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Emergence in Gnotobiotic Mice of Nontoxinogenic Clones of *Clostridium difficile* from a Toxinogenic One

G. CORTHIER* AND M. C. MULLER

Laboratoire d'Ecologie Microbienne, Institut National de Recherche Agronomique, Centre de Recherches de Jouy-en-Josas, 78350 Jouy-en-Josas, France

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In previous studies, we showed that diet composition or *Saccharomyces boulardii* ingestion could protect gnotobiotic mice against lethal *Clostridium difficile* infection. Using an original method, we detected nontoxinogenic clones from feces of protected mice challenged with a toxinogenic clone of *C. difficile*. These clones became established at the same level as the toxinogenic one after about 30 days. In these protected mice bearing nontoxinogenic clones, no enterotoxin production could be detected and cytotoxin titers were highly reduced. These nontoxinogenic clones were genetically stable because nontoxinogenic clones and clones that produce intermediate levels of toxins in vivo did not revert to toxin production, even after repeated culture in vitro. Furthermore, the nontoxinogenic clones were shown to arise from a single toxinogenic clone and were identical to that clone in metabolic patterns and antibiotic sensitivity tests. When mice fed a nonprotective diet were challenged with a nontoxinogenic or intermediate clone, they remained healthy and no toxin production could be detected in their feces. Moreover, these mice were protected against further infections with toxinogenic strains of *C. difficile*, and a strong antagonism between nontoxinogenic and toxinogenic clones was observed.

It is well known that *Clostridium difficile* is involved in human pseudomembranous colitis, and in some cases of diarrhea it is associated with antibiotic treatment (1, 9, 12). The pathogenicity of the strains is related to their in vivo production of toxins (14, 18). Whereas *C. difficile* can be commonly isolated from children, the strains are generally nontoxinogenic and children rarely develop the disease (7, 11, 20). Such strains are also nonpathogenic in experimental animal models, and various studies using clindamycin-treated hamsters or gnotobiotic mice suggest that they have a protective effect against infection by toxinogenic strains (4, 22). The origin of nontoxinogenic strains has not been established, and it is important to determine whether they arise from toxinogenic strains or have entirely different biological properties.

In previous studies, we showed that different diets (15) or ingestion of *Saccharomyces boulardii* (2) could protect gnotobiotic mice against *C. difficile* infection. One week after the challenge, a protective diet could be changed to a nonprotective one or *S. boulardii* could be removed from the drinking water with no subsequent pathology. Mice remained healthy while associated only with a toxinogenic *C. difficile* strain.

The aim of this study was to determine whether nontoxinogenic clones may be derived from a toxinogenic one in gnotobiotic mice protected against *C. difficile* infection and whether the nontoxinogenic clones can protect mice against the disease.

MATERIALS AND METHODS

Bacterial strains, *S. boulardii* strain, and counts. Two toxinogenic strains of *C. difficile* were used: VPI (Virginia Polytechnic Institute strain 10463) and Mara (5, 15). They were cultivated and enumerated in brain heart infusion medium (BHI; Difco Laboratories, Detroit, Mich.). Continuous-flow culture was performed in an anaerobic chamber as described by Freter et al. (8). *S. boulardii* was kindly provided in an aqueous suspension by Biocodex Laboratories, Montrouge, France.

Enterotoxin assay. Enterotoxin quantitation was performed by an immunoenzymatic assay previously described (15). The enterotoxin concentration was expressed in $\log_{10}$ nanograms per gram of feces. The limit of detection was about 0.2 ng of pure enterotoxin per ml, but the limit of detection in feces was 5 ng/g (0.7 $\log_{10}$ ng/g) because dilutions are required before testing.

Cytotoxicity assay. Cytotoxin activity was measured in a Chinese hamster ovary cell line (CHO-K1) (3). Titers corresponded to the $\log_{10}$ of the highest feces dilution able to develop a cytotoxic effect in our test.

Method for rapid detection of toxinogenic and nontoxinogenic *C. difficile* clones. Membrane filters (Millipore Corp., Bedford, Mass.) were incubated with a solution of antienterotoxin pig pooled immunoglobulin (2 μg/ml in bicarbonate buffer, pH 9.8, 0.1 M) for 2 h at 4°C and then washed twice in phosphate (0.01 M, pH 7) saline (0.15 M NaCl) buffer containing 0.05% Tween 20 (PBSTw). After another incubation with newborn calf serum (5% in PBSTw) for 1 h at room temperature, followed by three washes in PBSTw, filters were ready for use. They could be kept dried for 1 week at room temperature. *C. difficile* colonies were enumerated on petri dishes in an anaerobic chamber with BHI agar as the culture medium. Treated filters were layered on plates containing 100 to 1,000 colonies and incubated for 2 h at room temperature. Filters were then washed four times in PBSTw and incubated overnight at 4°C with rabbit serum antienterotoxin prepared as described previously (15) and diluted 1/500 in PBSTw. After four washes in PBSTw, filters were incubated for 2 h at 37°C with anti-rabbit immunoglobulin coupled to peroxidase (Institut Pasteur Diagnostic, Ville d'Avray, France; diluted 1/500). After they were washed four times in PBSTw, diaminobenzidine (0.5 mg/ml) was layered on the filters and incubated for 15 min at room temperature in darkness. The filters were washed twice in distilled water, and the enzyme reaction was stopped by 0.1 M HCl. The filters were dried in darkness at 42°C for 30 min.

* Corresponding author.

TABLE 1. Experimental *C. difficile* infections with toxinogenic clones in gnotobiotic mice

Strain which produced the infecting clones	Mean (SEM) $\log_{10}$[a]:			No. of dead mice/total
	C. difficile count/g	Cytotoxin titer	ng of enterotoxin/g	
VPI	8.3 (0.3)	5.8 (0.3)	2.8 (0.1)	10/10
Mara	8.1 (0.2)	6.0 (0.2)	3.4 (0.2)[b]	10/10

[a] In mouse feces at 2 days postinfection.
[b] Different from strain VPI at $P < 0.02$.

Enterotoxin-producing colonies could be identified by a brown spot and enumerated. They represented enterotoxinogenic clones. The number of nontoxinogenic clones was obtained by counting colonies without brown coloration. Absence of toxin production was checked after subculture of these colonies in BHI liquid medium. The detection limits were one nontoxinogenic clone among 10^3 colonies and one toxinogenic clone among 10^5 colonies.

Characterization of *C. difficile* clones. Media containing 50 different carbohydrates (API, Montalieu Vercieu, France) were used for studying the carbohydrate fermentation patterns of *C. difficile* clones. Antibiotypes were determined by using the disk method (Institut Pasteur) in BHI agar medium. The antibiotics used and their concentrations (micrograms per disk) were as follows: clindamycin, 15; cefoxitin, 30; lincomycin, 15; tetracycline, 30; kanamycin, 30; lividomycin, 60; erythromycin, 15; cephalothin, 30; rifampin, 30; minocycline, 30; ampicillin, 25; gentamicin, 30; nalidixic acid, 30; bacitracin, 10; neomycin, 30; colistin, 50; vancomycin, 30; chloramphenicol, 30; tobramycin, 30. Fermentation patterns were determined by gas-liquid chromatography from cultures in TGY medium incubated for 48 h at 37°C (10).

Animals used and treatments protective against *C. difficile* infections. C3H/He axenic adult mice were reared in a Trexler-type isolator fitted with a rapid-transfer system (La Calhène, Velizy, France) and distributed into three groups. The control group was fed the commercial diet RO3-40 (UAR, Villemoisson, France) ad libitum. In the other two groups, animals received either a simplified semisynthetic diet for 2 weeks or *S. boulardii* for 4 days in their drinking water as previously described (2, 16). The semisynthetic diet consisted of casein (22%), cellulose (11%), corn oil (5%), corn starch (44%), and saccharose (11%). *C. difficile* challenges with VPI or Mara toxinogenic clones were performed by the intragastric route with 1 ml of a 24-h culture in BHI (10^8/ml). Feces were individually collected from surviving mice for enumerating viable toxinogenic and nontoxinogenic *C. difficile* clones and *S. boulardii* and for determining enterotoxin concentrations and cytotoxin titers.

Statistical analyses. Student's *t* test was used for comparison of mean values of $\log_{10}$ *C. difficile* counts, $\log_{10}$ cytotoxin titers, and $\log_{10}$ nanograms of enterotoxin per milliliter.

RESULTS

Experimental infections in the control group. A toxinogenic clone from the VPI or Mara strain of *C. difficile* became established within 18 h in gnotobiotic mice; all of the animals died within 2 days. The clone of the Mara strain produced significantly more enterotoxin in vivo than did that of the VPI strain (Table 1).

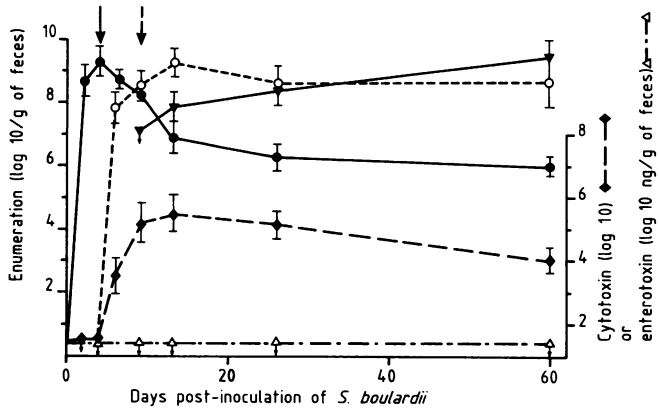

FIG. 1. Emergence of nontoxinogenic clones of *C. difficile* in *S. boulardii*-protected mice. Solid arrow, Inoculation of a toxinogenic clone of *C. difficile*; broken arrow, removal of *S. boulardii* from drinking water. A small arrow under an experimental value indicates that the true value is less than that shown. Symbols: ●, *S. boulardii*; ○, toxinogenic *C. difficile*; ▼, nontoxinogenic *C. difficile*.

Emergence of nontoxinogenic and intermediate clones. (i) Mice protected by *S. boulardii*. Prior ingestion of *S. boulardii* for 4 days afforded a good protection against challenges with clones from *C. difficile* VPI (20 of 24 survived) or Mara (10 of 20 survived). The number of *S. boulardii* decreased from $10^{9.5}$ to $10^{6.3}$/g of feces when *S. boulardii* was no longer given in the drinking water (Fig. 1). Compared with levels observed in dying mice (data not shown; cytotoxin titers, $>10^5$; enterotoxin titers, $>10^3$), the cytotoxin levels in surviving mice decreased, and the enterotoxin levels were below the limits of detection (Fig. 1). Nontoxinogenic clones of *C. difficile* VPI could be found on day 14, and their number increased in 3 weeks to a level as high as that of toxinogenic clones of *C. difficile*. Production of the toxins remained low throughout the 60-day experiment (Fig. 1). Similar results were obtained after infection with a toxinogenic clone of *C. difficile* Mara (data not shown).

(ii) Mice protected by diet. All mice fed the protective diet survived after *C. difficile* infection with clones from strain VPI or Mara. Figure 2 shows the modulation of enterotoxin and cytotoxin production after *C. difficile* VPI infection. One week after challenge, the protective diet was replaced by the

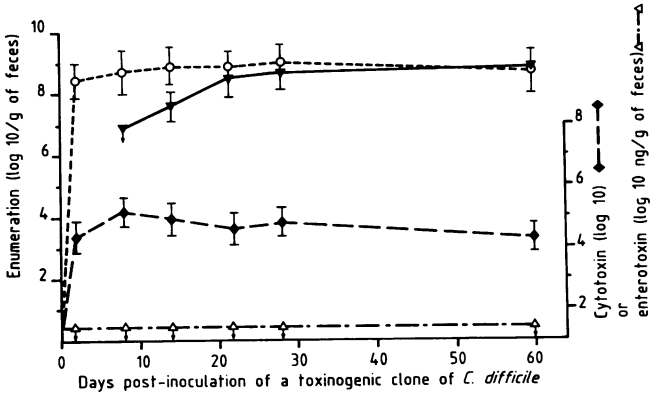

FIG. 2. Emergence of nontoxinogenic clones of *C. difficile* after treatment by a protective diet and challenge with a toxinogenic clone. A small arrow under an experimental value indicates that the true value is less than that shown. Symbols: ○, toxinogenic *C. difficile*; ▼, nontoxinogenic *C. difficile*.

TABLE 2. Characterization of *C. difficile* clones isolated in gnotobiotic mice

Strain or clone (no. tested)	Log_{10} ng of enterotoxin/g	Log_{10} cytotoxin titer	Filter test result
Initial			
VPI	3.3	5.1	+
Mara	3.8	5.8	+
Positive			
VPI (9)	3.1–3.5	5–5.2	+
Mara (10)	3.7–4	5.6–6	+
Intermediate			
VPI (6)	0.9–2.5	2.6–4.8	+
Mara (2)	2.4–3.3	3.9–4.5	+
Negative			
VPI (23)	<0.5	<1	−
Mara (13)	<0.5	<1	−

nonprotective commercial one. Mice did not develop disease, and production of the toxins remained low. Nontoxinogenic *C. difficile* clones could be detected on day 14 (Fig. 2), and they coexisted in equivalent numbers with toxinogenic clones of *C. difficile* 1 month after infection. This equilibrium persisted throughout the experiment (60 days). Similar data (not shown) were obtained with the clones of strain Mara.

Characterization of nontoxinogenic clones. Table 2 shows production of toxins by the different clones. Filter test-negative clones did not produce detectable amounts of cytotoxin or enterotoxin and remained toxin negative even after 10 successive subcultures. Most of the filter test-positive clones produced as much of the toxins as did the initial strains, but some of them appeared to be intermediate clones, i.e., they produced significantly less of the toxins ($P < 0.05$) than did the initial strains, although they remained positive in the filter test.

Clones of *C. difficile* obtained in mice were tested in vitro for their abilities to metabolize carbohydrates. All of the clones were similar to the initial strains and metabolized glucose, fructose, mannose, and *N*-acetylglucosamine. Antibiotypes were determined for one clone of each group from the VPI strain (positive, intermediate, and negative clones). The initial strain and the clones were resistant to clindamycin, cefoxitin, lincomycin, kanamycin, lividomycin, ampicillin, gentamicin, nalidixic acid, neomycin, and tobramycin and sensitive to all of the other antibiotics tested. All of the strains produce similar quantities of volatile fatty acids (acetic acid, 24%; isobutyric acid, 2.4%; butyric acid, 20%; isocaproic acid, 3.4%; isovaleric acid, 48% of the total volatile fatty acids).

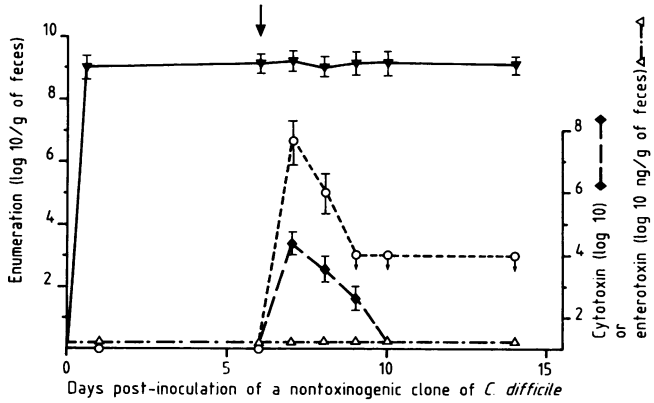

FIG. 3. Antagonistic effect of a nontoxinogenic clone of *C. difficile* on a toxinogenic clone. Large arrow, Inoculation of a toxinogenic clone of *C. difficile*. A small arrow under an experimental value indicates that the true value is less than that shown. Symbols: ○, toxinogenic *C. difficile*; ▼, nontoxinogenic *C. difficile*.

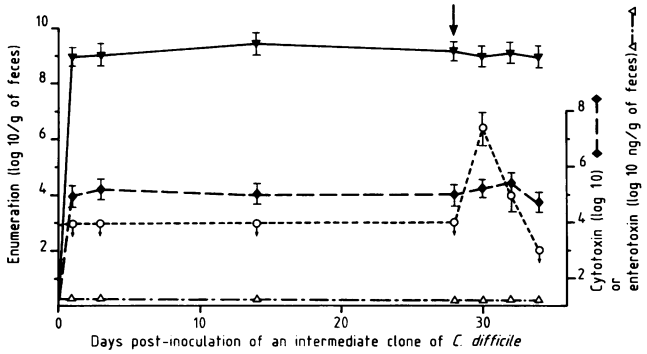

FIG. 4. Antagonistic effect of an intermediate clone of *C. difficile* on a toxinogenic clone. Large arrow, Inoculation of a toxinogenic clone of *C. difficile*. A small arrow under an experimental value indicates that the true value is less than that shown. Symbols: ○, toxinogenic *C. difficile*; ▼, nontoxinogenic *C. difficile*.

Some of the negative ($n = 4$) and intermediate ($n = 2$) clones were inoculated individually into axenic mice. None of them were lethal. These clones became established at levels similar to that of lethal *C. difficile* strains (Fig. 3 and 4). Even 1 and 4 weeks, respectively, after inoculation of negative and intermediate clones, no filter test-positive clones could be found and no enterotoxin could be detected in mouse feces (Fig. 3 and 4). Cytotoxin was detected only in mice infected with the intermediate clone (Fig. 4).

Antagonistic effect of nontoxinogenic and intermediate clones on toxinogenic ones. Mice were fed a nonprotective diet and inoculated with a nontoxinogenic clone from the VPI strain. When the inoculation was performed 18 h or more before challenge with the lethal VPI strain, all mice were protected, cytotoxin production was reduced, and no enterotoxin could be detected (Table 3). Similar data were observed with the lethal Mara strain, together with nontoxinogenic clones from the VPI or Mara strain (data not shown).

After inoculation of the toxinogenic VPI strain in mice associated only with a nontoxinogenic clone (Fig. 3), the VPI strain was detectable for only 2 days after inoculation. No enterotoxin production could be detected, and transient cytotoxin production was observed for only a few days after

TABLE 3. Effect of time delay between inoculation of nontoxinogenic clone and strain VPI challenge on mouse survival

Delay	Mean log_{10}[a]:		No. of surviving mice/total
	Cytotoxin titer (SEM)	ng of enterotoxin/g	
10 days	<1	<1	10/10
6 days	2.5 (0.4)	<1	10/10
18 h	3.1 (0.2)	<1	10/10
0 h	5.4 (0.2)	<1	6/10

[a] Levels in surviving mice 3 days postinfection with toxinogenic *C. difficile*.

challenge. Similar results were obtained with an intermediate clone, except for cytotoxin production, which remained elevated (Fig. 4).

Attempts to isolate nontoxinogenic clones in vitro. Attempts to obtain some nontoxinogenic clones of *C. difficile* VPI in vitro were unsuccessful. After 10 successive subcultures or after continuous-flow culture in an anaerobic chamber for 20 days, no nontoxinogenic *C. difficile* clones could be detected.

DISCUSSION

The results of experimental infections of gnotobiotic mice with toxinogenic clones from *C. difficile* VPI and Mara were similar to those of previous reports (15, 23; A. Vernet, G. Corthier, F. Dubos, P. Rapine, and A. Parodi, 9th Int. Symp. Gnotobiol., abstr. no. E25, p. 222, 1987). At death, large amounts of cytotoxin and enterotoxin were detected. As previously described (2, 15, 16, 19), rodents could be protected from the adverse effects of toxinogenic strains of *C. difficile* by *S. boulardii* ingestion or modification of the diet. Whatever the method of protection, reduction of cytotoxin production and suppression of enterotoxin under the detection limit were noticed, whereas no effect on the population level of *C. difficile* could be evidenced. Whereas other authors (13, 14) have reported that cytotoxin caused death in other animal models, persistent cytotoxin production did not kill mice in our gnotobiotic model. It may be that the cytotoxin levels were too low or the gnotobiotic mice were less sensitive to the cytotoxin effect. By contrast, we observed that death of mice always occurred with a large production of enterotoxin in the digestive tract.

S. boulardii could be removed or the diet could be changed without inducing subsequent mouse mortality. Low amounts of *S. boulardii* still remained in the digestive tract after yeast removal from the drinking water, but this level is not high enough to protect mice (2). Thus, after the end of protective treatment, animals were protected by a factor other than the small number of remaining *S. boulardii* organisms or the diet composition. Nontoxinogenic clones could be detected at that time, and it could be suggested that they prevent cytotoxin and enterotoxin production (and consequently mouse death), as observed in other experiments performed with axenic mice associated only with hare *C. difficile* (4) or *Escherichia coli* or *Bifidobacterium bifidum* (3). Such nontoxinogenic clones were not present in the inoculum, since a single filter test-positive clone was used for initial inoculation of the mice. Some intermediate clones were also isolated from protected mice. On the basis of fermentative profiles and volatile fatty acid production, all of these clones appeared to be *C. difficile*. In addition, the fact that nontoxinogenic clones shared the same antibiotypes as toxinogenic ones strengthened the idea that all of the negative clones were derived from the toxinogenic one used for mouse inoculation. This phenomenon of emergence of negative clones was not limited to strain VPI, since similar data were obtained with the Mara strain. These results suggest that several different conditions permit emergence of nontoxinogenic clones after infections by toxinogenic strains. Earlier, Wilkins et al. (21) demonstrated the emergence of a reduced toxin mutant from rats associated only with a toxin-positive strain and showed that this mutant was not pathogenic (5). Using an original method, we quantitated the emergence of nontoxinogenic clones and demonstrated that the appearance of nontoxinogenic clones, even in the presence of toxinogenic clones, confers protection.

Whereas no genetic analyses were performed on the nontoxinogenic clones, one can postulate that their origin is due to mutation or loss of genetic material (chromosome, phage, transposon, or plasmid) which can be unrelated to the antibiotic resistances tested. The passage from a toxinogenic clone to a nontoxinogenic one probably involved different irreversible steps, since we were able to find clones that produced intermediate levels of cytotoxin and enterotoxin. Each step must be considered irreversible, since the intermediate and nontoxinogenic clones did not revert to the initial toxinogenic type after in vitro subcultures or inoculation into gnotobiotic mice.

The limit of sensitivity of the filter test technique is 1 negative clone per 1,000 toxinogenic clones. Therefore, undetectably low numbers of nontoxinogenic clones may have existed after subcultures or continuous-flow cultures of toxinogenic clones in vitro. By contrast, in gnotobiotic animals the population level of nontoxinogenic clones was equal to that of the toxinogenic clones. This result suggests that in vivo, nontoxinogenic clones have an ecological advantage over toxinogenic clones. Indeed, nontoxinogenic or intermediate clones prevented further establishment of toxinogenic strains of *C. difficile*. Wilson and Sheagren (22) have already observed that a nontoxinogenic strain of *C. difficile* prevented mortality due to another toxinogenic *C. difficile* strain by an antagonistic effect of the former on the latter. We also reported earlier a partial protective effect of a low-toxinogenic strain isolated from hares on the toxinogenic VPI strain in the gnotobiotic mouse model (4). In the experiment described here, the nontoxinogenic clones were derived from the toxinogenic clones and the observable genetic variations were limited to toxin production genes. The similarity of the toxinogenic and nontoxinogenic strains may explain why nontoxinogenic clones exerted a strong antagonistic effect on toxinogenic ones when they were given to gnotobiotic mice before the toxinogenic clone. When the toxinogenic clone was given first (to protected mice), the nontoxinogenic ones exerted no antagonistic effect on the toxinogenic clone. When the two clones were given simultaneously, protection was only partial. Such ecological interactions between isogenic strains was previously described (6) in a gnotobiotic mouse model inoculated with *E. coli*.

When mice were fed a commercial nonprotective diet, a minimal time (18 h) was needed between inoculation of the nontoxinogenic clone and inoculation of the toxinogenic clone for a protective effect of the nontoxinogenic clone to become evident. This time was long enough for establishment of *C. difficile*. This result shows that a nontoxinogenic clone must be established before infection by a toxinogenic one. Wilson and Sheagren (22) had reported that nontoxinogenic strains must be given before the toxinogenic one, but the minimal time lapse was not determined.

From an ecological point of view, an important observation was that nontoxinogenic clones originate from toxinogenic clones. It could be suggested that the large number of nontoxinogenic strains of *C. difficile* detected in the feces of children (7, 11, 17, 20) originated from toxinogenic *C. difficile* strains. The frequent finding of nontoxinogenic clones of *C. difficile* in children may explain the low frequency of pseudomembranous colitis in this population. Furthermore, we detected some intermediate clones which were derived from the toxinogenic one. Similar intermediate strains isolated in humans may, like the nontoxinogenic clones, originate from the toxinogenic ones and may exert a

protective effect against further infection by toxinogenic *C. difficile* strains.

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