Isolation of a Toxin B–Deficient Mutant Strain of Clostridium difficile in a Case of Recurrent C. difficile–Associated Diarrhea


From the Department of Internal Medicine, Division of Infectious Diseases and Immunologic Diseases, University of California–Davis Medical Center, Sacramento, California

Clostridium difficile–associated diarrhea (CDAD) recurs in ~15%–20% of patients after discontinuation of metronidazole or vancomycin therapy. Most recurrences are believed to be endogenous relapses due to the persistence of spores. However, there is evidence that reinfection with a different strain is a cause of recurrence. We report the case of a patient with a history of multiple episodes of C. difficile colitis. The patient, a 56-year-old female, has had 5 years of repeated recurrences, each shortly after discontinuing vancomycin therapy. During the course of these episodes, three isolates were cultured from her stools at different times. These isolates were analyzed for the presence of toxin A and B gene sequences and genotyped by means of arbitrarily primed polymerase chain reaction (AP-PCR). The original two isolates contained the toxin A and B genes, as determined by PCR, and were of the same AP-PCR type. During her last relapse, a C. difficile strain lacking at least a portion of the toxin B gene was isolated. AP-PCR analysis of this isolate showed a different DNA banding pattern from that of the previous isolates. A vancomycin susceptibility assay revealed a slight decrease in vancomycin activity as compared with that against the prior isolate. This case demonstrates two unique features: (1) recurrent infections can be due to reinfections and (2) toxin B mutants can possibly cause CDAD. This study also raises concerns about long-term vancomycin use and the development of resistance of C. difficile to vancomycin.

Toxigenic Clostridium difficile is the most common cause of nosocomial diarrhea. At least two toxins are known to be responsible for the virulence of C. difficile: toxin A, an enterotoxin, and toxin B, a potent cytotoxin. These toxins act synergistically to cause extensive tissue damage and fluid accumulation [1]. Until recently it was thought that strains either have the genes for these toxins or lack them. A strain lacking at least part of the toxin A gene has been described and characterized [2]. This isolate, C. difficile 8864, has been found to be highly virulent in the animal model [3].

C. difficile–associated diarrhea (CDAD) recurs in 15%–20% of patients after discontinuation of vancomycin and metronidazole treatment. Until recently, it was believed that recurrences were endogenous in nature, due to the persistence of spores after eradication of the vegetative cells with therapy. In the past several years, reinfection with a new strain of C. difficile has been described as a cause of recurrent CDAD [4, 5]. O’Neill et al. studied 10 patients who had apparent relapses and found that more than half of the recurrences were due to infection with a different strain [4].

We report the case of a patient with a history of multiple CDAD recurrences, from whom two different strain types were isolated during the course of her recurrent episodes. Some genotypic characteristics of these isolates as well as their susceptibility to vancomycin are reported herein.

Case Report

A 56-year-old woman first had problems due to C. difficile in May 1989. She had received cefadroxil for the treatment of sinusitis. She had explosive watery diarrhea occurring up to 20 times per day. She was treated with vancomycin, and the diarrhea resolved but subsequent relapses occurred. The initial diagnosis was made on the basis of toxin assays and endoscopic proof of pseudomembranes. She had received vancomycin, metronidazole, bacitracin, cholestyramine, and oral lactobacillus, with relapses after initial response.

She was first seen at our center in 1990, at which time she was treated with vancomycin enemas. She was thought to have irritable bowel syndrome. She had been taking maintenance therapy with oral vancomycin at doses between 125 and 500 mg q.i.d. for years. In February 1995 she began having diarrhea again. The cytotoxin assay was once again positive for C. difficile toxin B. Since that time she has had intermittent episodes of diarrhea with C. difficile.

Materials and Methods

Isolation of C. difficile Strains

Over a period of 5 years, several stool specimens from this patient were cultured for C. difficile on the selective medium cycloserine-cefoxitin-fructose agar, as described elsewhere [6].
Isolates were identified as *C. difficile* by the latex agglutination test (Becton Dickinson, Cockeysville, MD). Isolates lacking the toxin B gene were further identified with the Analytical People Index Anaerobic Panel (Analytical Products, Plainview, NY).

**Amplification of the Toxin A and B Gene Sequences**

The presence of the toxin A and B genes was determined by PCR on all isolates. The primers and conditions for amplification of the toxin A and B genes are described elsewhere [7, 8]. In brief, the reaction mixtures were prepared in 1× PCR buffer (50 mM of KCl, 20 mM of Tris-HCl, 2.5 mM of MgCl₂, and 100 μg of bovine serum albumin per mL [pH, 8.4]) and contained 20 pmol of each of the primers, 0.1 mM each of deoxynucleoside triphosphates, 2 U of Taq polymerase, and 5 μL of the DNA preparation in each reaction.

**Genotyping of Isolates by Arbitrarily Primed PCR**

Arbitrarily primed PCR (AP-PCR) on three isolates obtained from this patient was performed with use of the arbitrary oligonucleotides T-7 and PG-05 as described previously. The DNA banding patterns of each isolate were compared by running PCR products on the same gel as described previously [9].

**Vancomycin Susceptibility**

*C. difficile* isolates were tested for susceptibility to vancomycin by means of the Etest (AB BIODISK, Solna, Sweden). The testing was performed as described for anaerobic organisms. *Streptococcus pneumoniae* ATCC 49619, incubated in 5%–10% CO₂ for ~24 hours, was used as a control organism.

**Results**

**C. difficile Isolates**

Three isolates were cultured from the stool of the patient during a 5-year period. The first two isolates (P-752 [1990] and P-760 [1993]), recovered 2½ years apart, contained the toxin A and B genes, as determined by PCR (figure 1). Table 1 lists some genotypic characteristics of these isolates. The DNA banding pattern of these isolates was identical (figure 2). Vancomycin susceptibility testing was performed on one of these isolates (P-760) and revealed that it was susceptible to vancomycin (MIC, 0.75 μg/mL).

An atypical *C. difficile* strain was isolated from this patient during her last relapse. The isolate (P-829 [1995]) lacked at least a portion of the toxin B gene, as determined by PCR.
Table 1. Characteristics of *Clostridium difficile* isolates recovered during different relapses of *C. difficile*-associated diarrhea in a 56-year-old woman.

<table>
<thead>
<tr>
<th>Test feature</th>
<th>Data per isolate (year of recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxin A in PCR</td>
<td>+</td>
</tr>
<tr>
<td>Toxin B in PCR</td>
<td>+</td>
</tr>
<tr>
<td>AP-PCR Type</td>
<td>A</td>
</tr>
<tr>
<td>Vancomycin susceptibility (MIC, μg/mL)</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE. AP-PCR = arbitrarily primed PCR; ND = not determined; + = positive; – = negative.

(table 1 and figure 1). Genotyping of this strain revealed a DNA banding pattern distinctly different from that of the previous two isolates (figure 2). In addition, this strain was found to be less susceptible to vancomycin (MIC, 2 μg/mL) than the earlier isolates.

Discussion

We report the isolation of two different *C. difficile* strains from a patient with multiple CDAD relapses. The first two strains, isolated 2 1/2 years apart, had the same AP-PCR genotype, suggesting that the nature of the relapse in this case was endogenous. These two isolates had toxin A and B gene sequences, as determined by PCR. The isolation of an atypical strain of *C. difficile* 2 years later is indicative of reinfection with a different isolate. However, it is also possible that both strain types were simultaneously present in this patient and that only the predominant strain type was isolated during culturing.

The clinical significance of a toxin B mutant strain isolated from this patient in a relapse episode is not known. However, the isolation of this strain after several purification passages in the selective medium indicates that the strain was present in high numbers. Even though we did not determine levels of toxin B in the stool specimen, we believe the presence of this strain in high density is significant and probably is the cause of the relapse in this patient.

It has been considered that toxin A is responsible for most of the intestinal damage observed in CDAD. However, Riegler et al. reported that toxin B is more important in the pathogenicity of *C. difficile* in humans, as implied by in vitro studies of toxicity and in human colonic cells in culture [10]. An isolate that lacks portions of the toxin A gene (*C. difficile* 8864) has been found to be more virulent than a toxigenic control strain. The toxin B of strain 8864 differed slightly from that of other toxigenic *C. difficile* strains.

The results obtained in this study demonstrate that recurrent *C. difficile* infections can be due to reinfection with a new strain. Isolate P-829, which lacks portions of the toxin B gene, is still virulent and can cause CDAD. This preliminary study suggests that long-term vancomycin use may result in resistance to vancomycin.

References