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Epidemiology of Recurrences or Reinfections of *Clostridium difficile*-Associated Diarrhea

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Approximately 15 to 35% of patients with a first episode of *Clostridium difficile*-associated diarrhea relapse within 2 months. Between 1994 and 1997, strains from 93 hospitalized patients with *C. difficile* recurrences were fingerprinted by using both serotyping and PCR-ribotyping. The results showed that 48.4% of clinical recurrences were, in fact, reinfections with a different strain of *C. difficile*. Rates of clinical recurrences could therefore be reduced by implementing strict isolation precautions.

Clostridium difficile is responsible for 15 to 25% of all cases of antibiotic-associated diarrhea and has been increasingly recognized as a major nosocomial pathogen (1, 2, 15, 16). Oral glycopeptides and metronidazole have been shown to be effective in the treatment of *C. difficile*-associated diseases, but symptomatic recurrences occur in 15 to 35% of cases (12, 20, 23). Recurrences of *C. difficile*-associated diarrhea are a serious, difficult, and still unsolved management problem, especially when patients have experienced three or more episodes, and they increase the length and overall cost of hospitalization (19). Physiopathology of recurrences (which is a clinical definition) may be explained either by the endogenous persistence of *C. difficile* spores or by the acquisition of a new strain from an exogenous source. Little is known about the relative frequency of each mechanism. Three reports have previously shown that 38 to 56% of recurrences of *C. difficile*-associated diseases were in fact due to reinfections (14, 18, 21). Nevertheless, these studies were conducted in one hospital where an endemic clone of *C. difficile* could be present and were based on small series of patients (10 to 27 participants) (14, 18). To better elucidate the mechanism of recurrences, strains isolated from 93 patients from 20 different hospitals presenting recurrences of *C. difficile*-associated diarrhea have been fingerprinted by using both serotyping and PCR ribotyping.

Between January 1994 and December 1997, the laboratory of Saint-Antoine hospital identified 93 patients with multiple recurrences of *C. difficile*-associated diarrhea. These patients were receiving treatment in 20 different hospitals, corresponding to 50 different clinical units. Diagnosis of *C. difficile*-associated diarrhea was based on a positive stool cytotoxicity assay or on the isolation of a toxigenic strain of *C. difficile*. Recurrences were defined as patients with resurgence of symptoms after cessation, at least 10 days after the first episode. Patients with positive repeat testing within 10 days were excluded.

Stools were plated on selective medium (taurocholate, cycloserine, cefoxitin agar) and were incubated in an anaerobic atmosphere for 48 h. *C. difficile* isolates were identified by colony morphology, Gram staining, odor, and Rapld 32A gallery (bioMérieux, La Balme-les-Grottes, France). Cytotoxicity assay was used to detect toxin B from stools. Briefly, fresh stool samples were diluted 1:10 in phosphate-buffered saline and were centrifuged at 2,500 × *g* for 30 min. The supernatants were filtered through a 0.45-μm-pore-size filter and were inoculated on MRC-5 cell monolayers and incubated at 37°C for 24 h. Specificity of the cytopathic effect was confirmed by seroneutralization with *Clostridium sordellii* antitoxin. Following culture of *C. difficile*, isolates were confirmed as being toxin B producers. Three to four colonies were inoculated into Trypticase yeast glucose broth (Diagnostics Pasteur, Marne-la-Coquette, France) and were incubated for 5 days under anaerobic conditions. The supernatant from this culture was inoculated onto MRC-5 cells as described above.

Serotyping was performed by an enzyme-linked immunosorbent assay by using 11 antisera corresponding to serogroups A1, A5, A8, A9, A10, C, D, F, G, H, and K according to the method described by Delmée et al. (10).

Strains were genotyped by using PCR ribotyping (4, 17). DNA was extracted from three large *C. difficile* colonies by the use of a Chelex resin-based commercial kit (InstaGene Matrix; Bio-Rad, Ivry, France) as recommended by the manufacturer. The primer sequences were 5′-GTG CGG CTG GAT CAC CTC CT-3′ (16S primer) and 5′-CCC TGC ACC CTT AAT AAC TTG ACC-3′ (23S primer) and corresponded to bases 1482 to 1501 of the 16S rRNA gene of *C. difficile* and bases 1 to 24 of the 23S rRNA gene of *C. difficile*, respectively. Amplification reactions were performed in a 100-μl volume containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dXTP (Pharmacia Biotech, Orsay, France), 50 pmol of each primer, 2.5 U of *Taq* polymerase (Pharmacia), and 10 μl of DNA extract (or distilled water as negative control). Amplifications were carried out in the thermal cycler (Perkin Elmer Cetus 480) for 1 cycle of 6 min at 94°C for denaturation, followed by 35 cycles (1 min at 94°C, 1 min at 57°C, and 1 min at 72°C) and a final extension of 7 min at 72°C. Amplification products were fractionated by electrophoresis through 3% Resophor agarose (Eurobio) during 6 h at 85 V in Tris-borate-EDTA with a distance of 24 cm between electrodes (3.5 V/cm) and were analyzed on a UV table after ethidium bromide staining.

Gel images were analyzed by Image Master software (Bio-Rad). Strains presenting at least one band of difference were assigned to separate groups.

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A total of 236 *C. difficile* strains isolated from 93 patients (48 females and 45 males) were studied. Ages of patients ranged from 1 to 96 years (59 ± 23 years). One, two, and more than two recurrences were observed in 57, 25, and 11 patients, respectively. Mean time elapsed between the first episode and the recurrence was 42 days (range, 10 to 211 days).

All the isolates were confirmed as being toxin producers. Serogroups most commonly found during the first episode of *C. difficile*-associated diarrhea were serogroups C (24.7%), H (17.2%), A (12.9%), K (11.8%), and G (8.6%). Strains isolated from the first episode and the recurrence belonged to two different serogroups in 21.5% of patients and to the same serogroup in 78.5% of cases. In this latter case, PCR-ribotyping was used to discriminate strains belonging to the same serogroup and showed a different pattern in 65.7% of cases. These results suggest that 45 of 93 (48.4%) clinical recurrences were in fact due to reinfections with a different strain. Delay of relapse and reinfection were 28 and 38 days (median), respectively. All the patients with reinfection, but only 20% of patients with relapse, were rehospitalized between the first and second episode of *C. difficile*-associated diarrhea. Patients with relapse had a shorter length of hospitalization between the first episode and the recurrence (median, 7 versus 20 days, respectively). Two patients presented relapse due to the same strain 6 months after the initial episode. We also observed two patients who presented an episode of reinfection followed by a second recurrence due to the initial strain.

Only scanty data are available on the mechanism of *C. difficile* recurrences. Two previous reports have shown that 38 to 50% of clinical recurrences were due to reinfection with a different strain (14, 18). Nevertheless, these results needed to be confirmed because they were based on a small number of patients (10 and 11) admitted in the same hospital, which may not be fully representative of others. More recently, Wilcox et al. (21) showed that 56% of recurrences were reinfections by using the random amplified polymorphic DNA method to fingerprint strains from 27 patients from six different hospitals. They showed, however, that an endemic clone of *C. difficile* accounted for 53% of all isolates, and they hypothesized that the frequency of reinfections was probably underestimated because of the reacquisition of the same strain from the hospital environment. Moreover, the random amplified polymorphic DNA method is known to lack reproducibility, which can hamper interpretation of fingerprints (11). Our results, based on a larger number of patients hospitalized in 20 different hospitals, corresponding to 50 different clinical wards, confirm that approximately half (48.4%) of clinical recurrences are in fact reinfections with a different strain. PCR ribotyping was used because this technique has been found to be easy and rapid to perform and highly discriminatory for *C. difficile* (4, 6, 9). We also showed that reinfections tend to occur later than relapses do and that patients could harbor the same strain for at least 6 months. Moreover, patients with reinfections spent more time in hospital than patients with relapses and were more frequently rehospitalized between their first episode and the recurrence. This observation confirms that length of hospital stay is a major risk factor for acquiring a new strain of *C. difficile* (16).

However, different biases should be pointed out. First, we cannot rule out the hypothesis of an elimination of the organism and a subsequent reinfection by the same organism: this situation may occur in units where *C. difficile* has become endemic and where a high incidence of recurrence is observed. This possible bias could be reduced in our series because patients were hospitalized in 50 different clinical units. Secondly, coinfection with two different strains could be possible, although this situation appears uncommon (5, 18, 21). This hypothesis is supported by the isolation, in two patients with multiple recurrences, of the same strain from the first and third episodes, whereas the isolate responsible for the second episode was different.

There is considerable interest to document the incidence of reinfections as opposed to relapses. Indeed, incidence of reinfections depends on the quality of infection control procedures such as handwashing, environmental decontamination, and enteric isolation. Contamination of the environment and persistence of the spores have been demonstrated and implicated in cross infections (7, 8, 13, 16, 22). The differences in environmental contamination could account for the different rates of recurrences reported from different institutions (3).

Our data support the yield of typing strains from patients with multiple recurrences of *C. difficile*-associated diarrhea in order to better determine the appropriate therapeutic strategy. These findings underline the need for culturing stool specimens to recover strains.

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