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An Epidemic, Toxin Gene–Variant Strain of *Clostridium difficile*

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ABSTRACT

BACKGROUND

Recent reports suggest that the rate and severity of *Clostridium difficile*–associated disease in the United States are increasing and that the increase may be associated with the emergence of a new strain of *C. difficile* with increased virulence, resistance, or both.

METHODS

A total of 187 *C. difficile* isolates were collected from eight health care facilities in six states (Georgia, Illinois, Maine, New Jersey, Oregon, and Pennsylvania) in which outbreaks of *C. difficile*–associated disease had occurred between 2000 and 2003. The isolates were characterized by restriction-endonuclease analysis (REA), pulsed-field gel electrophoresis (PFGE), and toxinotyping, and the results were compared with those from a database of more than 6000 isolates obtained before 2001. The polymerase chain reaction was used to detect the recently described binary toxin CDT and a deletion in the pathogenicity locus gene, *tcdC*, that might result in increased production of toxins A and B.

RESULTS

Isolates that belonged to one REA group (BI) and had the same PFGE type (NAP1) were identified in specimens collected from patients at all eight facilities and accounted for at least half of the isolates from five facilities. REA group BI, which was first identified in 1984, was uncommon among isolates from the historic database (14 cases). Both historic and current (obtained since 2001) BI/NAP1 isolates were of toxinotype III, were positive for the binary toxin CDT, and contained an 18-bp *tcdC* deletion. Resistance to gatifloxacin and moxifloxacin was more common in current BI/NAP1 isolates than in non-BI/NAP1 isolates (100 percent vs. 42 percent, $P < 0.001$), whereas the rate of resistance to clindamycin was the same in the two groups (79 percent). All of the current but none of the historic BI/NAP1 isolates were resistant to gatifloxacin and moxifloxacin ($P < 0.001$).

CONCLUSIONS

A previously uncommon strain of *C. difficile* with variations in toxin genes has become more resistant to fluoroquinolones and has emerged as a cause of geographically dispersed outbreaks of *C. difficile*–associated disease.

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CLOSTRIDIUM DIFFICILE IS A GRAM-positive, anaerobic, spore-forming bacillus that can cause pseudomembranous colitis and other *C. difficile*-associated diseases. Studies during the 1970s showed that two toxins, A and B, were involved in the pathogenesis of *C. difficile*-associated disease.¹⁻⁵ Transmission occurs primarily in health care facilities, where exposure to antimicrobial drugs (the major risk factor for *C. difficile*-associated disease) and environmental contamination by *C. difficile* spores are more common.⁶ Certain strains of *C. difficile* have a propensity to cause outbreaks, including multistate outbreaks in health care facilities.⁷ Because these outbreak-associated strains are resistant to certain antimicrobial agents, such as clindamycin, the use of such antimicrobial agents provides these strains with a selective advantage over strains that are not associated with outbreaks. Historically low rates of severe disease and death (3 percent or less) may have led to an underestimation of the importance of *C. difficile*-associated disease as a health care-associated infection⁸; however, each case of *C. difficile*-associated disease has been estimated to result in more than \$3,600 in excess health care costs, and these costs may exceed \$1 billion annually in the United States.⁹

Both the rate and the severity of *C. difficile*-associated disease may be increasing in U.S. health care facilities. An analysis of data from the National Nosocomial Infections Surveillance system identified an upward slope in *C. difficile*-associated disease rates from the late 1980s through 2001.¹⁰ Of greater concern is a reported increase of 26 percentage points between 2000 and 2001 in the proportion of patients discharged from nonfederal U.S. hospitals with *C. difficile*-associated disease listed as a diagnosis.¹¹

Indications of the increased severity of *C. difficile*-associated disease include reports from the University of Pittsburgh Medical Center, where the incidence of the disease in 2000 and 2001 was nearly twice as high as in 1990 through 1999. Twenty-six patients with severe disease required colectomy, and 18 patients died.¹²⁻¹⁴ In addition, in the past two years, the Centers for Disease Control and Prevention (CDC) has received an increased number of reports from health care facilities of cases of severe *C. difficile*-associated disease that have resulted in admissions to intensive care units, colectomies, and deaths. These reports have been confirmed by a nationwide survey of infectious-disease physicians in the Emerging Infections Network of the Infectious

Diseases Society of America, which found that approximately 39 percent of respondents noted an increase in the severity of cases of *C. difficile*-associated disease in their patient population.¹⁵

One explanation for an increase in both the rate and the severity of *C. difficile*-associated disease could be the emergence of an epidemic strain with increased virulence, antimicrobial resistance, or both. To examine this possibility, we characterized *C. difficile* isolates obtained from health care facilities that reported outbreaks from 2001 through 2003 and compared these isolates with historic isolates (obtained before 2001) with the use of strain typing, identification of genetic determinants of newly described virulence factors, and testing for antimicrobial susceptibility.

METHODS

HEALTH CARE FACILITIES AND ISOLATES FROM PATIENTS

Isolates were collected from patients in eight health care facilities that had reported an outbreak of *C. difficile*-associated disease since 2001 to investigators at either the CDC or the Hines Veterans Affairs (VA) Hospital. These facilities were located in six states (Georgia, Illinois, Maine, New Jersey, Oregon, and Pennsylvania); all were acute care hospitals, except for one long-term care facility in Georgia that was associated with a VA hospital.¹⁶ The isolates were obtained from patients who had received a diagnosis of *C. difficile*-associated disease on the basis of clinical history (e.g., diarrhea with recent receipt of an antimicrobial drug) and a positive clinical laboratory test for *C. difficile* toxin (e.g., cytotoxin assay or enzyme immunoassay). Isolates from current (since 2001) outbreaks were compared with isolates from a historic (pre-2001) database of more than 6000 *C. difficile* isolates maintained by Hines VA investigators. The isolates in the historic database were collected during the period from 1984 through 1990; all isolates were extensively characterized by *Hind*III restriction-endonuclease analysis (REA) and linked to clinical and epidemiologic data.

STRAIN TYPING

The isolates underwent REA typing and pulsed-field gel electrophoresis (PFGE), as previously described^{17,18}; software from BioNumerics 3.5 (Applied Maths) was used to perform dendrographic analysis of the PFGE results. In addition, toxino-

typing was performed according to the method of Rupnik et al., with modifications.¹⁹ Toxinotyping analyzes the restriction-fragment-length polymorphisms (RFLPs) of the genes encoding toxins A (*tcdA*) and B (*tcdB*), the surrounding regulatory genes (*tcdC* and *tcdD*), and a porin gene (*tcdE*) in a region of the *C. difficile* genome known as the pathogenicity locus (PaLoc) (Fig. 1). Because RFLP analysis of polymerase-chain-reaction (PCR) fragments A3 and B1 results in a pattern sufficient to identify most toxinotypes,¹⁹ we limited our analysis to these two fragments.

MOLECULAR MARKERS OF POTENTIALLY INCREASED VIRULENCE

In addition to the well-characterized A and B toxins, a binary toxin has been identified in about 6 percent of clinical *C. difficile* isolates obtained in the United States and Europe.^{20,21} The structure and function of this toxin (referred to as binary toxin CDT) are similar to those of other binary toxins, such as the iota toxin found in *C. perfringens*, and it is a suspected virulence factor in strains of *C. difficile* that carry the toxin.²² We detected the *C. difficile* binary toxin gene by using PCR for *cdtB*, which is located outside the PaLoc and encodes the beta subunit of the binary toxin (Fig. 1).²⁰

We also looked for deletions in *tcdC* by using PCR with the primers *tcdc1* and *tcdc2*, which were synthesized at the CDC Core Facility on the basis of published sequences.²³ The gene *tcdC* is located within the PaLoc downstream from the genes encoding toxins A and B, and it is transcribed in the opposite direction from these genes (Fig. 1). The *tcdC* protein is thought to function as a negative regulator of the production of toxins A and B. Recently, multiple alleles of *tcdC* have been described that include different-sized deletions, point mutations, and in one case, a nonsense mutation, all of which would result in a truncated *tcdC* protein.^{23,24} It has been hypothesized that mutations in *tcdC* may result in a loss of negative regulatory function, leading to increased toxin production and virulence.^{23,24}

TESTING FOR ANTIMICROBIAL SUSCEPTIBILITY

Susceptibility to clindamycin and the fluoroquinolones (levofloxacin, gatifloxacin, and moxifloxacin) was determined with the use of E-test strips (AB Biodisk), and the results were interpreted according to standard criteria.²⁵ Specific breakpoints for the interpretation of clindamycin-susceptibility results were available from the Clinical and Laboratory

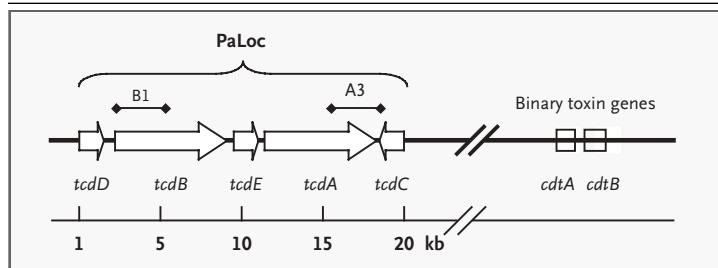


Figure 1. Major Genes in the Pathogenicity Locus (PaLoc) of *Clostridium difficile* and Relation to the Genes for Binary Toxin.

Genes *tcdA* and *tcdB* encode toxins A and B, respectively, whereas *tcdD* encodes a positive regulator of the production of toxins A and B. Gene *tcdE* encodes a protein that may be important for the release of toxin from the cell. Gene *tcdC* is a putative negative regulator of the production of toxins A and B. Genes *cdtA* and *cdtB* are located at an unknown distance from the PaLoc and encode the enzymatic and binding components, respectively, of binary toxin. B1 and A3 designate the location and relative size of the gene fragments that underwent polymerase-chain-reaction (PCR) amplification for toxinotyping.

Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards).²⁵ However, because no breakpoints have been set by the CLSI for *C. difficile* tested against these fluoroquinolones, the CLSI breakpoints for *C. difficile* tested against trovafloxacin were used. The validity of the trovafloxacin breakpoints was confirmed by identification of two distinct subpopulations in the distribution of minimum inhibitory concentrations (MICs) for apparently susceptible isolates, as compared with resistant isolates, tested against these fluoroquinolones; these subpopulations were demarcated by the trovafloxacin breakpoints. Quality control of antimicrobial-susceptibility testing was performed during each test run with the standard strains *Enterococcus faecalis* American Type Culture Collection (ATCC) 29212, *Pseudomonas aeruginosa* ATCC 27583, *Bacteroides fragilis* ATCC 25285, and *B. thetaiotaomicron* ATCC 29741.

STATISTICAL ANALYSIS

To compare the overall resistance patterns of current epidemic and nonepidemic isolates, a total of three (determined according to the availability of isolates) epidemic-strain (case) and three nonepidemic-strain (control) isolates, as determined by REA and PFGE, were randomly selected from each health care facility. Resistance was then compared by matched case-control analysis with the use of Epi Info software (version 6.02). This method was chosen to take into account possible geographic variation in resistance and to avoid bias resulting

from outbreaks with a larger number of isolates. In contrast, we used Fisher's exact test and the StatCalc function of Epi Info software (version 6.02) to make an unmatched comparison between current and historic epidemic isolates. All P values are based on a two-tailed comparison.

RESULTS

A total of 187 isolates were obtained from the eight health care facilities in which the outbreaks occurred. In each of the facilities, a strain composed of closely related isolates was identified by both PFGE and REA. This epidemic strain accounted for 50 percent or more of the isolates from five of the eight facilities (Table 1). The epidemic strain has been identified as belonging to REA group BI and North American PFGE type 1 (NAP1). Within this strain, characterized as BI/NAP1, the isolates have been further differentiated on the basis of minor differences in the band pattern into 14 REA subtypes, designated by numbers, in which at least 90 percent of the bands are identical.¹⁷ Similarly, several PFGE subtypes are included in the NAP1 designation. Five REA BI types (BI1 through BI5), dating back to 1984, were identified in the historic database. These represented 18 isolates obtained from 14 patients and consisted of 5 isolates of BI1 from 4 patients, 8 isolates of BI2 from 7 patients, 2 isolates of BI3 from 1 patient, 2 isolates of BI4 from 1 patient, and 1 isolate of BI5 from 1 patient.

One isolate from each of the five REA BI types in the historic database was selected for further ge-

netic testing, along with three BI/NAP1 and three non-BI/NAP1 current isolates from each health care facility. The PFGE results and the dendrogram of these representative isolates are shown in Figure 2, along with the toxinotype, the status of binary CDT, and the status of a deletion in the *tcdC* gene. According to dendrographic analysis, 25 of 29 of the combined current and historic BI/NAP1 isolates (86 percent) were 90 percent or more related, and all were more than 80 percent related. In contrast to this close relatedness among BI/NAP1 isolates across a wide geographic area, relatively few non-BI/NAP1 isolates were more than 80 percent related. All of the BI/NAP1 isolates were of toxinotype III, were positive for binary toxin CDT, and had an 18-bp deletion in *tcdC*; these features were largely absent among non-BI/NAP1 isolates (Fig. 2). Of the 24 non-BI/NAP1 isolates, 20 (83 percent) were toxinotype 0, none of which had binary toxin CDT or the *tcdC* deletion.

Susceptibility testing was performed on the 3 current BI/NAP1 and non-BI/NAP1 isolates from each health care facility, as well as on the 14 patient BI isolates available from the historic database. Among current isolates (obtained after 2000), all BI/NAP1 and only a fraction of the non-BI/NAP1 isolates were resistant to gatifloxacin and moxifloxacin (Table 2). Although both BI/NAP1 and non-BI/NAP1 isolates were largely resistant to clindamycin and levofloxacin, the MICs of levofloxacin were higher for BI/NAP1 isolates as a group (Fig. 3). All current BI/NAP1 isolates and no historic isolates (obtained before 2001) were resistant to gatifloxacin and moxifloxacin (Table 2).

Table 1. Isolates of *Clostridium difficile* According to Health Care Facility and the Proportion of Isolates Belonging to the BI/NAP1 Strain.

Health Care Facility	Date of Onset of Outbreak	No. of Isolates Tested	BI/NAP1 Strain no. (%)
Georgia	Oct. 2001	46	29 (63)
Illinois	July 2003	14	6 (43)
Maine, Facility A	March 2002	13	9 (69)
Maine, Facility B	July 2003	48	30 (62)
New Jersey	June 2003	12	9 (75)
Oregon*	April 2002	30	3 (10)
Pennsylvania, Facility A	2000–2001	18	7 (39)
Pennsylvania, Facility B	Oct. 2003	6	3 (50)
Total		187	96 (51)

* Isolates were not collected until after the peak of the outbreak.

DISCUSSION

An epidemic strain of *C. difficile* has been associated with outbreaks of *C. difficile*-associated disease in eight health care facilities since 2001. This strain is the same as the strain responsible for recent outbreaks outside the United States.^{26,27} It is classified by REA typing as BI and by PFGE as NAP1, and is distinct from the J strain (REA type J7/9) that was responsible for outbreaks during the period from 1989 through 1992.²⁸ Eighteen related isolates of the BI REA group, obtained from 14 known U.S. cases of *C. difficile*-associated disease that occurred between 1984 and 1993, were found in a database of more than 6000 isolates (representing more than 100 REA groups). According to PFGE dendrographic analysis, the majority of BI/NAP1 strain isolates

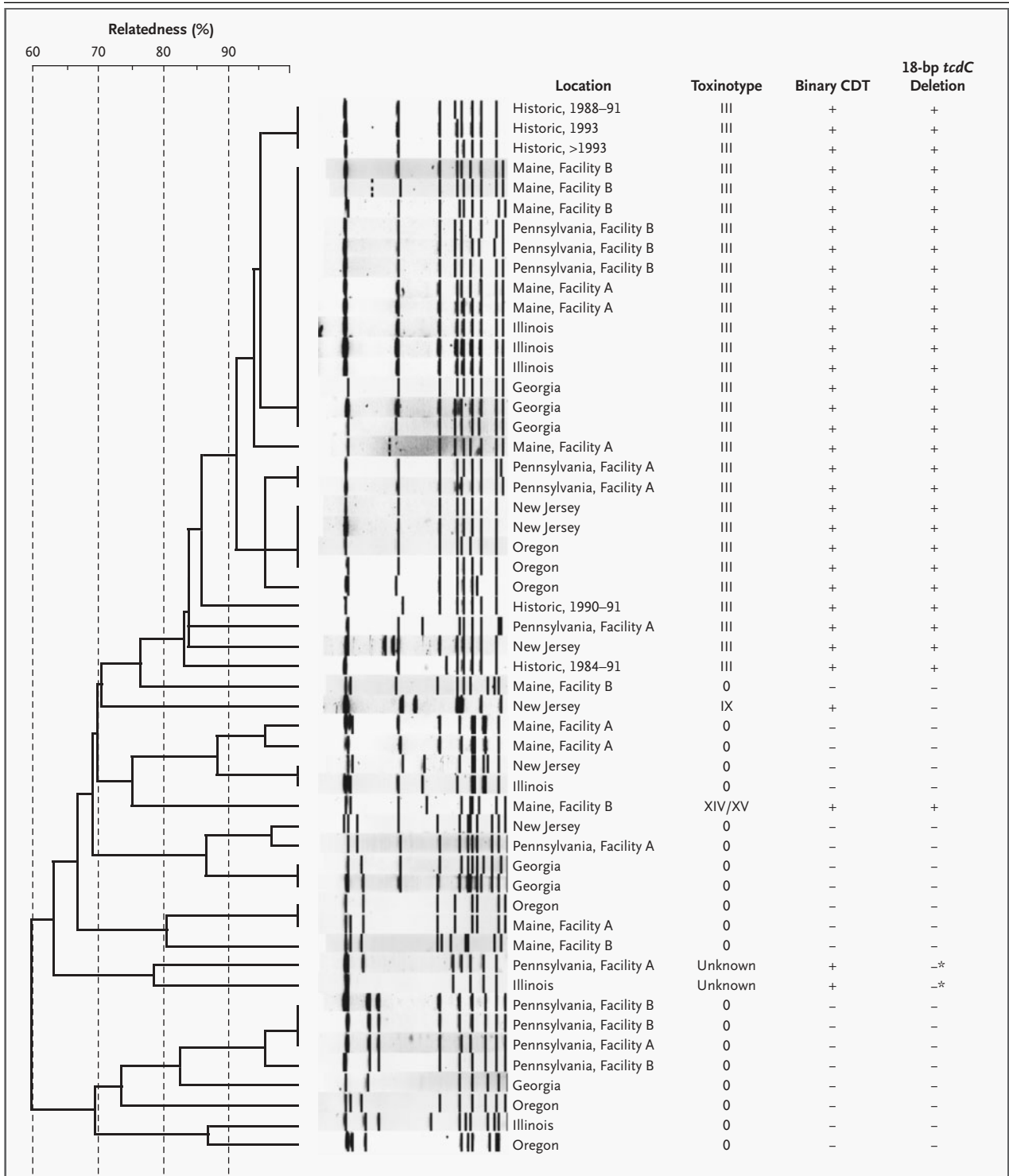


Figure 2. Pulsed-Field Gel Electrophoresis Results and Dendrographic Analysis of a Sample of BI/NAP1 and Non-BI/NAP1 Isolates from Current Outbreaks of *Clostridium difficile*-Associated Disease and of Isolates from a Historic Database.

The years listed for the historic isolates indicate years in which isolates of that type were recovered from patients, according to the database. The asterisk denotes the presence of a 39-bp deletion in *tcdC*.

Table 2. Resistance of Current BI/NAP1 *Clostridium difficile* Isolates, Current Non-BI/NAP1 Isolates, and Historic BI/NAP1 Isolates to Clindamycin and Fluoroquinolones.*

Antimicrobial Agent	Current BI/NAP1 Isolates (N=24) no. with intermediate resistance or resistant (%)§	Current Non-BI/NAP1 Isolates (N=24)	P Value†	Historic BI/NAP1 Isolates (N=14) no. with intermediate resistance or resistant (%)	P Value‡
Clindamycin	19 (79)	19 (79)	1.0	10 (71)	0.7
Levofloxacin	24 (100)	23 (96)	1.0	14 (100)	1.0
Gatifloxacin	24 (100)	10 (42)	<0.001	0	<0.001
Moxifloxacin	24 (100)	10 (42)	<0.001	0	<0.001

* The fluoroquinolones are levofloxacin, moxifloxacin, and gatifloxacin. Current BI/NAP1 isolates are those obtained since 2001, and historic BI/NAP1 isolates are those obtained before 2001.

† The P value is for the comparison between BI/NAP1 and non-BI/NAP1 isolates.

‡ The P value is for the comparison between current and historic BI/NAP1 isolates.

§ A minimal inhibitory concentration breakpoint of not more than 2 µg per milliliter was used for the definition of susceptibility, on the basis of the recommendations of the Clinical Laboratory Standards Institute for trovafloxacin.

(including historic BI isolates) were more than 90 percent related, and all were more than 80 percent related. Although current BI/NAP1 isolates shared with historic BI isolates the putative virulence factors of binary toxin and an 18-bp deletion in *tcdC*, the current isolates were more likely to be resistant to fluoroquinolones. Therefore, the increasing use of fluoroquinolones in U.S. health care facilities may have provided a selective advantage for this epidemic strain and promoted its widespread emergence.

The most compelling evidence of an increase in the severity of *C. difficile*-associated disease in the United States is found in the reports from Pennsylvania Facility A, where an increase in both the number of cases and the severity of the disease was noted in 2000 and 2001.¹²⁻¹⁴ In addition, there was evidence of higher white-cell counts and more severe disease in patients infected with BI/NAP1 strains than in those infected with non-BI/NAP1 strains at the Illinois facility in our study.²⁹ Another report from a Connecticut hospital indicates an increase in the number of cases of severe disease necessitating colectomy during a recent outbreak associated with the BI/NAP1 strain.³⁰ However, reports of other outbreaks, such as the outbreak in the Georgia long-term care facility included in our study, do not suggest increased disease severity.¹⁶ Even in the case of Pennsylvania Facility A, investigators were unable to find a significant association between the occurrence of severe *C. difficile*-associated disease and infection with the outbreak strain ($P=0.23$).¹⁴ Therefore, other factors, such as underlying host susceptibility, prevailing practices of the use of antimicrobial agents or approaches to the treatment of

C. difficile-associated disease, may have an important role in the causation of severe disease.

The importance of binary toxin CDT as a virulence factor in *C. difficile* has not been established; however, a similar toxin, iota toxin, is responsible for virulence in *C. perfringens*.²² In previous reports, binary toxin CDT was found in only about 6 percent of *C. difficile* isolates^{20,21,31}; therefore, our finding that the prevalence of this toxin is much higher in isolates from outbreaks associated with increased morbidity suggests that it could, indeed, affect the severity of *C. difficile*-associated disease. Previous studies have indicated that *C. difficile* strains with binary toxin CDT nearly always have polymorphisms in the PaLoc.²¹ Binary toxin CDT has been associated with several different toxinotype patterns³¹; in our isolates, it was associated with toxinotype III, which was infrequently found in previous clinical surveys. Pseudomembranous colitis is more frequent among patients infected with *C. difficile* of toxinotype III than among patients infected with *C. difficile* of other toxinotypes, suggesting that this toxinotype is associated with increased severity of the disease.^{19,21}

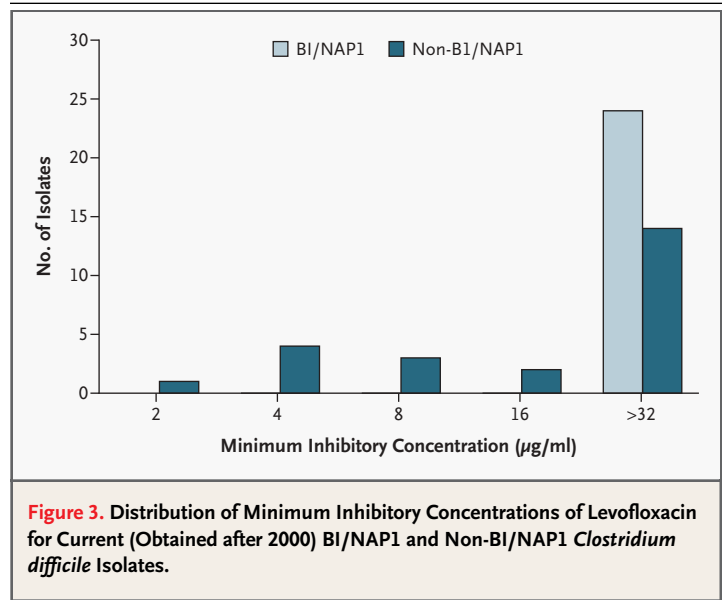
The importance of the 18-bp deletion in *tcdC* is currently unknown. Although *tcdC* is a proposed negative regulator of the production of toxins A and B, it is not known whether this 18-bp deletion would render a *tcdC* product nonfunctional and lead to increased production of toxins A and B.^{23,24} A recent report, however, indicates that BI/NAP1 isolates in vitro do, indeed, produce toxins A and B in considerably greater quantities and at higher rates than non-BI/NAP1 isolates.²⁷ Nonetheless,

additional research on the effects of binary toxin CDT and of *tcdC* deletions on the severity of *C. difficile*-associated disease appears warranted.

In addition to geographic variation in disease severity, there is variation in the role of particular fluoroquinolones as risk factors in these outbreaks. The outbreak in the Georgia long-term care facility occurred after a change in the formulary from levofloxacin to a C-8-methoxy fluoroquinolone, gatifloxacin.¹⁶ Gatifloxacin was an important risk factor for *C. difficile*-associated disease among patients, and the outbreak resolved after a formulary switch back to levofloxacin. The authors hypothesized that the higher antianaerobic activity of gatifloxacin than of levofloxacin led to a greater alteration in bowel flora and that this, combined with resistance to fluoroquinolone in the prevailing *C. difficile* strain, contributed to the outbreak.¹⁶

Similarly, in Pennsylvania Facility B, the outbreak started within three months after a switch in the formulary from levofloxacin to a C-8-methoxy fluoroquinolone (moxifloxacin); the preliminary results of a case-control study identify moxifloxacin as a risk factor for *C. difficile*-associated disease during the outbreak.³² In Pennsylvania Facility A, *C. difficile*-associated disease was associated with the use of levofloxacin, clindamycin, and ceftriaxone.¹³ However, a higher proportion of cases of *C. difficile*-associated disease was associated with levofloxacin (31 percent) than with clindamycin (10 percent) or ceftriaxone (7 percent).

The emergence of a previously uncommon strain of *C. difficile* that is more resistant and potentially more virulent than other strains indicates a need for inpatient health care facilities in North America to track the incidence of *C. difficile*-associated disease. Clinical outcomes of patients with *C. difficile*-associated disease should also be monitored, especially if an increase in rates is noted. If an increase in the proportion of severe cases is noted, special consideration should be given to the need for early diagnosis and treatment. Strict infection-control measures, including contact precautions, should be instituted for all patients with *C. difficile*-associated disease. In contact precautions, the patient is placed in a room alone or with another patient with *C. difficile*-associated disease, health care workers wear gloves and gowns when entering the room, and patient-care equipment (such as blood-pressure cuffs and stethoscopes) either is used only for the patient or is cleaned before it is used for another



patient.³³ Enhanced environmental cleaning with dilute bleach should be used to eliminate *C. difficile* spores.³⁴ Because alcohol is ineffective in killing *C. difficile* spores, it is prudent for health care workers to wash their hands with soap and water, rather than with alcohol-based waterless hand sanitizers, when caring for patients with *C. difficile*-associated disease during an outbreak.³⁵

Finally, an important method of controlling past outbreaks of *C. difficile*-associated disease has been restriction of the use of antimicrobial agents implicated as risk factors for the disease.³⁶ Whether a large-scale restriction of the use of these antimicrobial agents could slow the geographic spread of the BI/NAP1 strain is not known. Because fluoroquinolones have become a mainstay in the treatment of several common infections, a large-scale restriction of the use of these drugs would be quite difficult. However, if this epidemic strain continues to spread and to contribute to increased morbidity and mortality, it will be important either to reconsider the use of fluoroquinolones or to develop other innovative measures for controlling *C. difficile*-associated disease.

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