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Correlation of Disease Severity with Fecal Toxin Levels in Patients with *Clostridium difficile*-Associated Diarrhea and Distribution of PCR Ribotypes and Toxin Yields In Vitro of Corresponding Isolates

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We investigated in vivo and in vitro yields of toxins A and B from and PCR ribotypes of *Clostridium difficile* isolates from 164 patients with differing severities of *C. difficile*-associated diarrhea (CDAD) (patients were grouped as follows: <3 loose stools per day, $n = 45$; 3 to 10 per day, $n = 97$; >10 per day, $n = 22$). The median fecal toxin levels in each group were 0.5, 6.8, and 149 U/g feces ($P < 0.001$), respectively. Patients with severe diarrhea also had more-frequent occurrence of blood in stool and vomiting, but there was no association with fecal toxin levels per se. There was no correlation between fecal toxin level and toxin yield in vitro for the corresponding *C. difficile* isolate or between its PCR ribotype and disease severity. A broad range of toxin yields among isolates belonging to major PCR ribotypes indicated a presence of many subtypes. We hypothesize that bacterial and host factors that affect *C. difficile* toxin levels in feces are important determinants of symptoms in CDAD patients. An inverse correlation between toxin yield and spore count ($r = 0.66$) in stationary-phase cultures supported the notion that toxin production and sporulation represent opposite alternative survival strategies for *C. difficile* cells facing nutrient shortage.

Most strains of *Clostridium difficile* produce two toxins, A and B, that cause *C. difficile*-associated diarrhea (CDAD) with symptoms ranging from mild diarrhea to pseudomembranous colitis. CDAD is associated mainly with the use of antibiotics that reduce the protective microflora, which allows for overgrowth and toxin production by *C. difficile* (21), and chemostat and animal studies have verified that certain antibiotics induce *C. difficile* growth, toxin production, and toxin release (11, 29, 31). Furthermore, the age of the patient, underlying diseases, and levels of toxin-neutralizing antibodies are factors that affect attack rate, severity of disease, and the risk of relapse of CDAD (17, 18, 21, 38). In addition, factors that differ between toxin-producing *C. difficile* strains, e.g., the amount of toxin produced, the serogroup, and the surface layer protein composition, may affect symptoms (12, 14, 20, 25, 32, 33). For example, strains of *C. difficile* belonging to specific serogroups are highly virulent in animal models (3, 8), but whether *C. difficile* strain types differ in terms of virulence in humans is less clear.

The epidemiology and disease pattern of *C. difficile* strains have been studied by several methods, e.g., serotyping, toxinotyping, and PCR ribotyping (4). A cohort study showed no difference in the distributions of *C. difficile* immunoblot types between asymptomatic carriers and CDAD patients, suggesting that patient factors or bacterial numbers contribute to disease more than the properties of specific *C. difficile* strain types (24). In addition, for a total of 62 *C. difficile* strains isolated from 17 patients with CDAD and 11 carriers and typed by PCR ribotyping and by use of randomly amplified polymorphic DNA, no significant correlation between genotype and disease severity was found (5). In the United Kingdom, PCR ribotype 001 was shown to dominate in hospital settings nationwide but not in community-acquired CDAD, suggesting that this PCR ribotype (or a subtype) has an increased attack rate or greater ability for nosocomial spread (35). Another United Kingdom study showed that while many strain types of *C. difficile* were present in the hospital environment, only certain types were associated with infection (10). Recently, a dramatic increase of both the incidence and the mortality rate of CDAD was reported for hospitals in Quebec, suggesting local spread of a highly transmissible and virulent strain of *C. difficile* (30).

In this study, we investigated the relationship between fecal *C. difficile* toxin levels, PCR ribotypes and their toxin-producing characteristics, and the severity of disease in CDAD patients. The fecal specimens, *C. difficile* isolates, and patient data were collected during a 1-year study of CDAD at a teaching hospital where CDAD was endemic (36).

MATERIALS AND METHODS

Patients. Consecutive patients with *C. difficile* infection at Huddinge University Hospital, Stockholm, Sweden, diagnosed during 12 months and from whom *C. difficile* isolates were available for PCR ribotyping were included. The patients studied were part of a prospective evaluation of the CDAD rate and the nosocomial transmission and molecular characteristics of *C. difficile* (36). The patients were asked to answer a questionnaire regarding the date of the onset of diarrhea, underlying diseases, recent antibiotic treatment, and symptoms and signs. A study nurse monitored the patients and helped them to complete the questionnaires as far as possible. Of 227 patients included, 63 (28%) were excluded from the present analysis because the information on symptoms was insufficient. The total study population thus comprised 164 adult patients with CDAD. Also,

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selected *C. difficile* isolates from the remaining patients were included in the bacteriological analysis (see below). The study was approved by the ethics committee of the Karolinska Institute (Huddinge, Sweden).

Cytotoxin B detection and isolation of *C. difficile*. A fecal sample for *C. difficile* diagnostics was taken upon the decision of the treating physician, usually within 3 days of onset of diarrhea, and the specimens were handled according to the standard hospital routine. Thus, these were stored at 4°C prior to transport to the laboratory at 10:30 a.m. or 2:00 p.m. and analyzed for the presence of cytotoxin B by use of a McCoy cell assay and a toxin/antitoxin kit (TechLab, Blacksburg, VA). *C. difficile* was isolated based on colony morphology on cycloserine-cefoxitin fructose agar supplemented with sodium taurocholate (TCCFA). The isolates were tested for toxin B production using the McCoy cell assay. The remaining portions of the fecal specimens were stored at −70°C for a later quantitative enzyme-linked immunosorbent assay of *C. difficile* toxins (see below).

PCR ribotyping. Cell pellets from overnight cultures of *C. difficile* were boiled in 10% Chelex, and PCR amplification of DNA interspaced between the genes encoding 16S and 23S rRNA was performed using Ready-to-Go PCR tubes (Amersham Biosciences) with primer pairs and conditions as described by Stubbs et al. (35) with minor modifications. The primers used were 5′-CTGGGGTGA AGTCGTAACAAGG-3′ and 5′-GCGCCCTTTGTAGCTTGACC-3′. After an initial denaturation at 94°C for 5 min, the PCR amplification conditions were 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s, which were repeated for 30 cycles on a PTC-200 instrument (MJ Research Inc.). After the final cycle, samples were heated for 72°C for 7 min and cooled to 4°C. PCR products were separated on precast polyacrylamide gels (GeneGel Excel 12.5/24; Amersham Biosciences) and visualized by silver staining. In addition to the clinical isolates, the serogroup reference strains CCUG 37766 to 37787, corresponding to serogroups C, A2, A3, A4, A5, A6, A7, A8, A9, A10, S1, S3, S4, A, B, D, F, G, H, I, K, and X, respectively, were analyzed. Two reference strains (CCUG 37766 [serogroup C] and CCUG 37779 [serogroup A]) were included on each gel, and a 100-bp ladder was run in every fifth lane. Digitized gels were analyzed in Molecular Analyst Fingerprinting Plus 1.6 software (Bio-Rad) by cluster correlation and an unweighted pair group method using arithmetic averages. The clustering of band patterns was double checked manually, and each unique pattern was given a number and, when necessary, a suffix to indicate a closely related but distinct ribotype (see reference 36).

Growth of *C. difficile* isolates and assay of toxin yield in vitro and fecal toxin levels. Isolates from the 164 patients studied were grown for 24 h in PY medium containing 4.1 mM cysteine, diluted 1:100 in PY medium with 0.4 mM cysteine (see Fig. 2) or without added cysteine (see Fig. 3), and further grown for 24 h (final optical density at 600 nm [OD_{600}], 1.0 to 1.5). As cysteine suppresses toxin production by *C. difficile*, this procedure gives maximum toxin yields (15). Selected isolates of PCR ribotypes SE20 and SE30 from additional patients (see above) were used for comparison of major ribotypes (see Fig. 3). Cell and spore counts of selected *C. difficile* isolates were obtained by growth in PY medium with 0.4 mM cysteine for 48 h and microscopy using a Bürker chamber (see Fig. 4). Bacterial cells with and without visible spores were scored in 10 squares and the values averaged.

Total toxin yield (intracellular plus extracellular toxin) from 24-h cultures was measured using a Ridascreen *C. difficile* toxin A/B kit (r-Biopharm) after rupture of the bacterial cells by sonication and given as A_{450} units per ml culture (16). For assay of in vivo toxin levels, 0.1- to 0.5-g samples of feces from 20 consecutive patients from each disease group (total number, 60) were thawed and diluted in Ridascreen sample buffer, each to a final volume of 1.0 ml. After vortexing and centrifugation, toxin in the supernatant was measured and given as A_{450} units per g feces. Repeated assay of higher dilutions was done for samples with A_{450} scores of ≥2.0. All these samples showed A_{450} values between 2.0 and 1,900 when calculated back to undiluted feces, confirming that these samples had high toxin levels. The sensitivity and specificity of the Ridascreen toxin A/B kit, according to the kit manual, are 94.7% and 98.0% compared to a cytotoxin assay, based on 425 clinical stool samples tested at a Scandinavian reference center. The intravariation coefficient of the kit is 6.26% ($n = 5$), and the interassay variation coefficient is 11.16% ($n = 5$), as measured by use of three different kits from each of two different batches (kit manual).

Statistical analysis. Numerical data (e.g., toxin levels) were compared using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests and Student's *t* test after logarithmic transformation of values. Ages were compared using the Kruskal-Wallis method, and for categorical data, the chi-square test or Fisher's exact test was used.

TABLE 1. Diarrhea frequency related to other clinical parameters for CDAD patients

Parameter	Value for disease group[a]			*P* value[b]
	Mild	Moderate	Severe	
Total no. of patients	45	97	22	
Male gender[c]	23/45 (51)	45/97 (46)	4/22 (18)	0.0067
Median age[d]	76 (2–93)	69 (1–95)	57 (1–83)	0.034
Abdominal pain[c]	7/36 (19)	30/78 (38)	11/20 (55)	0.022
Blood in stool[c]	4/39 (10)	19/84 (23)	12/22 (55)	<0.0001
Vomiting[c]	7/40 (18)	30/89 (34)	10/21 (48)	0.013
Fever > 38°C[c]	12/40 (30)	32/82 (39)	11/20 (55)	0.17
Mortality[c]	13/45 (29)	33/97 (34)	1/22 (5)	0.022

[a] The mild, moderate, and severe disease groups were defined as patients having <3, 3 to 10, and >10 loose stools per day, respectively.
[b] Chi-square test. For comparisons of age, the numerical Kruskal-Wallis method was applied.
[c] Values indicate the number of patients positive for the given parameter. Data are given in the form n/n_{total} because of missing data. Values in parentheses are percentages.
[d] Ages are given in years. Values in parentheses are ranges.

RESULTS

Clinical symptoms and fecal toxin levels. The 164 CDAD patients were distributed into three disease severity groups having mild, moderate, or severe diarrhea (Table 1). Symptoms such as abdominal pain, blood in stool, vomiting, and fever were more common as the diarrhea frequency increased, although the correlation with fever was not statistically significant (Table 1). Patient age and the proportion of males and deceased patients were lower in the severe diarrhea group. The difference in mortality rates may be related to age, while the reason for the gender discrepancy is not known.

The fecal toxin levels in the initial (diagnostic) specimens assayed in 20 consecutive patients per group were associated with diarrhea frequency, with median levels of 0.5, 6.8, and 149 U/g in the mild, moderate, and severe groups, respectively ($P < 0.0001$) (Fig. 1A). Toxin levels were associated also with abdominal pain ($P = 0.014$) (Fig. 1B) but not with blood in stool ($P = 0.58$) (Fig. 1C), vomiting ($P = 0.38$) (Fig. 1D), or fever (data not shown), despite the apparent correlation between these parameters and diarrhea frequency (Table 1).

Distribution of PCR ribotypes in patients with differing levels of severity of CDAD. The overall distribution of *C. difficile* PCR ribotypes was similar in the three patient groups having differing diarrhea frequencies (Table 2). The 10 most common types comprised 67%, 45%, and 59% of the groups with mild, moderate, and severe diarrhea, respectively. Although certain PCR ribotypes were more common among patients with low or high diarrhea frequencies (e.g., SE3 was absent in the severe diarrhea group), none of these associations were statistically significant. The PCR ribotype of the infecting *C. difficile* strain and the severity of CDAD remained unrelated after the data were combined to obtain a higher statistical power, e.g., by comparing patients with mild or moderate diarrhea with those with severe diarrhea (Fisher's exact test; data not shown).

In vitro toxin yields from clinical isolates. In contrast to the fecal toxin levels, the maximum toxin yields in vitro from *C. difficile* isolates from patients with mild ($n = 20$) and severe ($n = 20$) diarrhea were similar, with mean values of 72 (range,

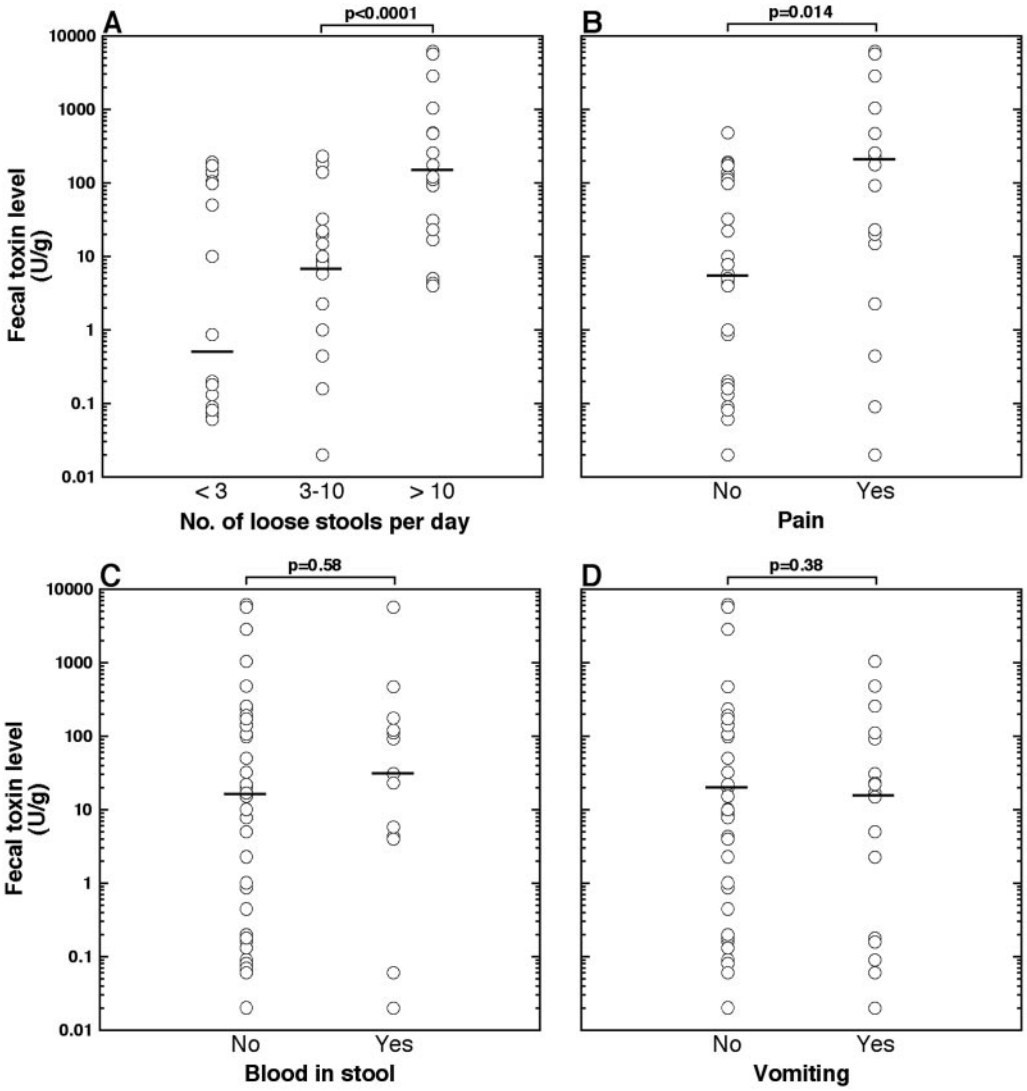

FIG. 1. Toxin levels in feces versus symptoms in CDAD patients. (A) Toxin levels (U/g feces) in groups of patients having differing diarrhea frequencies. Fecal samples from 20 consecutive patients per group were analyzed. Horizontal bars indicate median values. (B to D) Toxin levels in the same patients as for panel A but divided into groups having abdominal pain (No/Yes, $n = 30/18$; missing data, $n = 12$) (B), blood in stool (No/Yes, $n = 40/15$; missing data, $n = 5$) (C), or vomiting (No/Yes, $n = 35/18$; missing data, $n = 7$) (D). Statistical P values were obtained by one-way ANOVA followed by Bonferroni post hoc test (A) or Student's t test (B to D) of logarithmic toxin level values.

0.01 to 799) and 65 (range, 1 to 534) U/ml, respectively ($P = 0.97$). Consequently, there was also no correlation between toxin yields in vitro of the infecting *C. difficile* isolates and the patients' fecal toxin levels (Fig. 2). The median toxin yield for 38 isolates belonging to four major PCR ribotypes after growth in a medium supporting high toxin production was 164 U/ml (range, 15 to 3,222). Ribotypes SE21 and SE30 had the lowest and highest median toxin yields, respectively (42 versus 314 U/ml; $P = 0.007$). Dramatic differences in toxin yields were observed for isolates belonging to major PCR ribotypes, such as SE30 and SE20 (up to >100-fold) (Fig. 3). No toxin was detected in vitro from five SE3 isolates tested. SE3 was not isolated in severe CDAD (see above), and its PCR ribotype pattern was identical to that of strain CCUG37781, a toxin A- and toxin B-negative reference strain belonging to serotype D.

Relationship between *C. difficile* toxin yield and sporulation in vitro. The relationship between *C. difficile* toxin yield and sporulation in vitro was studied for 17 isolates belonging to the major PCR ribotypes SE20, SE21, SE22, and SE25. The mean cell count by microscopy was 3.2×10^9 (range, 2.2×10^9 to 5.4×10^9) per ml after 48 h of growth in PY medium with 0.4 mM cysteine added. No correlation between total cell count and toxin yield was observed ($r = 0.096$), showing that toxin regulation rather than bacterial counts determined the marked differences in toxin yield in vitro between isolates (see above). In contrast, there was a correlation between toxin yield and count of vegetative bacterial cells ($r = 0.78$) and an inverse correlation between toxin yield and spore count ($r = 0.66$) (Fig. 4). There was also a negative correlation between the number of vegetative cells and the spore count ($r = 0.82$, not shown).

TABLE 2. Distribution of *C. difficile* PCR ribotypes in groups of patients having differing frequencies of diarrhea

PCR ribotype[a]	No. of isolates per diarrhea group (%)[c]			
	Mild (n = 45)	Moderate (n = 97)	Severe (n = 22)	Total (n = 164)
SE21 (H)	7 (16)	11 (11)	2 (9.1)	20 (12)
SE21b (A8)	3 (6.8)	9 (9.4)	2 (9.1)	14 (8.5)
SE22	3 (6.8)	5 (5.2)	2 (9.1)	10 (6.1)
SE20 (G)	5 (11)	3 (3.1)	1 (4.5)	9 (5.5)
SE3 (D)	4 (9.0)	3 (3.0)	0 (0.0)	7 (4.3)
SE25	0 (0.0)	5 (5.2)	1 (4.5)	6 (3.7)
SE30	2 (4.5)	1 (1.0)	3 (14)	6 (3.7)
SE12 (A2)	2 (4.5)	2 (2.1)	1 (4.5)	5 (3.1)
SE16 (A)	1 (2.2)	3 (3.1)	1 (4.5)	5 (3.1)
SE29b	3 (6.8)	2 (2.1)	0 (0.0)	5 (3.1)
Other[b]	15 (33)	53 (55)	9 (41)	77 (47)

[a] The serotype of the reference strain having an identical PCR ribotype pattern is indicated within parenthesis.
[b] Other PCR ribotypes were represented by four isolates (patients) or less.
[c] For definitions of diarrhea groups, see Table 1, footnote *a*.

This supported the hypothesis that toxin production and sporulation represent opposite alternative survival strategies for *C. difficile* entering stationary phase, i.e., facing nutrient shortage.

DISCUSSION

A new finding of this study was that fecal *C. difficile* toxin levels from CDAD patients correlated with diarrhea frequency and abdominal pain. As this was not the case for blood in stool or vomiting, these diarrhea-related symptoms were apparently also dependent on other factors. There was no association between the PCR ribotype of the infecting strain and the severity of disease. In accord with this result, others found no particular PCR ribotype predominant in cases of severe or relapsing CDAD (5, 27, 36, 39), and the distributions of *C. difficile* immunoblot types were similar in symptomatic patients and in healthy carriers (24). However, Fawley et al. (10) reported that a total of 17 strain types were isolated from both symptomatic patients and the hospital environment during a 4-year period, but only 4 of 17 types resulted in CDAD, suggesting a correlation between type and virulence. Also, a *C. difficile* clone recently emerging in Canada appears to be associated with high transmission, attack, and mortality rates (30).

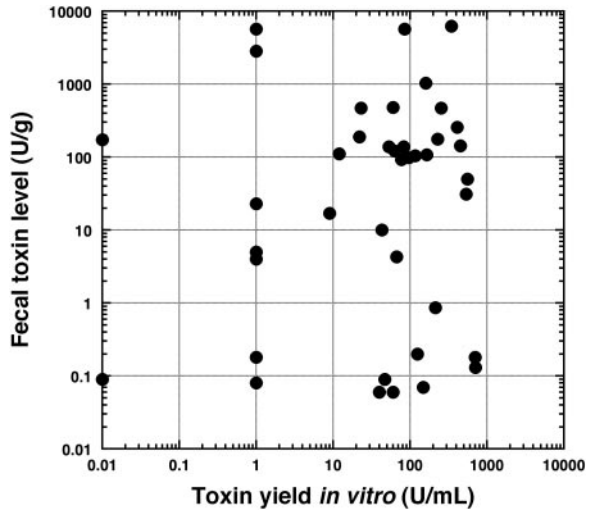

FIG. 2. Toxin yields in vitro from infecting *C. difficile* isolates versus fecal toxin levels in corresponding CDAD patients having mild or severe diarrhea (n = 40). For high-level toxin production, the isolates were grown for 24 h in PY medium with 0.4 mM cysteine added (final OD_{600}, 1.0 to 1.5).

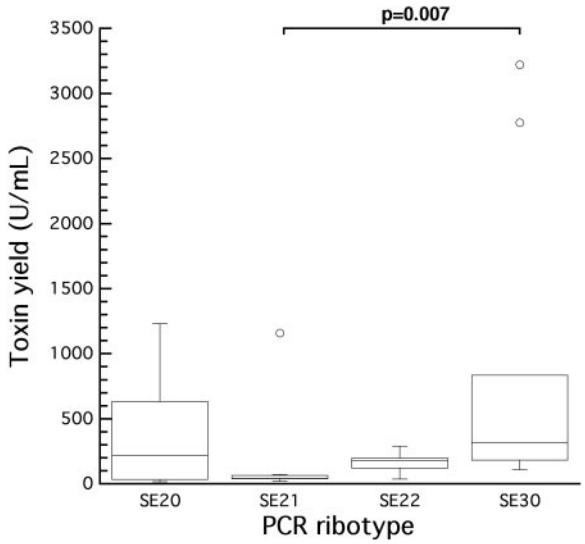

FIG. 3. Toxin yields in vitro from *C. difficile* isolates belonging to major PCR ribotypes. SE21, SE30 (n = 9 each), SE20, and SE22 (n = 10 each) isolates were grown in PY medium without cysteine added for 24 h for maximum toxin production (final OD_{600}, 1.0 to 1.5). Each box encloses 50% of the values, and the median is represented by a horizontal line. Error bars include values that extend to the upper quartile value plus 1.5 times the interquartile distance and to the lower quartile value minus 1.5 times the interquartile distance, and open circles represent values outside these ranges (outliers). The P value was derived as described in the legend for Fig. 1 (ANOVA).

The lack of correlation between CDAD symptoms and PCR ribotype of the infecting *C. difficile* strain may be due to the highly variable toxin production capacities in vitro here found among isolates belonging to a given PCR ribotype (Fig. 3), as has been reported also for immunoblot types (24), indicating a presence of subtypes within major PCR ribotypes. Several typing methods for *C. difficile* have been developed, and PCR ribotyping, pulsed-field gel electrophoresis, and immunoblot typing offer a high discriminatory power compared to serotyping, but a direct comparison between all methods or systematic combinations of these has to our knowledge not yet been performed (1, 4, 19, 32, 33). Further typing of the dominating PCR ribotype in United Kingdom hospitals (UK001, serogroup G) revealed several subtypes (9, 10, 28). Thus, combinations of typing methods or new methods for typing or subtyping are needed to better define the epidemiology of distinct *C. difficile* strains and for studies of their clinical virulence. Toxinotyping results in fewer types than PCR ribotyping but is more directly related to the variability within the toxin genes (32, 33). Toxinotypes may differ by up to 10^6-fold in terms of toxin yield, but the basis for this variability is not known (2). Interestingly, the degrees of cytoxicity between toxins belonging to different toxinotypes may differ (2), making the relation between strain type and severity of CDAD even more complex.

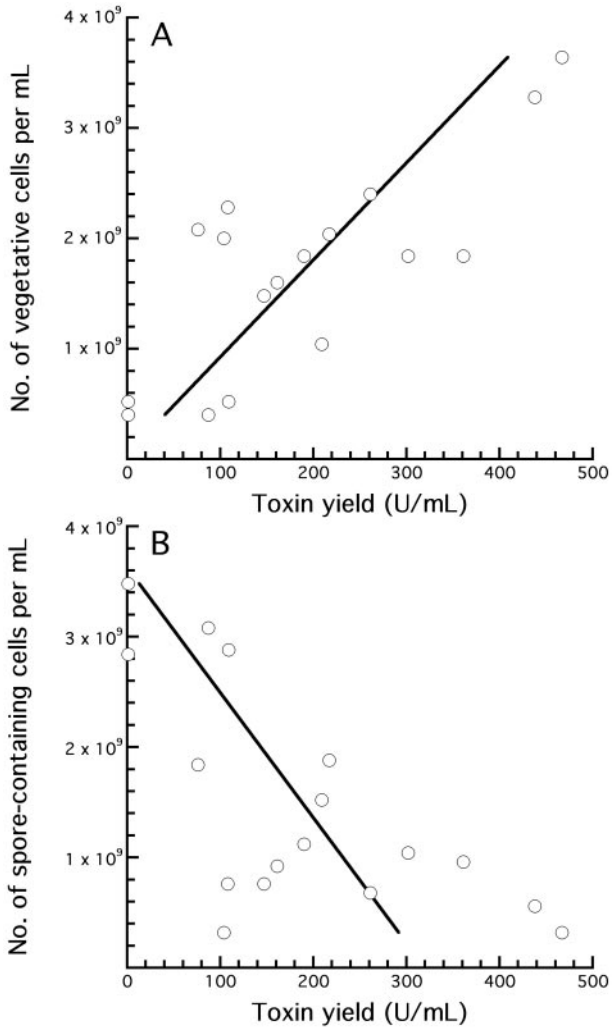

FIG. 4. *C. difficile* toxin yield in vitro related to count of vegetative bacterial cells (A) and spore count (B). Isolates belonging to PCR ribotypes SE20, SE21 ($n = 5$ each), SE22 ($n = 3$), and SE25 ($n = 4$) were grown for 48 h in PY medium with 0.4 mM cysteine added for high-level toxin production. Counts of vegetative bacteria and cells containing visible spores were obtained by microscopy and were plotted against the toxin yields from the same cultures.

Seven isolates of a PCR ribotype belonging to the toxin-negative serotype D (SE3) were here found exclusively in patients with mild or moderate diarrhea, and although rare, toxin-negative strains have been isolated even from patients with pseudomembranous colitis (26). This may be due to the fact that such patients were also infected by a second *C. difficile* strain present in low numbers, and thus not detected, but able to produce toxins.

We found no correlation between *C. difficile* toxin yields in vitro and fecal toxin levels. One possible explanation is the variation of *C. difficile* counts in vivo, as these correlated with fecal toxin levels in mice (31) and were on average 100-fold higher in CDAD patients than in asymptomatic *C. difficile* carriers (24). However, although broth cultures of individual isolates generated similar and reproducible optical densities and bacterial counts, we could not reliably determine *C. difficile* counts in feces using the selective medium TCCFA. For example, we found a plating efficiency on TCCFA that was 10- to 1,000-fold reduced (depending on the isolate tested) from that on blood agar containing taurocholate by using ethanol preparations of spores from batch cultures (unpublished). As certain amino acids dramatically affect *C. difficile* toxin yields in vitro (15, 16), nutrients present in the colonic lumen may also greatly affect toxin levels in vivo and need to be examined further (23, 37). In gnotobiotic mice colonized with strain VPI 10463, a change of diet reduced fecal toxin A levels by up to 100-fold without affecting bacterial numbers, and the mortality due to CDAD decreased significantly (22). The correlation between the low spore counts, high counts of vegetative cells, and high toxin yields in vitro found here (Fig. 4) indicated that bacterial and external factors that modulate sporulation may also have an impact on *C. difficile* toxin levels in vivo. Similarly, the high-toxin-level-producing strain VPI 10463 produces very few spores in vitro (cf. reference 25), and we have found one clinical isolate sharing the PCR ribotype, an extremely high toxin yield, and a low sporulation efficiency with VPI 10463 (unpublished).

The situation is even more complex in vivo, where toxin levels are affected not only by the strain and counts of *C. difficile* and nutrients in the colon but also by diarrhea frequency, by toxin degradation by proteolytic digestive enzymes, and by competing microbiotas affecting *C. difficile*. For example, colonizing strains of *Escherichia coli* and *Bifidobacterium bifidum* reduced cytotoxin levels and CDAD mortality in gnotobiotic mice infected with *C. difficile* VPI 10463 (7), elderly patients tend to have low numbers of protective bacterial species in the colon (13), and suppression of the colonic flora by antibacterial agents is fundamental for the expansion of *C. difficile* and the production of its toxins and thus for the development of CDAD. The broad range of fecal toxin levels observed even within each disease severity group (Fig. 1A) may be due to our practical but rather coarse measure of diarrhea frequency, to the fact that the toxin levels represented only one time point per patient during infection, and/or to the effect of neutralizing antibodies in terms of reducing the impact of the *C. difficile* toxins in some patients (17, 18, 38).

This study supported the notion that toxin levels in feces, influenced by both bacterial and patient factors, contribute to the severity of symptoms in CDAD patients. The clinical value of determining the strain type of *C. difficile* by PCR ribotyping, e.g., for prediction of the outcome of CDAD, appeared to be limited. For the assay of fecal toxin levels to be useful during the management of CDAD patients, as has been suggested previously (6, 34), larger evaluations and further studies, e.g., of the kinetics of toxin levels in CDAD patients, are required. The variable toxin yields within a PCR ribotype found here together with other reports of subtypes support the notion that molecular epidemiology tools should be used only to exclude rather than to confirm transmission.

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