 2011 and 1 March 2012. Stool specimens were processed at the NIIDMB, by spreading samples onto selective cycloserine-cefoxitin-fructose agar plates (CLO agar, BioMérieux, Marcy l'Etoile, France) and incubating at 35±2°C in an anaerobic atmosphere (85% nitrogen, 10% hydrogen, 5% carbon dioxide) for 48 hours.

Putative *C. difficile* colonies were selected based on morphological criteria, Gram-stain results and odour, and identified with the Vitek 2 ANC card (BioMérieux, Marcy l'Etoile, France). Isolates were stored in cryo-bank vials (MastDiagnostika, Reinfeld, Germany) at -80°C. In September 2012, 64 isolates could be revitalised and transferred to the Austrian Reference Centre for *Clostridium difficile* in Vienna (Austria).There, species diagnosis was confirmed by testing for the common antigen (*C. difficile* Agglutination Test kit; Microgen, Camberley, United Kingdom) and by mass spectrometry (MALDI-TOF Biotyper; Bruker Daltonics, Bremen, Germany). Toxin production of strains isolated on Columbia blood-agar (bioMérieux, Marcy l'Etoile, France) was tested using a Toxin A+B ELFA (Enzyme Linked Fluorescent Assay) test (Vidas, bioMérieux). All isolates were ribotyped and tested for genes encoding for toxin A, toxin B, and binary toxin, as described elsewhere [7]. Isolates belonging to ribotype 027 were further subtyped by multilocus variable-number tandem repeat analysis (MLVA), as described by van den Berg *et al.* [8].

The MLVA repeat numbers were analysed using BioNumerics software version 6.6 (Applied Maths, Brussels, Belgium) and by using the unweighted pair-group method with arithmetic mean (UPGMA) with arithmetic averages with the categorical similarity coefficient. All markers were given an equal weight, irrespective of the number of repeats. Strains were considered to be related when they had an equal number of repeats in six out of seven markers (tolerance = 1). The minimum-spanning-tree analysis of the MLVA data from was done using two priority rules (1-locus variants with a weight of 10000 and 2-locus variants with weight of 10) nodes closer than 1 were put into the same partition.

Results

Between 1 March 2011 and 1 March 2012, 200 patients were diagnosed as defined or possible CDI. In accordance with SHEA & IDSA definitions, for 64 CDI cases the origin could not be determined or was unknown. Of the remaining 136 cases with known medical history, 114 cases were considered healthcare-associated CDI (83.8%) and 22 cases were community-associated (16.2%) [10]. Among 136 patients with healthcare-associated CDI, 130 patients (95.6%) were previously admitted in 17 other hospitals or nursing homes from the metropolitan area of Bucharest or surrounding counties.

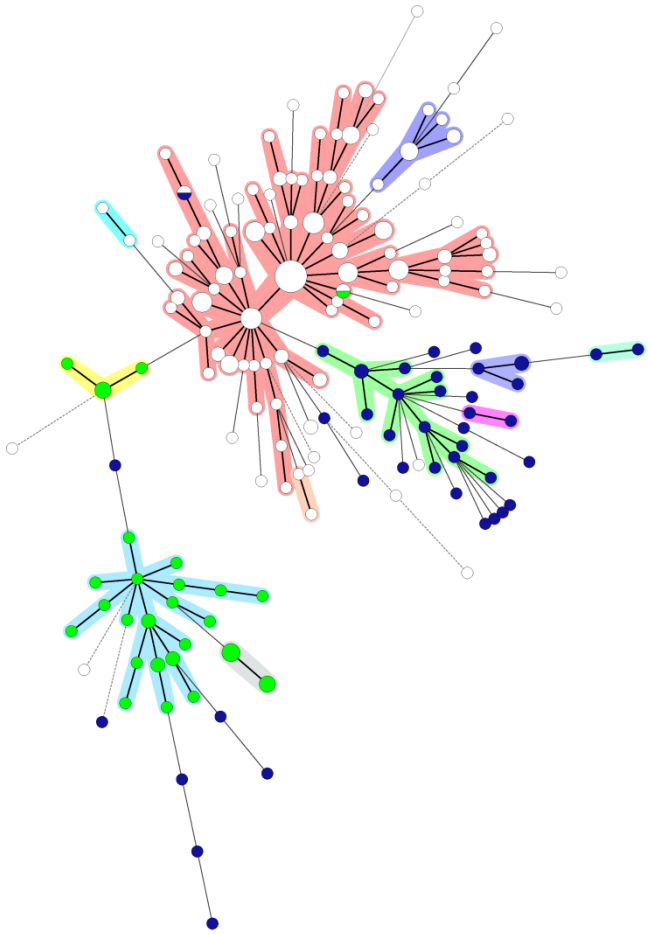
Stool cultures were performed for 157 patients and and yielded 81 non-duplicate *C. difficile* isolates. Of these, only 64 isolates could be sub-cultured and additionally tested at the Austrian Reference Centre

for *Clostridium difficile* in Vienna (Austria). This study includes data for these 64 patients from whom the 64 isolates were collected.

Demographic data were available for 58 of the 64 patients. These were aged between 23 and 88 years (mean: 63.4; median: 68.5), and 33 (56.9%) of them were female. Information on epidemiological case classification was available for 36 cases: 34 (94.4%) cases were healthcare-associated (two cases from nursing homes) and two cases were community-associated. Detailed history of antibiotic consumption was available for 34 patients. Thirty-three cases had received antimicrobial therapy in the three months prior to CDI diagnosis. Besides recent antibiotic administration, patients had other classical risk factors for CDI: 41 (70.7%) were aged 60 years and older and 36 (62%) had comorbidities, including carcinoma (solid tumours, hemato-oncological diseases) (n = 11), local and systemic inflammatory diseases (n = 13), cardiovascular diseases (stroke, myocardial infarction) (n = 7) and diabetes mellitus (n = 5).

Of the 64 *C. difficile*-isolates, all but one produced toxins. An isolate of PCR-RT 031 lacked the genes for toxin A, toxin B, and binary toxin. All 63 toxigenic strains harboured toxin A and toxin B; 44 (69%) isolates were also positive for binary toxin genes. Ribotype 027 was the dominant ribotype, accounting for 43 (68%) of the 63 toxigenic strains. The remaining 20 isolates were ribotype 018 (n = 9), 012 (n = 3), and one isolate each of: 014, 031, 081, 416, 433, 500, 507 and PR03035 (new ribotype). Pre-discharge hospital mortality was documented for 62 of the 64 patients and four patients (6.4%) deceased. Recurrence was documented for 11 (18.3%) of the 60 patients with information available. Among patients with ribotype 027 the mean age was 64.9 years (range 25-86 y) and was 59.6 years (range 23-88 y) for all other patients. MLVA subtyping of the 43 *C. difficile* isolates with ribotype 027 revealed clustering of the Romanian isolates**,** with clear differences in patterns compared to 177 ribotype 027-isolates from Austria and 36 from Italy (Figure 1).

Figure 1. Minimum spanning tree analysis of 270 *C. difficile* type 027 isolates by MLVA. Each circle represents either one unique isolate or more isolates that have identical MLVA types. Thick lines represent single-locus variants, thin lines represent double-locus variants, and the interrupted lines represent triple-locus variants between MLVA types. Grey shadings indicate portioning of groups of more the two single-locus variants.



Discussion

The global emergence of *C. difficile* infection (CDI) in the past decade followed highly-publicized *C. difficile* outbreaks in the United States and Canada that were associated with increased rates of disease recurrence and mortality [11-13]. These outbreaks were caused by a previously uncommon, fluoroquinolone-resistant *C. difficile* strain, genotyped as ribotype 027.

In our study, the PCR ribotype 027 was found to be the most dominant type, accounting for 68% of the Romanian isolates tested during this 12-month period. To the best of our knowledge, this is the first report on the occurrence of PCR ribotype 027 in Romania. A study performed in another region of Romania between 2008 and 2009 by the University of Târgu-Mureş found that *C. difficile* accounted for 22.7% of the positive stool culture results in patients with acute gastroenteritis but did not identify the PCR ribotype 027 [14].

Our data do not allow for an estimation of the CDI incidence in Romania. However, the surprisingly high percentage of PCR ribotype 027 could be an indication of a recent outbreak in Bucharest. Moreover, even if the recent increase in CDI incidence was initially due to an outbreak, the number of hospitals from which

these cases originated suggests endemic level of disease in Bucharest area.

There are several inherent limitations of our data due to certain special characteristics of Romanian health care system. Resource constrains hamper adequate laboratory diagnosis of CDI**.** The existence of a national network of Infectious Diseases Hospitals (especially in the bigger cities of Romania) which take care a lot of patients transferred with hospital-acquired infections (HAI) from other general hospitals, including patients with *Clostridium difficile* infection (CDI), hampers comparability with the situation in other countries. Second, there is no surveillance system for CDI in place in Romania and the incidence of the disease cannot be evaluated.

Conclusion

This study indicates two major epidemiological changes, of interest in a Europe with free circulation of patients: CDI is a serious problem with an emerging disease in Romania and the high level of 027 strains among *Clostridium difficile* isolates in Romania. Our study proved the occurrence of ribotype 027 in Eastern Europe. Despite some limitations of our study design, an ongoing outbreak of CDI due to ribotype 027 in the Bucharest area seems highly likely.

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References

1. Pepin J, Valiquette L, Alary ME, Villemure P, Pelletier A, Forget K, Pepin K, Chouinard D (2004) Clostridium difficile-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. CMAJ. 171: 466-472.
2. Zilberberg MD, Shorr AF, Kollef MH (2008) Increase in adult Clostridium difficile-related hospitalizations and case-fatality rate, United States, 2000-2005. Emerg Infect Dis. 14: 929-931.
3. Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, Frost E, McDonald LC (2005) Toxin production by an emerging strain of Clostridium difficile associated with outbreaks of severe disease in North America and Europe. Lancet. 366: 1079-1084.
4. Smith A (2005) Outbreak of Clostridium difficile infection in an English hospital linked to hypertoxin-producing strains in Canada and the US. Euro Surveill. 10(26):pii=2735. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=2735. Accessed 27 March 2013
5. Bauer MP, Notermans DW, van Benthem BH, Brazier JS, Wilcox MH, Rupnik M, Monnet DL, van Dissel JT, Kuijper EJ; ECDIS Study Group (2011) Clostridium difficile infection in Europe: a hospital-based survey. Lancet 377: 63-73.
6. Kuijper EJ, Barbut F, Brazier JS, Kleinkauf N, Eckmanns T, Lambert ML, Drudy D, Fitzpatrick F, Wiuff C, Brown DJ, Coia JE, Pituch H, Reichert P, Even J, Mossong J, Widmer AF, Olsen KE, Allerberger F, Notermans DW, Delmée M, Coignard B, Wilcox M, Patel B, Frei R, Nagy E, Bouza E, Marin M, Akerlund T, Virolainen-Julkunen A, Lyytikäinen O, Kotila S, Ingebretsen A, Smyth B, Rooney P, Poxton IR, Monnet DL (2008) Update of Clostridium difficile infection due to PCR ribotype 027 in Europe, 2008. Euro Surveill.13(31):pii=18942. Available at: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=18942. Accessed 27 march, 2013.
7. Indra A, Huhulescu S, Schneeweis M, Hasenberger P, Kernbichler S, Fiedler A, Wewalka G, Allerberger F, Kuijper EJ (2008) Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. J Med Microbiol 57: 1377-1382.
8. van den Berg R, Schaap I, Templeton KE, Klaassen CHW, Kuijper EJ (2007) Typing and Subtyping of *Clostridium difficile* Isolates by Using Multiple-Locus Variable-Number Tandem-Repeat Analysis J Clin Microbiol 45: 1024–1028.
9. Bauer MP, Kuijper EJ, van Dissel JT; European Society of Clinical Microbiology and Infectious Diseases (2009) European Society of Clinical Microbiology and Infectious Diseases (ESCMID): treatment guidance document for Clostridium difficile infection (CDI). Clin Microbiol Infect 15: 1067-1079.
10. Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, Pepin J, Wilcox MH; Society for HealthcareEpidemiology of America; InfectiousDiseases Society of America (2010) Clinical Practice Guidelines for Clostridium difficile Infection in Adults: 2010 Update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA). Infect Control Hosp Epidemiol 31: 431-455
11. McDonald LC, Killgore GE, Thompson A, Owens RC Jr, Kazakova SV, Sambol SP, Johnson S, Gerding DN (2005) An epidemic, toxin gene-variant strain of *Clostridium difficile*. N Engl J Med. 353: 2433-2441.
12. Muto CA, Pokrywka M, Shutt K, Mendelsohn AB, Nouri K, Posey K (2005) A large outbreak of *Clostridium difficile*-associated disease with an unexpected proportion of deaths and colectomies at a teaching hospital following increased fluoroquinolone use. Infect Control Hosp Epidemiol. 26: 273-280.
13. Pepin J, Valiquette L, Cossette B (2005) Mortality attributable to nosocomial *Clostridium difficile*-associated disease during an epidemic caused by a hypervirulent strain in Quebec. CMAJ. 173: 1037-1042.
14. Mare A, Szekely E, Man A, Toma F, Bilcă D, Ureche R (2009) Diagnosis of *Clostridium difficile* associated disease. Revista de Medicinăşi Farmacie – Orvosies Gyogyszereszeti Szemle55: 188-190.
15. Benea S, Popescu GA, Badicut I, Florea D, Petrache D, Gavriliu L, Rafila A (2012) *Clostridium difficile* infections hospitalized in Romanian Institute of Infectious Diseases during the first three months of 2012. Poster Presentation, 4th International C. difficile Symposium, 2012 September 20th to 22th, Bled, Slovenia.

16. Kuijper E, van Dissel J, Wilcox M (2007) *Clostridium difficile*: changing epidemiology and new treatment options. Curr Opin Infect Dis 20:376–383
17. Søes LM, Brock I, Persson S, Simonsen J, Pribil Olsen KE, Kemp M (2012) Clinical features of *Clostridium difficile* infection and molecular characterization of the isolated strains in a cohort of Danish hospitalized patients. Eur J Clin Microbiol Infect Dis 31: 185-192

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