Molecular analysis of relapses or reinfections of *Clostridium difficile*-associated diarrhea

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SUMMARY

Recurrence is a major complication of *Clostridium difficile*-associated diarrhea and occurs in 15 to 20% of patients after discontinuation of therapy. Strains from 53 patients with *Clostridium difficile* recurrences were fingerprinted by PCR ribotyping. Reinfection with a different strain occurred in 15 out of 53 patients (28,3%), while 38 patients relapsed. These data suggest the need to perform molecular typing for implementation of infection control procedures and for a more appropriate therapeutic strategy.

KEY WORDS: Clostridium difficile, Recurrences, RAPD

Received February 21, 2011 *Accepted* May 05, 2011

Clostridium difficile is the most common cause of nosocomial antibiotic-associated diarrhea (Johnson and Gerding, 1998). Treatment with metronidazole and vancomycin has proved effective but approximately 15-20% of patients have recurrences (Wilcox and Spencer, 1992) that may occur in up to 42% of patients in high-risk groups such as the elderly (Borriello and Larson, 1981). Recurrences may be explained by endogenous persistence of *C. difficile* spores (relapse) or by the acquisition of a new strain from an exogenous source (reinfection).

This increases the costs of treatment, the risks of cross-infection and the duration of hospitalization. The aim of our study was to analyze *C. difficile* isolates obtained from patients with recurrent diarrhea to distinguish between relapse and reinfection.

A total of 130 *C. difficile* strains isolated from 53 patients presenting recurrence of *Clostridium difficile* infection (CDI) and collected over a period of 1 year (May 2009-April 2010) were included in this study (Table 1). A recurrence rate of 41% was found during the period of observation. Before CDI diagnosis, all patients were treated with proton pomp inhibitors (PPI) a known risk factor for recurrences (Cadle *et al.*, 2007). Multiple *C. difficile*-positive faeces (at least two) were obtained from each patient during hospitalization. These

TABLE 1 - *Epidemiology of recurrent C. difficile-associated diarrhea.*

Patient population	*No. of patients*	*Time range between recurrences*
Total	53	2-5 weeks
With relapse	38	
With reinfection	15	
Gender (M:F)	34:19	
Ward		
Cardiology	18	
Medicine	22	
Cancer	13	

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patients were inpatients at a long-term facility (ASP Istituti Milanesi Martinitt e Stelline e Pio Albergo Trivulzio, Milan; Italy). Recurrences were defined as patients with a resurgence of symptoms after cessation, at least 10 days after the first episode. All faecal specimens from patients suspected of having CDI were tested for toxin A/B using the enzyme-linked immunosorbent assay (ImmnunoCard Toxins A&B; Meridian) according to the manufacturer's instructions.

Toxins A and B positive stools were plated on selective cycloserine-cefoxitin-fructose agar plates (CLO agar; bioMérieux, Italy) and incubated in an anaerobic atmosphere for 48 h. Species identity was confirmed using an automatic system (Anaerobic Card-Vitek; bioMérieux, Italy). Strains were fingerprinted using PCR ribotyping (Bidet *et al*., 1999) and toxinotyping (Rupnik *et al*., 1997). DNA was extracted from a single *C. difficile* colony using a Chelex resin-based commercial kit (InstaGene Matrix; Bio-Rad) as recommended by the manufacturer.

For PCR ribotyping, amplification reactions were performed in a 50 µl volume containing 1x reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP (Sigma), 50 pmol of each primer, 1.25 U of *Taq* polymerase (Sigma) and 5 µl of DNA extract (or distilled water as negative control). After a denaturation of 5 min at 94°C, the DNA was amplified by 35 cycles of 1 min at 94°C, 1 min at 57°C, and 30 s at 72°C). At the end, samples were held at 72°C for 7 min.

Amplification products were electrophoresed in an 3% agarose (SeaKem LE; Lonza) and visualized under UV light following ethidium bromide staining. PCR ribotype profile analysis was carried out using BioNumerics software (version 4.0; Applied Maths). Strains were considered to be "indistinguishable" when showed ≥97% similarity and no banding differences. We compared the patterns with those obtained by the use of clinical strains with known ribotypes. For toxinotyping we followed the methods reported in the toxinotyping home page (http://www.mf.uni-mb.si/mikro/tox).

The determination of toxin genes were performed by PCR as previously described (Kato *et al.*, 1998) using 5 µl of crude DNA extract. Because the spread of hypervirulent *C. difficile* strains, ribotype 027 (Loo *et al*., 2005; McDonald *et al*., 2005) and 078 (Goorhuis *et al*., 2008), the presence of binary toxin was also investigated by PCR, as previously described (Stubbs *et al*., 2000).

Ages of patients ranged from 76 to 91 years (86±4 years).

All the isolates were confirmed as being toxin A and B producers, and to belong to toxinotype 0; no strain harboured binary-toxin gene.

During the period of study, the strains circulating in the wards of the long-term facility belonged to 5 different ribotypes; the most frequent type, ribotype 018, accounted for 78% of all the strains, followed by ribotype 002 (10%), ribotype 106 (8%), ribotype 014 (2%), and ribotype 001 (2%).

Fifteen of the 53 recurrences (28.3%) were due to reinfection with exogenous *C. difficile* strains, while 38 (71.7%) were relapses (Figure 1). Our results are in agreement with those obtained by Alonso *et al.* (2001).

Ribotypes found during the first episode of reinfection were: 018 (66.7%), 002 (20%), 106 (6.7%) and 014 (6.7%). Strains isolated from the next episodes belonged to ribotypes 018 and 004. In particolar ribotype 108 was found in 86.01% of patients.

For the relapses, 26 (68.5%) samples belonged to ribotype 018, four (10.6%) to ribotype 002, four to ribotype 106 (10.6%), two (5.3%) to ribotype

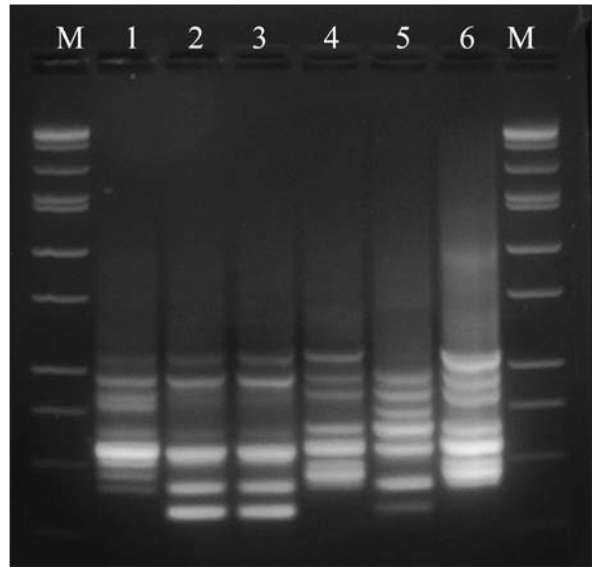

FIGURE 1 - *PCR ribotyping of selected C. difficile isolates from patients with recurrent CDAD. Lanes 1 and 4, and lines 5-6, DNA-banding patterns of isolates from two patients with recurrent CDAD due to reinfection; and lanes 2-3, DNA fingerprint of one patient with relapse.*

014, one (2.7%) to ribotype 186 and one (2.7%) to ribotype 001.

So, the patients from whom identical isolates were recovered for various disease episodes cannot conclusively be said to have undergone relapse due to an endogenous source of the organism or therapeutic failure. It is possible that these patients re-acquired *C. difficile* from the hospital environment (even without having isolated an associated environmental strain due to the absence of samples taken during the period under consideration), or through horizontal transmission. In fact, 17 of our patients with relapses harboured strains with the same pattern found in other patients of the same ward.

We therefore hypothesized that the frequency of reinfection was probably underestimated because of the reacquisition of the same strain from the hospital environment. Previous reports have shown that reinfections tend to occur later than relapses (Barbut *et al*., 2000; Johnson *et al*., 1989). Interestingly, all our cases of recurrences showed no significant delay of relapse and reinfection (Table 1). We hypothesized that in this category of patients, it was probably due to the concomitant presence of different risk factors: advanced age, long hospital stay, serious underlying diseases and continuous treatments with broad-spectrum antibiotics and PPI. In our experience, we can conclude that for recurrences, the time elapsed between two or more episodes is not sufficient to establish if they are reinfection or relapse. So the use of methods to distinguish relapses from re-infections seems essential to choose alternative drug regimens or to implement adequate control measures. For this purpose, appropriate genotyping methods are needed. Although Killgore *et al.* (2008) reported that PCR ribotyping is not able to recognize subtypes under primary types, we used this technique because is easy and rapid to perform (Bidet *et al*., 1999; Bidet *et al*., 2000; Stubbs *et al*., 1999) and shows excellent concordance and equal discriminatory ability with other *C. difficile* typing methods such as tandem repeat sequences analysis (Zaiß *et al*., 2009).

Our conclusion agree with Johnson *et al.* (Johnson *et al*., 1989) who reported that, in order to contain recurrent CDI, the documentation of reinfection as opposed to relapse is important because the incidence of reinfection may be reduced on the basis of several infection control procedures (Wilcox *et al*., 1998); a more rational use of antibiotics has also to be considered. The differences in environmental contamination could affect the different rates of recurrences reported from different institutions.

In conclusion, after our study, additional infection control measures have been reinforced, including an increase in environmental cleaning with chlorine, bedside tables and toilet seats treatment with sporicidal agents, strict regulations about handwashing of health care workers, and early isolation of patients with diarrhoeal illness. Furthermore, it has been established to avoid, when possible, the use of antibiotics shown to have a particular predisposition to cause CDI.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. C. Farina and Dr. G. Russello for their technical and scientific support. This work was supported by financial grant from University (PUR, Programma dell' Università per la Ricerca).

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