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BRIEF REPORT

Clostridium difficile infection: monoclonal or polyclonal genesis?

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Abstract *Clostridium difficile* is considered to be a leading cause of hospital-acquired diarrhea. *C. difficile* (CDI) infection shows a high rate of recurrence. There would have to be a predominantly monoclonal mechanism of CDI within individual patients in order for molecular epidemiologic tools such as polymerase chain reaction (PCR) ribotyping to be useful in outbreak investigation or differentiation between infection relapse versus re-infection. It was the aim of our study to determine whether CDI is of monoclonal or of polyclonal genesis. Between December 2009 and June 2010, 11 patients with nosocomial CDI were chosen arbitrarily. Five individual colonies of *C. difficile* were picked from each of the primary culture plates. Of 55 isolates gained, 47 were available for PCR ribotyping (eight isolates failed attempts to re-culture). Among these 47 isolates, eight different PCR ribotypes were identified. Only one of the 11 patients had a stool sample that yielded more than one ribotype (PCR ribotypes 438 and 232); this 67-year-old female cancer patient was already suffering from recurring diarrhea prior to the fatal episode of colitis which was subsequently investigated. We conclude that polyclonal infections may occasionally occur in patients with CDI. Our findings of predominantly monoclonal origin of CDI within patients suggest that molecular epidemiologic investigations can be used reliably for outbreak investigations or discrimination between relapse and re-infection.

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Introduction

Clostridium difficile, a Gram-positive, anaerobic, sporogenic bacterium, is considered to be the leading cause of hospital-acquired and antibiotic-associated diarrhea [1, 2]. It colonizes the intestinal tract in up to 3% of healthy adults and in up to 80% of healthy newborns [3–5]. Clinical symptoms range from watery diarrhea to pseudomembranous colitis and toxic megacolon [1]. *C. difficile* infection (CDI) occurs mainly sporadically; however, outbreaks are not uncommon [6, 7]. The incidence and severity of CDI has been increasing since 2002 [8–12]. *C. difficile* strains of polymerase chain reaction (PCR) ribotypes 027 and 078 are known to cause more severe disease and are associated with higher morbidity, higher mortality, and an increased risk of relapse [12–16]. Recent studies also indicate an increase in the severe outcomes of community-acquired CDI [17–20].

van den Berg et al. [21] postulated that the coexistence of multiple PCR ribotypes of *C. difficile* in fecal samples limits the value of PCR ribotyping for epidemiologic studies. As patients often develop recurrence of CDI, using PCR ribotyping to differentiate between relapse versus re-infection is only feasible if CDI within an individual is usually monoclonal. It was the aim of this study to elucidate whether CDI is of monoclonal or polyclonal genesis.

Materials and methods

From December 2009 until June 2010, 11 patients who developed nosocomial CDI >48 h after admission to the 1,252-bed, tertiary care hospital in Salzburg, Austria, were chosen arbitrarily. During that time period, this institution was experiencing its usual baseline rate of CDI without any signs of clustering. Demographic data and information on clinical presentation and outcome were gained by clinical visits, chart reviews, and telephone calls. The study was presented to the local ethics committee; in view of the legal obligation for the surveillance of CDI, no formal approval was required.

Stool samples had been screened for *C. difficile* toxins A and B using enzyme-linked immunoassay (RIDASCREEN Clostridium difficile Toxin A/B ELISA, R-Biopharm AG, Darmstadt, Germany). Toxin-positive stool samples were spread onto cycloserine/cefoxitin agar plates (bioMérieux, Marcy l'Etoile, France) and incubated for 48 h at 35°C in an anaerobic atmosphere produced by AnaeroGen (Oxoid, Hampshire, England, GB). Five individual colonies from different areas of each primary agar plate ($n = 11$) were picked and subcultured. In pilot experiments, we often experienced difficulties to find more than five clearly distinct colonies per primary culture plate.

The 55 subcultured isolates were sent to the National Reference Centre for *Clostridium difficile* of the Austrian Agency for Health and Food Safety (AGES) in Vienna, Austria, using BBLPort-A-Cul-Tubes (BD Diagnostic Systems, Sparks, CA, USA). A total number of 47 isolates originating from 11 CDI patients were finally available (eight isolates failed attempts to reculture) (refer to Table 2).

Capillary gel electrophoresis-based PCR ribotyping and testing for toxin genes was performed in a blinded manner with primers as described elsewhere [22]. In brief, for PCR ribotyping, the 16S primer was labeled at the 5′ end with tetrachlorofluorescein. Twenty-five µl HotStar Taq Master Mix (Qiagen, Hilden, Germany) was used with 0.3 µl (10 pmol/µl) of each primer, 20.7 µl water, and 1.5 µl DNA. Amplification was done with a PCR thermocycler running a 15-min 95°C initial enzyme activation, 22 cycles of 1 min at 95°C for denaturation, 1 min at 57°C for annealing, 1 min at 72°C for elongation, and a 30-min 72°C final elongation step. PCR fragments were analyzed using an ABI 310 (Applied Biosystems, Foster City, CA, USA) genetic analyzer, with a 41-cm capillary loaded with a POP4 gel (Applied Biosystems, Foster City, CA, USA). A 50–625-bp TAMRA ladder (CHIMERx, Milwaukee, WI, USA) was used as an internal marker for each sample. Injection was done with 5 kV over 5 s, with a total running time of 28 min at the 15-kV run voltage. The size of each peak was determined by Peakscanner software 1.0 (Applied Biosystems, Foster City, CA, USA). To confirm the production of toxins A and B by the 47 toxigenic isolates, the Vidas *C. difficile* Toxin A & B (CDAB) assay (bioMérieux, Marcy l'Etoile, France) was utilized.

Results

Cultures for *C. difficile* were grown from the fecal samples of 11 CDI patients. The 11 patients had a median age of 67 years (range 25–90; mean age 68.7 years) and 4 (36.4%) were male. Symptoms of nosocomial CDI ranged from mild diarrhea to fatal pseudomembranous colitis. The patients' salient clinical features are summarized in Table 1.

The 47 isolates yielded eight different PCR ribotypes: Ribotype 053 (12 isolates, 25.5%) from three patients; Ribotype 001 (10 isolates, 1.3%) from two patients; Ribotype 018 (7 isolates,14.9%) from two patients; Ribotype 538 (5 isolates, 10.6%), Ribotype 002/2 (5 isolates,10.6%), Ribotype 438 (4 isolates,8.5%), Ribotype 014 (3 isolates, 6.4%), and Ribotype 232 (1 isolate, 2.1%) were found in only one patient each (Table 2).

Of the 11 patients, only one (9.1%) had more than one different PCR ribotype on her primary stool culture plate: four subcultures of patient 4 contained isolates that were PCR ribotype 438 and one subculture was PCR ribotype 232.

Four isolates (PCR ribotype 438) tested positive for toxin A, toxin B, and binary toxin. The other 43 isolates (92.5%) tested positive for toxin A plus toxin B only.

Discussion

Indra et al. [22] suggested PCR ribotyping to be a valuable tool for recognizing related cases of CDI in healthcare facilities. Also, McDonald et al. [9] postulated that the clustering of certain ribotypes in a healthcare setting should prompt an in-depth epidemiological investigation of a possible nosocomial outbreak and emphasized the need to develop a surveillance system for CDI. However, the molecular typing of *C. difficile* can support the detection and spread of clusters of CDI only if the pathogenesis is monoclonal. PCR ribotyping is also helpful in differentiating recurrence from re-infection in the individual patient only if polyclonal genesis is a relatively rare event.

There was controversy about the occurrence of the simultaneous existence of different strains in one fecal sample [21, 23]. While van den Berg et al. [21] described 23 patients with a first episode of CDI, of which two harbored two different PCR ribotypes within one fecal sample, another study group postulated monoclonal rather than

Table 1 Summarized data on patients and isolates from the primary stool culture plate available for polymerase chain reaction (PCR) ribotyping

Patient	Age	Sex	No. of available isolates ($n = 47$)	Underlying disease	CDI manifestation
1	61	M	4	Peripheral vascular disease	Watery diarrhea
2	78	M	5	CLL	Fatal colitis
3	71	F	5	Knee joint arthrosis	Watery diarrhea
4	67	F	5	Ovarian neoplasm	Fatal colitis after numerous episodes of diarrhea
5	86	F	5	Spontaneous sigma perforation	Colitis
6	60	F	3	Necrotizing fasciitis	Watery diarrhea
7	64	M	4	COPD + pleural empyema	Pseudomembranous colitis
8	67	M	3	Ulcerative colitis	Watery diarrhea
9	87	F	3	Pneumonia	Watery diarrhea
10	90	F	5	Pneumonia	Watery diarrhea
11	25	F	5	Peripartum hypertensive crisis + brain hemorrhage	Watery diarrhea

Table 2 Summarized results on the PCR ribotypes and toxin types

Patient	No. of isolates	PCR ribotype	Toxins
1	4	053	A + B
2	5	538	A + B
3	5	053	A + B
4	4	438	A + B + binary toxin
	1	232	A + B
5	5	002/2	A + B
6	3	014/0	A + B
7	4	018	A + B
8	3	053	A + B
9	3	018	A + B
10	5	001	A + B
11	5	001	A + B

polyclonal origin of CDI. To investigate the possibility of multiple-strain carriage, O'Neill et al. [24] assessed ten patients by taking ten colonies from a primary culture plate for restriction enzyme analysis (REA) typing. All isolates from each patient were indistinguishable from each other, which indicates that the carriage of multiple *C. difficile* strains is a rather rare event. Devlin et al. [23] also used REA for analyzing multiple isolates of *C. difficile* in a single stool sample of various patients and found that the REA patterns to be indistinguishable from each other for the individual patients.

Recurrences of CDI can either be a relapse caused by persisting *C. difficile* spores that are resistant to antibiotic therapy or a re-infection with a new strain. Without the subtyping of isolates, this differentiation is impossible to achieve. Recurrences occur in approximately 25% of CDI patients [25]. The persistence of *C. difficile* spores after the elimination of vegetative cells by standard antibiotic treatment regimens, i.e., genuine relapses, are supposed to be the dominant mechanism [17, 25]. Recurrence can also be due to the exogenous acquisition of a new strain (re-infection), which seems to be the reason in up to 50% of relapsed CDI cases [26]. Using REA, O'Neill et al. typed multiple isolates from ten patients with recurrent CDI and showed that less than half of those patients relapsed due to the same organism. Our patient 4, a 67-year-old female cancer patient, suffered from recurring diarrhea even before the fatal episode of colitis under study. The fact that this patient was the only one that harbored two different strains and was also the only one with proof of recurrent diarrhea is of special interest. Testing was only performed during the recurrent episode. We hypothesize that one of the two strains was present during the initial untested episode; the fatal episode could then be explained by a superinfection with a new strain. The other ten patients had a documented first manifestation of CDI. This phenomenon of double infection seems similar to what is known for, e.g., tuberculosis [27, 28]. We conclude that the possibility of double infections should be considered also in patients with CDI.

Dale Gerding recently patented the use of non-toxigenic *C. difficile* strains as a vaccine capable of preventing colonization and illness with toxigenic *C. difficile* (European Patent EP0952773), wherein the non-toxigenic strain "is administered after initiation of antibiotic treatment, in an amount sufficient to establish colonization of the gastrointestinal tract of a subject to prevent *C. difficile* associated disease" (Colonization is unlikely to occur if antibiotics have not been given, because the normal bacterial flora of the gastrointestinal tract can prevent colonization). This concept of protective colonization is highly dependent on a

predominantly monoclonal mechanism of CDI within individual patients.

PCR ribotyping is presently the dominant *C. difficile* typing method used in Europe. Multilocus variable number tandem repeat analysis (MLVA) has been shown to be more discriminatory than other typing methods [29, 30]. However, this method is clearly prone to over-discrimination: Tanner et al. investigated the presence of several subtypes of 027 isolates in the same fecal sample and found that 5 of 39 samples yielded at least one strain with a different MLVA pattern [31]. The fact that, in our study, *C. difficile* infections of all but one patient were caused by a monoclonal organism underscores the considerable potential of PCR ribotyping to support epidemiological outbreak investigation and to differentiate between relapse and re-infection.

Conflict of interest None.

References

1. Vonberg R-P, Kuijper EJ, Wilcox MH, Barbut F, Tüll P, Gastmeier P; European C difficile-Infection Control Group; European Centre for Disease Prevention and Control (ECDC), van den Broek PJ, Colville A, Coignard B, Daha T, Debast S, Duerden BI, van den Hof S, van der Kooi T, Maarleveld HJ, Nagy E, Notermans DW, O'Driscoll J, Patel B, Stone S, Wiuff C. Infection control measures to limit the spread of *Clostridium difficile*. Clin Microbiol Infect. 2008;14:2–20.
2. Bidet P, Lalande V, Salauze B, Burghoffer B, Avesani V, Delmée M, Rossier A, Barbut F, Petit J-C. Comparison of PCR-ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for typing *Clostridium difficile*. J Clin Microbiol. 2000;38:2484–7.
3. Al-Jumaili IJ, Shibley M, Lishman AH, Record CO. Incidence and origin of *Clostridium difficile* in neonates. J Clin Microbiol. 1984;19:77–8.
4. Ushijima H, Shinozaki T, Fujii R. Detection of *Clostridium difficile* enterotoxin in neonates by latex agglutination. Arch Dis Child. 1985;60:252–4.
5. Indra A, Schmid D, Huhulescu S, Hell M, Gattringer R, Hasenberger P, Fiedler A, Wewalka G, Allerberger F. Characterization of clinical *Clostridium difficile* isolates by PCR ribotyping and detection of toxin genes in Austria, 2006–2007. J Med Microbiol. 2008;57:702–8.
6. Indra A, Huhulescu S, Fiedler A, Kernbichler S, Blaschitz M, Allerberger F. Outbreak of *Clostridium difficile* 027 infection in Vienna, Austria 2008–2009. Eurosurveillance; 2009:14. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19186.
7. Kleinkauf N, Weiss B, Jansen A, Eckmanns T, Bornhofen B, Kühnen E, Weil H-P, Michels H. Confirmed cases and report of clusters of severe infections due to *Clostridium difficile* PCR ribotype 027 in Germany. Eurosurveillance; 2007;12. http://www.eurosurveillance.org/viewarticle.aspx?articleid=3307.
8. Vonberg R-P, Schwab F, Gastmeier P. *Clostridium difficile* in discharged inpatients, Germany. Emerg Infect Dis. 2007;13:179–80.
9. McDonald LC, Owings M, Jernigan DB. *Clostridium difficile* infection in patients discharged from US short-stay Hospitals, 1996–2003. Emerg Infect Dis. 2006;12:409–15.
10. Kuijper EJ, Coignard B, Tüll P; ESCMID Study Group for Clostridium difficile; EU Member States; European Centre for Disease Prevention and Control. Emergence of *Clostridium difficile*-associated disease in North America and Europe. Clin Microbiol Infect. 2006;12:2–18.
11. 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. Update of *Clostridium difficile* infection due to PCR ribotype 027 in Europe, 2008. Eurosurveillance; 2008:13. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=18942.
12. Hemminger J, Balada-Llasat JM, Raczkowski M, Buckosh M, Pancholi P. Two case reports of *Clostridium difficile* bacteremia, one with the epidemic NAP-1 strain. Infection. 2011; Apr 21. [Epub ahead of print].
13. Goorhuis A, van der Kooi T, Vaessen N, Dekker FW, van den Berg RJ, Harmanus C, van den Hof S, Notermans DW, Kuijper EJ. Spread and epidemiology of *Clostridium difficile* polymerase chain reaction ribotype 027/toxinotype III in The Netherlands. Clin Infect Dis. 2007;45:695–703.
14. Keel K, Brazier JS, Post KW, Weese S, Songer JG. Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. J Clin Microbiol. 2007;45:1963–4.
15. Goorhuis A, Bakker D, Corver J, Debast SB, Harmanus C, Notermans DW, Bergwerff AA, Dekker FW, Kuijper EJ. Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. Clin Infect Dis. 2008;47:1162–70.
16. Pépin J, Alary M-E, Valiquette L, Raiche E, Ruel J, Fulop K, Godin D, Bourassa C. Increasing risk of relapse after treatment of *Clostridium difficile* colitis in Quebec, Canada. Clin Infect Dis. 2005;40:1591–7.
17. Naggie S, Frederick J, Pien BC, Miller BA, Provenzale DT, Goldberg KC, Woods CW. Community-associated *Clostridium difficile* infection: experience of a veteran affairs medical center in southeastern USA. Infection. 2010;38:297–300.
18. Pépin 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.
19. Rupnik M. Is *Clostridium difficile*-associated infection a potentially zoonotic and foodborne disease? Clin Microbiol Infect. 2007;13:457–9.
20. Centers for Disease Control and Prevention (CDC). Severe *Clostridium difficile*-associated disease in populations previously at low risk—four states, 2005. MMWR. 2005;54:1201–5.
21. van den Berg RJ, Ameen HAA, Furusawa T, Claas ECJ, van der Vorm ER, Kuijper EJ. Coexistence of multiple PCR-ribotype strains of *Clostridium difficile* in faecal samples limits epidemiological studies. J Med Microbiol. 2005;54:173–9.
22. Indra A, Huhulescu S, Schneeweis M, Hasenberger P, Kernbichler S, Fiedler A, Wewalka G, Allerberger F, Kuijper EJ. Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. J Med Microbiol. 2008;57:1377–82.
23. Devlin HR, Au W, Foux L, Bradbury WC. Restriction endonuclease analysis of nosocomial isolates of *Clostridium difficile*. J Clin Microbiol. 1987;25:2168–72.
24. O'Neill GL, Beaman MH, Riley TV. Relapse versus reinfection with *Clostridium difficile*. Epidemiol Infect. 1991;107:627–35.

25. Bauer MP, van Dissel JT, Kuijper EJ. *Clostridium difficile*: controversies and approaches to management. Curr Opin Infect Dis. 2009;22:517–24.
26. Barbut F, Richard A, Hamadi K, Chomette V, Burghoffer B, Petit J-C. Epidemiology of recurrences or reinfections of *Clostridium difficile*-associated diarrhea. J Clin Microbiol. 2000;38:2386–8.
27. Pavlic M, Allerberger F, Dierich MP, Prodinger WM. Simultaneous infection with two drug-susceptible *Mycobacterium tuberculosis* strains in an immunocompetent host. J Clin Microbiol. 1999;37:4156–7.
28. Yeh RW, Hopewell PC, Daley CL. Simultaneous infection with two strains of *Mycobacterium tuberculosis* identified by restriction fragment length polymorphism analysis. Int J Tuberc Lung Dis 1999;3:537–9.
29. Killgore G, Thompson A, Johnson S, Brazier J, Kuijper EJ, Pepin J, Frost EH, Savelkoul P, Nicholson B, van den Berg RJ, Kato H, Sambol SP, Zukowski W, Woods C, Limbago B, Gerding DN, McDonald LC. Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. J Clin Microbiol. 2008;46:431–7.
30. Marsh JW, O'Leary MM, Shutt KA, Sambol SP, Johnson S, Gerding DN, Harrison LH. Multilocus variable-number tandem-repeat analysis and multilocus sequence typing reveal genetic relationships among *Clostridium difficile* isolates genotyped by restriction endonuclease analysis. J Clin Microbiol. 2010;48:412–8.
31. Tanner HE, Hardy KJ, Hawkey PM. Coexistence of multiple multilocus variable-number tandem-repeat analysis subtypes of *Clostridium difficile* PCR ribotype 027 strains within fecal specimens. J Clin Microbiol. 2010;48:985–7.