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Frequent Emergence of Resistance in *Clostridium difficile* during Treatment of *C. difficile*-Associated Diarrhea with Fusidic Acid

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Samples from patients with *Clostridium difficile*-associated diarrhea (CDAD) that were randomized to fusidic acid ($n = 59$) or metronidazole ($n = 55$) therapy for 7 days were cultured for *Clostridium difficile* in feces on days 1, 8 to 13, and 35 to 40. Of the patients who were culture positive only before treatment, 77% (36/47) were permanently cured (no treatment failure and no clinical recurrence), compared to 54% (22/41) of those with persistence of *C. difficile* at one or both follow-ups ($P = 0.03$). A similar association between bacterial persistence and a worse outcome of therapy was seen in both treatment groups. Resistance to fusidic acid was found in 1 of 88 pretherapy isolates available, plus in at least 1 subsequent isolate from 55% (11/20) of patients who remained culture-positive after fusidic acid therapy. In 10 of these 11 patients, the resistant follow-up isolate(s) belonged to the same PCR ribotype as the susceptible day 1 isolate, confirming frequent emergence of resistance to fusidic acid during treatment. Despite this, 5 of these 11 patients were permanently cured with fusidic acid, relative to 5 of 9 patients with susceptible *C. difficile* at follow-up ($P = 1.0$). None of the 36 PCR ribotypes of *C. difficile* identified was associated with any particular clinical outcome or emergence of fusidic acid resistance. In conclusion, culture positivity for *C. difficile* was common after both fusidic acid and metronidazole therapy and was associated with treatment failure or recurrence of CDAD. Development of resistance in *C. difficile* was frequent in patients given fusidic acid, but it was without apparent negative impact on therapeutic efficacy in the actual CDAD episode.

Treatment of human infections with antibiotics that disrupt the normal colonic flora often results in diarrhea. The role of toxigenic *Clostridium difficile* as the etiological agent in about one-third of cases with antibiotic-associated diarrhea is well established (3, 17, 18). Over the past 20 years, the incidence of *Clostridium difficile*-associated diarrhea (CDAD) has increased dramatically in the developed world (12). In Sweden, about 50 cases of CDAD/100,000 inhabitants were diagnosed in 1995, outnumbering all diagnosed domestic cases of other bacterial and parasitic diarrhea taken together (15), but an incidence of 97/100,000 was reported for 1999 to 2000 in the central part of Sweden (26). CDAD is usually treated with metronidazole or oral vancomycin, resulting in similar cure and relapse rates (16, 33, 38). However, due to the risk of emergence of vancomycin-resistant enterococci in the gut flora and the cost of vancomycin, metronidazole has become established as the drug of choice for CDAD. The importance of drug resistance in *C. difficile* for the outcome of CDAD treatment appears to be small, as clinical isolates usually have been highly susceptible both to vancomycin and metronidazole as well as to suggested alternative agents such as rifampin (1) and fusidic acid (8, 19). However, metronidazole resistance has been reported in 9% of clinical isolates from Spain (28) but only sporadically among isolates from France (2) and the United Kingdom (5). Recently, "relatively poor" outcome of metronidazole therapy in CDAD was reported from the United States (11, 23) and in patients infected with the high-level toxin-producing and hypervirulent PCR ribotype UK027 (NAP1/027) strain of *C. difficile*, which is spreading in the United States, Canada, and Europe (21, 22, 36). These observations emphasize the need for alternative therapeutic agents in CDAD.

Fusidic acid has been used in Europe for the treatment of staphylococcal bone and soft-tissue infections since the early 1960s, and due to the general susceptibility of *C. difficile* isolates to this agent, fusidic acid also has occasionally been evaluated in CDAD with a clinical efficacy comparable to that of metronidazole and vancomycin (9, 38, 40). The in vitro activity of fusidic acid against clinical isolates of *C. difficile* is generally good (19), and it is generally good for Swedish isolates as well ($n = 638$; MIC median, 0.25 mg/liter; unpublished data). The fusidic acid levels in feces correspond to 2% of the given dose, or around 0.3 mg/liter after an oral dose of 250 mg (30), but like metronidazole a significant intraluminal secretion due to inflammation would, in analogy, result in local therapeutic concentrations in CDAD patients (4). A propensity for emergence of resistance to fusidic acid in staphylococci during monotherapy, usually due to mutations in the *fusA* gene, has been reported (6, 7). As such, mutants appear to exhibit a growth disadvantage; this biological cost apparently is not readily compensated for (24), and their clinical importance remain unclear (27, 34). Whether resistance develops in *C. difficile* during treatment with fusidic acid and possibly hampers its future use in CDAD has not been studied.

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MATERIALS AND METHODS

Study population and *C. difficile* isolates. A prospective randomized double-blind trial comparing metronidazole (400 mg) and fusidic acid (250 mg) given

TABLE 1. Clinical outcome in CDAD patients in relation to bacteriological data

Fate of *C. difficile*[a]	Outcome[c]							
	Permanent cure		Failure		Recurrence		All patients	
	n[d]	%[e]	*n*[d]	%	*n*[d]	%	*n*[d]	%
Persistence at follow-up								
Fusidic acid therapy								
Susceptible follow-up isolate	5 (4/1)	56e	1 (1/0)	11	3 (1/2)	33	9 (6/3)	100
Resistant follow-up isolate[b]	5 (4/1)	46f	3 (3/0)	27	3 (2/1)	27	11 (9/2)	100
Metronidazole therapy	12 (8/4)	57g	1 (1/0)	5	8 (7/1)	38	21 (16/5)	100
Both treatment groups	22 (16/6)	54h	5 (5/0)	12	14 (10/4)	34	41 (31/10)	100
Permanent eradication								
Fusidic acid therapy	19	79i	2	8	3	13	24	100
Metronidazole therapy	17	74k	1	4	5	22	23	100
Both treatment groups	36	77l	3	6	8	17	47	100

[a] Persistence refers to culture positivity on days 8 to 13 and/or days 35 to 40. Culture negativity for *C. difficile* on both these occasions was defined as permanent eradication.

[b] One cured patient carried a resistant mutant of the initial PCR ribotype on days 8 to 13 but later acquired reinfection due to a susceptible strain of a different ribotype (patient 7 in TABLE 2).

[c] Permanent cure was defined as no diarrhea on days 8 to 13 or later, failure was persistence of diarrhea on days 8 to 13, and recurrence as cure on days 8 to 13 followed by diarrhea before day 40.

[d] The numbers in parentheses refer to patients with identical/new PCR ribotype of *C. difficile* compared to that of the pretherapy isolate.

[e] The *P* values were the following: e versus f, $P = 1.0$; e plus f versus g, $P = 0.76$; i versus k, $P = 0.74$; e plus f plus i versus g plus k, $P = 1.0$; h versus l, $P = 0.03$.

orally three times a day for 7 days for treatment of CDAD was conducted in nine hospitals in Sweden from September 1999 to May 2002 (40). The lower dose and shorter duration of the fusidic acid treatment used compared to those of other trials were based on the authors' previous clinical experience with this regimen and was applied to minimize the ecological impact of the drug. Antibiotic-associated diarrhea and *C. difficile* toxin in feces were the main inclusion criteria, and of 131 patients enrolled, 114 could be evaluated for clinical efficacy. Fecal samples were obtained just before start of treatment (day 1) and at scheduled follow-up on days 8 to 13 and days 35 to 40, and they were cultured for *C. difficile*.

Of the 114 patients originally included, 88 were culture positive for *C. difficile* on day 1 and could be further evaluated. Of the 26 patients here excluded, 19 could not be evaluated due to lacking specimen ($n = 13$) or negative culture on day 1 ($n = 6$), and 7 patients could not be evaluated because their bacteriological data set (MIC plus the PCR ribotype of the isolate; see below) was incomplete. We thus studied the single pretreatment *C. difficile* isolate obtained from 47 patients who remained culture negative at follow-up as well as the pre- and posttherapy isolates from 41 patients who were culture positive at one or both follow-up visits (Table 1). The patients yielded a total of 139 *C. difficile* isolates that were subjected to PCR ribotyping and MIC determination of fusidic acid and metronidazole.

Culture of *C. difficile* and toxin testing. Fecal samples were cultured anaerobically on cycloserine-cefoxitin-fructose agar at 37°C for 48 h in anaerobic jars (Becton Dickinson Gas-Pac system; BBL, Cockeysville, Md.), and purified *C. difficile* isolates were harvested and stored at −70°C in preservation broth (trypticase soy broth [BBL] and yeast extract [Difco] plus 30% horse serum) until analysis. Feces were analyzed for *C. difficile* toxin B or using a McCoy cell assay (one hospital) or for toxin A plus B using the Premier enzyme immunoassay (eight hospitals; Meridian Diagnostics). The latter test also was applied to all *C. difficile* isolates.

PCR ribotyping of *C. difficile* isolates. Preparation of template, PCR, separation of PCR products, and analysis of banding patterns were performed according to Stubbs et al. (32) and improved by us as described elsewhere (26). For this reason, we used our own nomenclature for the PCR ribotypes identified (using the prefix "SE," for "Sweden"). The clustering of the banding patterns obtained was rechecked visually, and each pattern was given a number (SE type) plus, occasionally, a suffix indicating a closely related but distinct PCR ribotype.

Determination of MICs. Stored *C. difficile* isolates were thawed and cultured anaerobically on fastidious anaerobic agar (FAA; Lab M Ltd., Bury, United Kingdom) at 37°C for 48 h and suspended in nutrient broth (Oxoid) to a turbidity of 1.0 using the McFarland scale. This suspension was seeded on Iso-Sensitest agar (Oxoid) with defibrinated horse blood (5%) and 20 mg/liter β-nicotinamide adenine dinucleotide (Sigma), and Etest strips (Biodisk AB, Solna, Sweden) containing metronidazole or fusidic acid were placed on top, followed by anaerobic incubation at 37°C for 48 h. The MICs were read as recommended on the scale of the test strip where the elliptic zone of growth inhibition intersected. As no systematic breakpoints for fusidic acid are yet established for *C. difficile*, we used microbiological ones. Thus, drug MICs for typical (normal) isolates of ≤0.75 mg/liter were defined as susceptible, and higher MICs were defined as resistance.

Because the reference method for susceptibility testing of anaerobic bacteria is agar dilution and because discrepancies between Etest and agar dilution MICs for metronidazole have been reported (29), 25 random isolates also were tested against metronidazole using agar dilution. The *C. difficile* isolates were here grown anaerobically on FAA with 5% horse blood and suspended to a turbidity of McFarland 0.5. Plates containing a series of doubling metronidazole concentrations were then inoculated with the bacterial suspension, incubated anaerobically, and read according to the guidelines of the Clinical Laboratory Standards Institute (formerly NCCLS) (25).

Statistical methods. The chi-square test or Fisher's exact test was used where appropriate.

RESULTS

Origin and PCR ribotype of *C. difficile* isolates. A total of 94/114 (83%) patients were culture positive for *C. difficile* pretherapy, and 88 (fusidic acid treatment, $n = 44$; metronidazole treatment, $n = 44$) could be studied further (Table 1). Of patients receiving fusidic acid and metronidazole, 20% and 16% were culture positive for *C. difficile* also on days 8 to 13 ($P = 0.63$) and 22% and 31% at follow-up on days 35 to 40 ($P = 0.29$), respectively, yielding 41 patients who remained culture positive and 47 who were negative for *C. difficile* at follow-up. All fecal samples from patients with treatment failure or recurrence and their corresponding *C. difficile* isolates were toxin positive. Among the 88 day 1 isolates, a total of 31 distinct PCR ribotypes were identified, including 17 shared types and 14 unique types (each found in only one patient), without any difference in type distribution between treatment groups (data not shown). The 41 patients who were culture positive for *C. difficile* also at follow-up contributed an additional five PCR ribotypes, indicating new colonization or reinfection (data not shown).

Emergence of fusidic acid resistance in *C. difficile*. As the metronidazole MICs determined with agar dilution were fully

TABLE 2. Bacteriological data and clinical outcome for CDAD patients who were treated with fusidic acid and were culture positive for *C. difficile* on day 1 and later

Patient	Fusidic acid MIC (mg/liter) on day(s)[a,b]:			PCR ribotype (SE) on day(s)[b]:			Outcome on day(s)[c]:	
	1	8–13	35–40	1	8–13	35–40	8–13	35–40
1	0.25	>256	>256	22	22	22	+	
2	0.38	0.25	>256	19c	19c	19c		+
3	0.38	>256		25	25			
4	0.75		>256	21a		21a		
5	0.38	1	4	7	43	43		
6	0.75	>256		21b	21b		+	
7	0.5	24	0.25	16	16	41		+
8	0.75	48		12	12		+	
9	0.75	32		57	57			
10	0.125		48	29b		29b		+
11	0.125		>256	17		17		
12	0.5	0.38		12	12		+	
13	0.25		0.5	16e		29b		+
14	0.38	0.25		30	30			
15	0.25	0.25	0.25	25	25	25		+
16	0.25		0.19	1		19		+
17	0.25		0.25	16		16		
18	0.19		0.125	21		21		
19	0.125	0.25		28	28			
20	0.25		0.25	21b		2		

[a] The fusidic acid MIC was >256 mg/liter for 1 pretreatment isolate of 88 (metronidazole group). The metronidazole MICs of all the above and other isolates tested ($n = 139$) were <1 (median, 0.125; range, 0.016 to 0.5) mg/liter.
[b] An empty cell indicates that the patient was culture negative for *C. difficile* on that occasion.
[c] Diarrhea at follow-up is indicated by a plus sign.

consistent with the Etest readings (maximum difference, one dilution step; data not shown) for a subset of isolates ($n = 25$), Etest was used thereafter for metronidazole. The 139 pre- and posttherapy isolates of *C. difficile* available were uniformly susceptible to metronidazole (MIC < 1 mg/liter; see footnote a in Table 2). Of the day 1 isolates from 88 patients, 87 were susceptible to fusidic acid (MIC < 1 mg/liter). The drug MIC for one isolate was >256 mg/liter, but the corresponding CDAD patient was randomized to metronidazole and successfully treated. In 11 of 20 (55%) patients who remained culture positive after fusidic acid therapy, one or both follow-up isolates were resistant to fusidic acid, corresponding to 22% of all patients given fusidic acid (median MIC, >256; range, 1 to >256 mg/liter; Table 2). In 10 of these 11 patients, the resistant follow-up isolate(s) belonged to the same PCR ribotype as the susceptible day 1 isolate, confirming frequent emergence of fusidic acid resistance in the infecting *C. difficile* strain during treatment.

Relationship between bacteriological data and clinical outcome. (i) Culture positivity for *C. difficile*. Among patients who were culture positive on day 1 and again at follow-up(s), the failure rate and recurrence rate were 12% (5/41) and 34% (14/41), respectively, compared to 6% (3/47) and 17% (8/47), respectively, for those who had become culture negative at follow-up (Table 1). Thus, persistence of *C. difficile* at follow-up was associated with a lower rate of permanent cure (no failure or recurrence) compared to apparent clearance of the organism (54% versus 77% of patients; $P = 0.03$; Table 1). A similar association between bacterial persistence and a worse outcome of therapy was seen in both treatment groups. In all five patients who had culture-positive treatment failure, the *C. difficile* isolate belonged to the same PCR ribotype as the day 1 isolate, whereas 29% (4/14) of patients with culture-positive recurrence and 27% (6/22) of patients who were clinically permanently cured but remained *C. difficile* positive had acquired a new genotype.

(ii) PCR ribotypes. PCR ribotyping showed that in both treatment groups, the initial strain persisted in 76% (31/41) of the patients who remained culture positive at follow-up, whereas 24% (10/41) of such patients had acquired a new strain of *C. difficile* (five patients in each treatment group; Table 1). No particular PCR ribotype was associated with cure, treatment failure, recurrence (relapse or reinfection; data not shown), or emergence of fusidic acid resistance (Table 1).

(iii) Fusidic acid resistance. In 7 of the 11 patients whose *C. difficile* strain acquired fusidic acid resistance, this resistance was present on days 8 to 13 and was associated with failure in three cases, but in 4 patients it was not isolated until days 35 to 40 and was associated with recurrence in three cases (Table 2). Thus, of patients positive for *C. difficile* resistant to fusidic acid after treatment, 46% (5/11) were nevertheless permanently cured with this agent, compared to 56% (5/9) of patients who had fusidic acid-susceptible *C. difficile* at follow-up ($P = 1.0$).

DISCUSSION

The main result of the present study was the finding of both preexisting and rapid emergence of fusidic acid resistance in *C. difficile* during therapy. Thus, true development of resistance in the infecting strain was observed in 10 of the 20 patients able to be evaluated who were given fusidic acid. Furthermore, one patient had a resistant strain at follow-up not belonging to the initial PCR ribotype and, thus, was acquired either from the hospital environment or by emergence of fusidic acid resistance in a second strain of *C. difficile* carried (35). Interestingly, the single patient who carried a high-level resistant strain on day 1 (footnote a in Table 2) had received fusidic acid in the orthopedic surgery unit 2 weeks prior to his CDAD episode. On the other hand, the lack of fusidic acid resistance among 638 isolates of *C. difficile* collected before onset of this trial (unpublished data) suggests that the use of fusidic acid against staphylococci has had little long-term impact on this organism. The emergence of resistance in the infecting strain of *C. difficile* recorded in 55% of patients given fusidic acid therapy and who remained culture positive at follow-up (and, thus, able to be evaluated for resistance) is remarkable. One possible explanation for why this did not seem to affect the short-term result of fusidic acid therapy is that the resistant subpopulations selected were apparently too small to play any significant role in the outcome of the actual CDAD episode. However, the emerging resistant strains may easily be transmitted between patients and hamper any future wide-spread use of this drug for CDAD.

The mechanisms of fusidic acid resistance in *C. difficile* remain unknown. We have, however, recently identified a putative *fusA* gene in the *C. difficile* strain 630 genome (unpublished data). The infecting *C. difficile* strain was here shown to change from fully sensitive to highly resistant in six patients, indicating possible selection of a subpopulation with a single crucial *fusA* mutation, whereas in five other patients it had acquired mod-

erate-level resistance, suggesting less significant mutations in this gene or genetic up-regulation of an efflux system able to handle fusidic acid.

We used PCR ribotyping, the current reference method for typing of *C. difficile*, for comparison of initial and follow-up isolates from each patient studied. As expected, persistence of the initial strain was typical in patients who remained culture positive for *C. difficile* at follow-up, including 10 of the 11 patients whose *C. difficile* strain was fusidic acid resistant after therapy. Furthermore, our data did not indicate any clear association between PCR ribotype and outcome of CDAD therapy, supporting the notion that other factors, such as the size of the *C. difficile* population or immunity to *C. difficile* toxins and other factors related to the patient, were more important in this respect. It should be noted that the clinical part of this study was performed from 1999 to 2000, i.e., before the occurrence of the new epidemic *C. difficile* strain (PCR ribotype UK027, pulsed-field gel electrophoresis type NAP1; see Introduction) in Europe.

Another interesting finding was the frequent persistence of *C. difficile* in the gut in almost half of the patients after treatment, irrespective of the drug received, which also has been observed by others (10). This high persistence rate may reflect that the spores of the organism were refractory to the drug and was associated with an average rate of permanent cure (no therapeutic failure or recurrence within a month) of CDAD of only 54%, compared to 77% of the patients who became culture negative for *C. difficile* ($P = 0.03$). Similarly, bacterial persistence posttherapy is commonly observed also for non-spore-forming diarrhea pathogens, such as in campylobacteriosis and salmonellosis, irrespective of clearance of symptoms or emergence of bacterial resistance to the therapeutic agent given (39). Apart from this, factors other than the impact of antimicrobial agents may also influence the outcome of CDAD. Thus, the *C. difficile* strain itself, including its capacity for toxin production (36) and spore formation and its counts in the colon, could be important. In addition, host factors, such as the levels of nutrients in the gut content crucial to the regulation of toxin production (13, 14) and immunity, including preexisting and formation of new toxin-neutralizing antibodies, may contribute to the outcome of therapy for CDAD and the elimination of *C. difficile* (20, 31, 37).

In conclusion, the frequent posttherapy emergence of fusidic acid resistance in *C. difficile* supported the notion that fusidic acid is not suitable for widespread use in CDAD. Nevertheless, fusidic acid monotherapy could be an option in units with a vancomycin-resistant enterococci problem (vancomycin restricted), e.g., in CDAD patients who need treatment but cannot tolerate metronidazole. Other possible indications include adjunctive therapy in severe acute or recurring CDAD episodes when the second antimicrobial agent also could minimize an emergence of fusidic acid-resistant *C. difficile*. In contrast, metronidazole apparently remains a first-line drug associated with little or no resistance in *C. difficile*.

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