Molecular Epidemiology of *Clostridium difficile* in a Tertiary Medical Center in Israel: Emergence of the Polymerase Chain Reaction Ribotype 027

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ABSTRACT: **Background:** The rate of infection with *Clostridium difficile* colitis and its associated mortality have been increasing in the last decade. The molecular epidemiology of *C. difficile* in Israel has not been studied.

Objectives: To screen for the existence of the 027 and 078 ribotypes and determine the longitudinal molecular epidemiology of the circulating clinical *C. difficile* isolates in a large hospital in central Israel.

Methods: Polymerase chain reaction (PCR) ribotyping was performed on *C. difficile* isolates obtained from hospitalized patients from November 2003 to May 2004 (first study period) and September 2009 (second study period). Isolates with PCR ribotype patterns, unlike those of the available reference strains (078 and 027), were labeled with letters. Forty-six isolates from the first study period and 20 from the second were analyzed.

Results: PCR strain typing of *C. difficile* isolates yielded approximately 26 unique ribotypes. During the first study period, ribotype A and B accounted for 30% and 28%, respectively, whereas ribotype E and K accounted for 6.5% for each. During the second study period, ribotypes A, E and K disappeared, and the incidence of ribotype B decreased from 28% to 15%. One isolate (1/20, 5%) emerged during the second period and was identified as ribotype 027. Moxifloxacin resistance was found in 93% of ribotype A isolates, 81% of the ribotype B group, and in 44% of other ribotypes.

Conclusions: The predominant ribotypes circulating in our institution were diverse and changing. This is the first report on the emergence of the 027 ribotype in Israel.

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Clostridium difficile-associated diarrhea is the most common nosocomial diarrhea in adults in the developed world [1]. Disease symptoms range from watery diarrhea to severe life-threatening pseudomembranous colitis. This infection is also recognized in Israel as a major cause of in-hospital morbidity and mortality [2]. The *Clostridium difficile* polymerase chain reaction ribotypes 027 and 078 have been increasingly recognized in Canada, the United States, The Netherlands, and other European countries as prevalent causes of severe cases of this disease [3-5]. Recently, we observed an increase in the prevalence and severity of *Clostridium difficile*-associated diarrhea cases in our institution. The molecular epidemiology of *C. difficile* in Israel has not been studied. The objectives of our study were to screen for the existence of the 027 and 078 ribotypes and determine the longitudinal molecular epidemiology of the circulating *C. difficile* isolates in a tertiary medical center in Israel during two study periods.

PATIENTS AND METHODS

The study sample included consecutive patients with *Clostridium difficile*-associated diarrhea hospitalized in our institution during two study periods. Patients hospitalized from November 2003 to May 2004 were enrolled in the first study period; patients hospitalized during September 2009 were enrolled in the second study period. *C. difficile* isolates from stool samples of patients in the first study period were stored at room temperature in Chopped Meat medium (HyLabs, Israel). Details of the patients' clinical characteristics and the antibiotic resistance of these isolates have been published previously [6]. *Clostridium difficile*-associated diarrhea was defined as diarrhea unattributable to any other cause and associated with positive *C. difficile* Toxin A/B. Diarrhea was defined as the passage of three or more unformed stools for at least two consecutive days [6]. Each patient was interviewed by one of the investigators, and data were entered into a questionnaire on demographics, underlying condi-

PCR = polymerase chain reaction

tions, previous drug and antibiotic therapy, and clinical findings.

TOXIN ASSAY

The enzyme immunoassay TOX A/B (Techlab Inc., Blacksbury, VA, USA) was used for toxin detection and performed according to the manufacturer's instructions. Stool samples were tested either on the day of collection or, if delayed, were stored at 4ºC overnight prior to testing the following day.

BACTERIAL CULTURE

Toxin A/B-positive stool specimens were treated for 1 hour with 95% ethanol [6]. The treated suspensions were cultured on tryptic soy agar + 5% sheep blood and incubated in anaerobic conditions at 37°C for 48 hours. *C. difficile* isolates were identified by colony morphology, Gram stain, API 20A and API ID 32A.

Antimicrobial susceptibilities of all isolates were tested by the disk diffusion method on Muller-Hinton agar + 5% sheep blood. In addition to the previously reported antimicrobial susceptibility to metronidazole, vancomycin, rifampicin, fusidic acid, doxycycline, and linezolid applied on 46 isolates from the first study period [6], all isolates from the two periods were tested for ciprofloxacin and moxifloxacin resistance. Isolates were considered sensitive if the zone of inhibition was greater than 35 mm. Minimum inhibitory concentration was determined by the Etest (AB Biodisk, Solona, Sweden) if disk diffusion was in the inhibition zone of less than 35 mm. If the results were inconclusive, the Etest was performed twice for each antibiotic.

BACTERIAL DNA EXTRACTION

Genomic DNA was extracted from isolated colonies using an *AccuPrep* genomic DNA extraction kit (Bioneer, Korea) according to the manufacturer's protocol.

PCR RIBOTYPING

PCR ribotyping was performed using the method described by Stubbs et al. [7] with slight modifications. Briefly, 40 ng of genomic DNA were added to the *AccuPower* HotStart PCR PreMix with 10 pmol of each primer CTGGGGTGAAGTCGTAACAAGG (positions 1445 to 1466 of the 16S rRNA gene) and GCGCCCTTTGTAGCTTGACC (positions 20 to 1 of the 23S rRNA gene). Reaction mixtures were subjected to 40 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 min and extending at 72°C for 1 min. Amplification products were run on a 3% high-resolution agarose gel, stained by ethidium bromide and photographed under ultraviolet light. Ribotype patterns were compared visually with *C. difficile* PCR ribotypes 027 and 078 (Control strains 027 and 078 were kindly provided by David Lyerly, from TECHLAB Inc., Blacksburg, VA, USA). To enable normalization of all ribotype patterns, a molecular size standard (100 base pairs, Bioneer, Korea) was run in parallel. Isolates with PCR ribotype patterns, unlike those of the available reference strains (027 and 078), were labeled with letters.

PCR FOR TOXIN TYPING

Toxigenicity of *C. difficile* strains was performed by PCR amplification of *tcdA* and *tcdB* as described in previous studies. The segment of the toxin B gene was amplified by nested PCR for the *tcdB* gene as previously described [8,9]. The *tcdC* gene was PCR-amplified with primers flanking the 18 base-pair deletions, associated with type 027 [10]. The specificity of the PCR products of amplified toxins was confirmed by sequencing using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Inc.) on the ABI PRISM 3730 Genetic Analyzer. Obtained sequences were aligned and compared with archived NCBI sequences.

For statistical analysis, dichotomous variables were compared using the chi-square test. Continuous variables were compared using the Mann-Whitney or *t*-test as appropriate. Statistical analysis was performed using the SPSS software.

RESULTS

Overall, 66 *C. difficile* patient unique isolates were analyzed: 46 from the first study period and 20 from the second. No significant differences between patients of the two groups were found regarding demographics, residence, and use of antibiotics, proton-pump inhibitors, H_2-blockers and corticosteroids prior to the *Clostridium difficile*-associated diarrhea episode. Underlying conditions were comparable between the two groups, except for chronic renal failure and malignancy, which were more prevalent among patients from the second study period (45% vs. 15%, and 40% vs. 15%, $P = 0.014$ and $P = 0.05$ respectively). Previous abdominal surgery was reported in 26% of patients from the first study period and 0% in the second ($P = 0.013$). Fever was observed more frequently in the first group, while abdominal pain was observed more frequently in the second, 67% vs. 25%, and 30% vs. 9%, $P = 0.001$ and $P = 0.03$ respectively.

PCR strain typing of *C. difficile* isolates yielded 26 unique ribotypes. The most prevalent ribotypes were: ribotype B found in 16 isolates (24.2%), ribotype A in 14 (21.2%), followed by ribotypes E and K (4.5%) for each [Figure 1]. During the first study period, ribotype A and B were the most dominant and accounted for 30% and 28% of all isolates, respectively. Ribotype E and K accounted for 6.5% of each isolate. During the second study period, ribotypes A, E and K disappeared, and the prevalence of ribotype B decreased from 28% to 15% [Figure 1]. None of the isolates was found to be compatible with the 078 ribotype pattern. We found one isolate (1/20, 5%) containing an 18 bp deletion in *tcdC* that

Figure 1. Distribution of the *C. difficile* ribotypes (in columns) in the entire study group (total), and during two study periods (first period 2003–2004, second study period 2009)

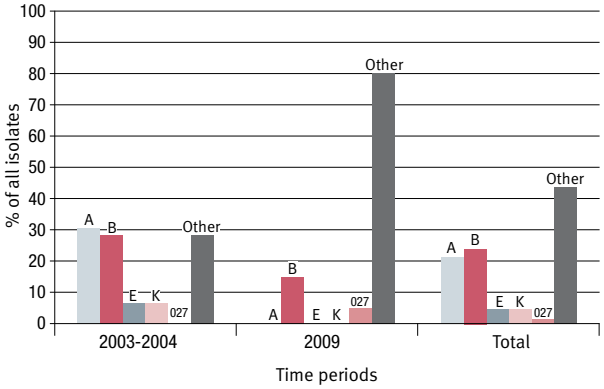
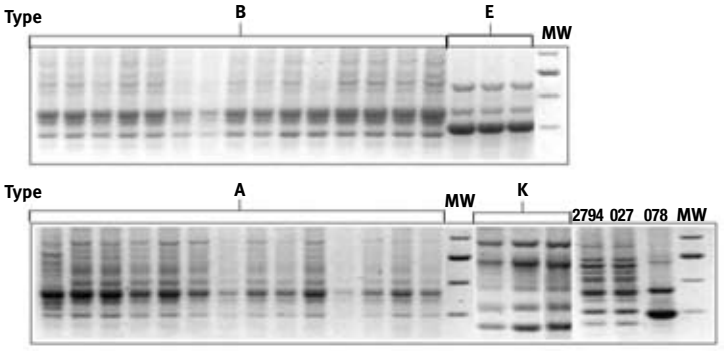


Figure 2. PCR ribotyping of *C. difficile* isolate representative groups. Isolates with PCR ribotype patterns different from the available reference strains 027 and 078 were labeled with consecutive letters type A, B, E and K. Isolate 2794 represents an identical pattern to 027. Molecular weight (MW) 100 bp molecular weight marker



corresponded to that previously described in ribotype 027 [Figures 1 & 2]. This strain was isolated from a 90 year old man, born in Israel, with no recent travel history, residing in a nursing home. His medical history included chronic atrial fibrillation, moderate left ventricular dysfunction, hyperthyroidism and recurrent urinary tract infections. On 23 September 2009, he was hospitalized in another department due to a fall and head trauma. Brain computed tomography confirmed multiple subarachnoid and subdural hemorrhages. During hospitalization, the patient contracted pneumonia and received antibiotic treatment with piperacillin/tazobactam. Several days after initiation of treatment, the patient became febrile and had diarrhea. Treatment with piperacillin/tazobactam was discontinued and his stool was sent for a *C. difficile* toxin enzyme-linked immunosorbent assay test. Concomitantly, the patient received treatment with metronidazole. The toxin test returned positive and the patient continued treatment for 14 days. Stool culture was positive for *C. difficile* resistant to both ciprofloxacin and moxifloxacin. Diarrhea resolved after treatment and no further complications occurred relating to the *C. difficile* infection.

All isolates were positive for both *tcdA* and *tcdB* genes, except the K ribotype isolates, which were all toxin A-negative and B-positive. Resistance to moxifloxacin and ciprofloxacin was observed in 36 (54.5%) and 46 (69.7%) isolates, respectively. Moxifloxacin resistance was found in 93% of ribotype A isolates, 81% of the ribotype B group, and in 44% of other ribotypes.

DISCUSSION

The molecular epidemiology of *Clostridium difficile*-associated diarrhea in our institution is diverse and dynamic. We identified more than 26 different ribotypes of *C. difficile* characterized by a change in the predominant ribotypes over time. During the years 2003–2004, ribotype A and B accounted for 30% and 28%, respectively, followed by ribotype E and K, while in the second study period, ribotypes A, E and K disappeared, and the incidence of ribotype B decreased from 28% to 15%. More importantly, the most striking finding of our report was that for the first time in Israel the 027 ribotype emerged during the second study period.

According to recent reports [11], PCR ribotypes 014 and 002 are the most common types in Hungary, while in Poland, ribotype 017 dominates, followed by ribotypes 014 and 046 [12]. Ribotype 106 and 001 are the most common strains currently prevalent in England [13]. Outbreaks of the *C. difficile* ribotype 027 have appeared in France, Belgium, England, The Netherlands, and recently in Austria [14] and Denmark [15].

This ribotype continued to spread to other continents such as Australia, Asia and Central America in the last few years [16]. The 027 ribotype was linked to large outbreaks and is considered hypervirulent due to overproduction of toxins, production of the binary toxin, and increased sporulation [17]. However, other studies found no evidence for this ribotype's hypervirulence and questioned its relation to the increasing incidence and severity of *C. difficile*-associated colitis [18]. In our study, we identified ribotype 027 in 1/20 isolates (5%) from the second study period.

Increased use of newer quinolones is considered a major factor contributing to the increase in *Clostridium difficile*-associated diarrhea [15]. The rates of resistance of our isolates to fluoroquinolones were higher than those reported in the literature. Indra et al. [14] reported a 39% resistance to moxifloxacin among their tested isolates. The European Study Group on *C. difficile* recently reported that only 20% of *C. difficile* strains circulating in European hospitals were resistant to moxifloxacin [19].

Our findings should be interpreted with caution. This is a single-institution study, based on a small sample size of strains isolated during two short study periods. Large-scale prospec-

tive studies from different centers in the country are needed to assess the extent of the spread of the 027 ribotype and to confirm the changing predominant circulating ribotypes of *C. difficile* in the country. In addition, due to the small sample size, the clinical impact of the 027 ribotype and other ribotypes on the course of the disease cannot be assessed.

This is the first report on the molecular epidemiology of *C. difficile* in Israel. The predominant ribotypes circulating in our institution were diverse and changing. For the first time, the emergence of the 027 ribotype was documented in Israel. Further restriction of fluoroquinolones is needed.

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References

1. Drummond LJ, McCoubrey J, Smith DGE, Star JM, Poxton IR. Changes in sensitivity patterns to selected antibiotics in *Clostridium difficile* in geriatric in-patients over an 18-month period. *J Med Microbiol* 2003; 52: 259-63.
2. Zafrir B, Laor A, Bitterman H. Nonagenarians in internal medicine: characteristics, outcomes and predictors for in-hospital and post-discharge mortality. *IMAJ Isr Med Assoc J* 2010; 12 (1): 10-15.
3. Goorhuis A, van der KT, Vaessen N, et al. Spread and epidemiology of *Clostridium difficile* polymerase chain reaction ribotype 027/toxinotype III in The Netherlands. *Clin Infect Dis* 2007;45: 695-703.
4. Pepin J, Valiquette L, Cossette B. Mortality attributable to nosocomial *Clostridium difficile*-associated disease during an epidemic caused by a hypervirulent strain in Quebec. *CMAJ* 2005;173: 1037-42.
5. Kuijper EJ, Coignard B, Tull P. Emergence of *Clostridium difficile*-associated disease in North America and Europe. *Clin Microbiol Infect* 2006; 12 (Suppl 6): 2-18.
6. Bishara J, Bloch Y, Garty M, Behor J, Samra Z. Antimicrobial resistance of *Clostridium difficile* isolates in a tertiary medical center, Israel. *Diagn Microbiol Infect Dis* 2006; 54: 141-4.
7. Stubbs SLJ, Brazier JS, O'Neill GL, Duerden BI. 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* 1999; 37: 461-3.
8. Kato H, Yokoyama T, Kato H, Arakawa Y. Rapid and simple method for detecting the toxin B gene of *Clostridium difficile* in stool specimens by loop-mediated isothermal amplification. *J Clin Microbiol* 1998; 43: 6108-12.
9. Lemee L, Dhalluin A, Testelin S, et al. Multiplex PCR targeting tpi (triose phosphate isomerase), tcdA (toxin A), and tcdB (toxin B) genes for toxigenic culture of *Clostridium difficile. J Clin Microbiol* 2004; 42: 5710-14.
10. Spigaglia P, Mastrantonio P. Comparative analysis of *Clostridium difficile* clinical isolates belonging to different genetic lineages and time periods. *J Med Microbiol* 2004; 53: 1129-36.
11. Terhes G, Brazier JS, Urban E, Soki J, Nagy E. Distribution of *Clostridium difficile* PCR ribotypes in regions of Hungary. *J Med Microbiol* 2006; 55: 279-82.
12. Pituch H, Brazier JS, Obuch-Woszczatynski P, Wultanska D, Meisel-Mikolajczyk F, Luczak M. 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* 2006; 55: 207-13.
13. Brazier J, Patel B, Pearson A. Distribution of *Clostridium difficile* PCR ribotype 027 in British hospitals. *Eur Surveill* 2007; 12: E070426.2.
14. Indra A, Huhulescu S, Hasenberger P, et al. First isolation of *Clostridium difficile* PCR ribotype 027 in Austria. *Eur Surveill* 2006; 11: E060914.3.
15. Søes L, Mølbak K, Strøbaek S, et al. The emergence of *Clostridium difficile* PCR ribotype 027 in Denmark – a possible link with the increased consumption of fluoroquinolones and cephalosporins? *Eur Surveill* 2009; 14: 19176.
16. Clements AC, Magalhaes RJ, Tatem AJ, Paterson DL, Riley TV. *Clostridium difficile* PCR ribotype 027: assessing the risks of further worldwide spread. *Lancet Infect Dis* 2010; 10 (6): 395-404.
17. Deneve C, Janoir C, Poilane I, Fantinato C, Collignon A. New trends in Clostridium difficile virulence and pathogenesis. *Int J Antimicrob Agents* 2009; 33 (Suppl 1): S24-8.
18. Morgan OW, Rodrigues B, Elston T, et al. Clinical severity of *Clostridium difficile* PCR ribotype 027: a case-case study. *PLoS One* 2008; 3 (3): e1812.
19. Spigaglia P, Barbanti F, Mastrantonio P, et al. Fluoroquinolone resistance in *Clostridium difficile* isolates from a prospective study of *C. difficile* infections in Europe. *J Med Microbiol* 2008; 57 (Pt 6): 784-9.