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In Vitro Activities of 15 Antimicrobial Agents against 110 Toxigenic *Clostridium difficile* Clinical Isolates Collected from 1983 to 2004[▽]

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The incidence and severity of *Clostridium difficile*-associated disease (CDAD) is increasing, and standard treatment is not always effective. Therefore, more-effective antimicrobial agents and treatment strategies are needed. We used the agar dilution method to determine the in vitro susceptibility of the following antimicrobials against 110 toxigenic clinical isolates of *C. difficile* from 1983 to 2004, primarily from the United States: doripenem, meropenem, gatifloxacin, levofloxacin, moxifloxacin, OPT-80, ramoplanin, rifalazil, rifaximin, nitazoxanide, tizoxanide, tigecycline, vancomycin, tinidazole, and metronidazole. Included among the isolates tested were six strains of the toxinotype III, NAP1/BI/027 group implicated in recent U.S., Canadian, and European outbreaks. The most active agents in vitro were rifaximin, rifalazil, tizoxanide, nitazoxanide, and OPT-80 with MICs at which 50% of the isolates are inhibited (MIC_{50}) and MIC_{90} values of 0.0075 and 0.015 μg/ml, 0.0075 and 0.03 μg/ml, 0.06 and 0.125 μg/ml, 0.06 and 0.125 μg/ml, 0.125 and 0.125 μg/ml, respectively. However, for three isolates the rifalazil and rifaximin MICs were very high (MIC of >256 μg/ml). Ramoplanin, vancomycin, doripenem, and meropenem were also very active in vitro with narrow MIC_{50} and MIC_{90} ranges. None of the isolates were resistant to metronidazole, the only agent for which there are breakpoints, with tinidazole showing nearly identical results. These in vitro susceptibility results are encouraging and support continued evaluation of selected antimicrobials in clinical trials of treatment for CDAD.

Clostridium difficile is the major identified infectious cause of nosocomial diarrhea, occurring mainly in patients previously administered antibiotics (2, 25). Vancomycin and metronidazole are first-line therapy for treatment of *C. difficile*-associated disease (CDAD) based on previous studies demonstrating equivalence of therapeutic outcomes (33, 35). However, recent data have shown increased CDAD rates and increased disease severity, as well as a higher risk of treatment failure and CDAD recurrence after treatment with metronidazole (20, 22, 29). In addition, the Centers for Disease Control and Prevention has discouraged vancomycin administration for treatment of CDAD in the hospital setting to minimize the risk of vancomycin resistance in enterococci and staphylococci (11). This places in question the adequacy or suitability of current treatments and warrants investigation of new antimicrobial agents active against *C. difficile*.

OPT-80 (previously known as tiacumicin B, proposed name difimicin) is a minimally absorbed, novel 18-membered macrocycle antibiotic that is currently under development for treatment of CDAD (7). Tinidazole is a structural analogue of metronidazole, with similar bioavailability (100%) and fewer drug-related adverse effects, but has similar in vitro activity against *C. difficile* (4, 9). Rifalazil and rifaximin are both rifamycin derivatives. Rifalazil is an orally absorbed systemic antibiotic with a broad spectrum of activity and has been shown to prevent and treat CDAD recurrence in a hamster model (1). Rifaximin, a nonsystemic antibiotic approved by the U.S. Food and Drug Administration for travelers' diarrhea, is currently under evaluation for treating CDAD (15). Ramoplanin, a poorly absorbed glycolipodepsipeptide evaluated for the prevention of vancomycin-resistant enterococci, has good in vitro activity against *C. difficile* (8). Nitazoxanide, a nitrothiazolide and metabolic precursor of tizoxanide, has broad-spectrum activity against helminths and protozoa, as well as bacterial enteric pathogens, including *C. difficile* (21). Nitazoxanide was recently shown comparable to metronidazole for CDAD treatment in a prospective, randomized, double-blinded clinical trial (23).

The susceptibility of other antimicrobials to *C. difficile* may relate to the propensity of that agent to precipitate CDAD (14). Quinolones have emerged as a major risk factor for CDAD during hospital outbreaks and the development of resistance to moxifloxacin and gatifloxacin has been associated with epidemic spread of the current NAP1/BI/027 strain of *C. difficile* (16, 19, 24, 30). Doripenem, a carbapenem (34), and tigecycline, a glycylcycline antibiotic structurally related to minocycline (10), are examples of new broad-spectrum agents that may have several clinical indications and whose use in the hospital setting may have an impact on the rates of CDAD. The purpose of the present study was to compare the in vitro activity of these agents against a large collection of toxigenic, clinical *C. difficile* isolates.

MATERIALS AND METHODS

***C. difficile* isolates.** *C. difficile* clinical isolates were selected based upon temporal, geographic, and genetic uniqueness considerations using restriction endonuclease analysis (REA) to identify strains from an international collection of over 6,000 isolates collected from 1983 to 2004 from the United States, South

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TABLE 1. Distribution of antimicrobials tested against 110 toxigenic clinical *C. difficile* isolates

Antimicrobial agent	No. of strains for which the antimicrobial agent MIC (μg/ml) was:															
	≤0.0019	0.0039	0.0078	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64[a]
Rifalazil	14	20	45	27	1											3*
Rifaximin	12	1	73	20	1											3*
OPT-80				9	11	29	50	11								
Tizoxanide				1	7	83	18		1							
Nitazoxanide					4	71	33		2							
Metronidazole					2	3	70	32	3							
Tinidazole					1	2	66	35	5	1						
Ramoplanin						1	19	45	45							
Tigecycline						2	89	17	1	1						
Vancomycin						2			34	68	5	1				
Doripenem										78	31	1				
Meropenem										16	89	5				
Gatifloxacin									3	73	17		1	9	6	1†
Moxifloxacin										57	38		1	8	6	
Levofloxacin											9	82	1		5	13‡

[a] *, Three strains had MICs of >256 μg/ml for rifalazil and rifaximin; †, one strain had an MIC of >32 μg/ml for gatifloxacin; ‡, thirteen strains had an MIC of >32 μg/ml for levofloxacin.

America, and Europe. All isolates were confirmed to be toxigenic by clinical fecal toxin testing and confirmed by REA typing to be in toxigenic *C. difficile* groups (5). Sixty-four REA unique isolate types were selected from the period from 1983 to 1998 largely from U.S. hospitals, but including seven isolates from Europe and Argentina. The remaining 46 isolates were selected from the period from 2000 to 2004 largely from U.S. hospitals reporting CDAD outbreaks. Five of the isolates were representatives of the new toxinotype III epidemic "BI" or NAP1/027 group. A BI group historic isolate from 1988, BI1, was included in the original group of 64 unique isolates for comparison with the more recent epidemic BI strains. Included in the 2000-to-2004 group were nine toxinotype V, REA BK group isolates that have been associated with animal disease but were isolated from humans (12). Also included were six unique isolates from the REA J group, including J7 and J9 responsible for multiple outbreaks in U.S. hospitals in the 1990s (14). In all, U.S. isolates were obtained from 10 hospitals in seven states located in the northern, northeastern, western, midwestern, and southwestern United States. Strains ATCC 25285 (*Bacteroides fragilis*), ATCC 29741 (*Bacteroides thetaiotaomicron*), and ATCC 700057 (*C. difficile*) were tested as controls.

Antimicrobial agents. The following antibiotics were tested: OPT-80 (Optimer Pharmaceuticals, Inc., San Diego, CA), metronidazole (Sigma-Aldrich, St. Louis, MO), tinidazole (Mission Pharmacal, San Antonio, TX), levofloxacin (Johnson & Johnson, Raritan, NJ), doripenem (Johnson & Johnson, Raritan, NJ), gatifloxacin (Bristol-Myers Squibb, New York, NY), moxifloxacin (Bayer, West Haven, CT), vancomycin (Sigma-Aldrich, St. Louis, Mo), rifalazil (ActivBiotics, Lexington, MA), rifaximin (Salix Pharmaceuticals, Inc., Morrisville, NC), ramoplanin (Oscient Pharmaceuticals, Waltham, MA), meropenem (AztraZeneca Pharmaceuticals, Wilmington, DE), tigecycline (Wyeth Research, Pearl River, NJ), nitazoxanide (Romark Laboratories L.C., Tampa, FL), and tizoxanide (Romark Laboratories L.C., Tampa, FL). The antimicrobials were tested using the following MIC ranges: OPT-80/PAR-101, metronidazole, and tinidazole, 0.0019 to 16 μg/ml; nitazoxanide and tizoxanide, 0.0039 to 16 μg/ml; rifalazil, rifaximin, and ramoplanin, 0.0039 to 32 μg/ml (rifalazil and rifaximin isolates with >32 μg/ml were retested at concentrations up to 256 μg/ml); vancomycin, 0.0078 to 16 μg/ml; doripenem and meropenem, 0.015 to 16 μg/ml; levofloxacin, gatifloxacin, and moxifloxacin, 0.03 to 32 μg/ml; and tigecycline, 0.015 to 32 μg/ml. The Clinical Laboratory and Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards) guidelines were used to dissolve and dilute all antimicrobial agents with the exception of rifalazil and OPT-80 (6). Both rifalazil and OPT-80 were dissolved in dimethyl sulfoxide (DMSO) and diluted in water exactly as described for metronidazole (6). Of note, the thiazolides were initially dissolved in DMSO, followed by further dilution in DMSO using a dilution scheme to achieve final desired concentrations of antibiotic when added to agar deeps and a maximum concentration of 0.5% DMSO. Control plates containing 0.5% DMSO were included with each test run to ensure their growth matched the growth control medium plates without antibiotics.

Agar dilution susceptibility testing. The CLSI-recommended reference agar dilution method for anaerobes (M11-A6) was used for susceptibility testing (26). Brucella agar supplemented with 5% laked sheep blood, 5 μg of hemin/ml, and 1 μg of vitamin K_1/ml was the test medium. Prior to testing, all isolates were subcultured twice onto enriched brucella agar plates. Standardization with a Vitek colorimeter was used to prepare each inoculum to the equivalent of a 0.5 McFarland standard, approximating 10^5 CFU per spot for *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 and 10^4 CFU/spot for *C. difficile* ATCC 700057 (6). All antibiotics were prepared and tested along with vancomycin and metronidazole as controls. The interpretation of endpoints was conducted according to CLSI guideline M11-A6 (26).

RESULTS

The MIC distributions for all antimicrobials are provided in Table 1 and the MIC_{50}, MIC_{90}, range, and geometric mean MIC data are shown in Table 2. All antibiotics tested fell within MIC quality control ranges that were recently established by the CLSI for *C. difficile* ATCC 700057 (6). All *C. difficile* strains were inhibited by metronidazole at concentrations of ≤0.5 μg/ml (100% susceptible), and all but one strain were inhibited by vancomycin at a concentration of 2.0 μg/ml. One strain had an MIC to vancomycin of 4 μg/ml and was recovered from an 84-year-old male as part of a study con-

TABLE 2. MICs for antimicrobial agents tested against 110 toxigenic clinical *C. difficile* isolates

Antimicrobial agent	MIC (μg/ml)			
	Range	MIC_{50}	MIC_{90}	Geometric mean
Rifaximin	0.0038–>16	0.0075	0.015	0.009
Rifalazil	0.0019–>16	0.0075	0.03	0.0067
Tizoxanide	0.015–0.5	0.06	0.125	0.0652
Nitazoxanide	0.03–0.5	0.06	0.125	0.076
OPT-80	0.015–0.25	0.125	0.125	0.081
Tigecycline	0.06–1.0	0.125	0.25	0.142
Metronidazole	0.025–0.5	0.125	0.25	0.149
Tinidazole	0.03–1.0	0.125	0.25	0.165
Ramoplanin	0.06–0.5	0.25	0.5	0.291
Vancomycin	0.06–4.0	1.0	1.0	0.801
Doripenem	0.5–4.0	1.0	2.0	1.19
Meropenem	1.0–4.0	2.0	2.0	1.87
Gatifloxacin	0.5–64	1.0	16	1.752
Moxifloxacin	0.5–32	1.0	16	1.90
Levofloxacin	2.0–64	4.0	32	5.801

ducted in 1983 (13). Rifalazil and rifaximin were the most active agents in vitro, inhibiting *C. difficile* strains at lower concentrations (≤0.0019 to 0.03 μg/ml, respectively) compared to the other antimicrobials tested and demonstrating low geometric mean MICs (0.0067 and 0.009 μg/ml, respectively). For only three *C. difficile* strains, did both rifalazil and rifaximin demonstrate high MICs (≥256 μg/ml). Nitazoxanide and tizoxanide had nearly identical in vitro activity against all strains tested, with an MIC_{50} and an MIC_{90} of 0.06 and 0.125 μg/ml, respectively. OPT-80 also showed very good activity against all *C. difficile* strains (MIC_{90} of 0.125 μg/ml). Tigecycline, tinidazole, and metronidazole had identical MIC_{50} and MIC_{90} values (0.125 and 0.25 μg/ml, respectively) and highly similar geometric mean MICs. Ramoplanin exhibited slightly greater in vitro activity than vancomycin, with an MIC_{90} of 0.5 μg/ml. Doripenem and meropenem showed good activity, both with an MIC_{90} of 2.0 μg/ml, and similar geometric mean MICs. Among the fluoroquinolones, levofloxacin demonstrated the highest MICs against *C. difficile* (MIC_{90} = 32 μg/ml). Of 110 isolates, 18 (16%) had levofloxacin MICs of ≥32 μg/ml, whereas seven and six isolates had MICs of ≥32 μg/ml for gatifloxacin and moxifloxacin, respectively. Specifically, for the five epidemic BI strains, the three quinoline MICs were high (≥32 μg/ml), whereas for the single historic BI1 isolate the MICs for these agents were low (≤2 μg/ml). The frequency of levofloxacin MICs of ≥32 μg/ml in isolates collected between 1983 and 1998 was 11% and was not statistically different from the 24% found in isolates from 2000 to 2004 ($P > 0.10$). However, isolates for which the levofloxacin MICs were ≥32 μg/ml were found only since 1990.

DISCUSSION

In this study, 15 antibiotic agents, including the two antimicrobials currently used as standard therapy for CDAD, vancomycin and metronidazole, were evaluated for in vitro activity against 110 toxigenic clinical *C. difficile* isolates. All 110 isolates were susceptible to metronidazole, the only antibiotic for which breakpoints are established, and vancomycin MICs tested within a very narrow low range. Pelaez et al. (28) reported resistance to metronidazole and vancomycin among isolates of *C. difficile*, a finding not confirmed in the present study. Six of the strains here were of the toxinotype III, REA BI group (PFGE type NAP1) implicated in recent epidemic CDAD outbreaks. For all of the epidemic BI and J group isolates, low antimicrobial agent MICs were found with the potential treatment agents, including OPT-80, ramoplanin, rifalazil, rifaximin, nitazoxanide, tizoxanide, and tinidazole. Similar low antimicrobial agent MICs were found for all BK group, toxinotype V isolates associated with animal disease (12). The rifamycin derivatives, rifalazil and rifaximin, were the most active against *C. difficile* in our study. Rifalazil was previously shown by Anton et al. to achieve cure and prevent relapse in a hamster model of CDAD (1). Rifaximin also demonstrated very good in vitro activity against *C. difficile* and was successful in treating nine of ten CDAD patients in a small clinical trial ($n = 20$) in Italy (3). For three *C. difficile* isolates, rifalazil and rifaximin demonstrated high MICs (>256 μg/ml). Two of these resistant *C. difficile* isolates were obtained from Argentina in 1998, and the third was from Chicago in 1995. These results could impact the clinical use of these two antibiotics if resistance were to become more widespread. However, in vitro resistance selection studies suggest that *C. difficile* has a particularly low incidence of spontaneously resistant rifaximin mutants (18).

The present study shows that relatively low concentrations of the structurally related agents, nitazoxanide and tizoxanide, are needed to inhibit growth of *C. difficile* (MIC_{90} of 0.125 μg/ml), a finding comparable to the nitazoxanide and tizoxanide MIC_{90} of 0.06 μg/ml for 21 *C. difficile* strains determined in another in vitro study (27). Nitazoxanide was found to be noninferior to metronidazole in a randomized, double-blind prospective patient trial ($n = 110$), confirming a clinical efficacy consistent with these in vitro susceptibility results (23). Similarly, the minimally absorbed oral agent OPT-80 exhibited a low MIC_{90} in the present study and has been shown to be highly effective in a hamster model of CDAD and in the preliminary report of a phase II trial for the treatment of patients with CDAD (17, 32). The in vitro activity of another poorly absorbed oral agent, ramoplanin, closely resembles that of vancomycin in our study. This may explain prior data showing that both ramoplanin and vancomycin were similarly effective at reducing cytotoxin production in a human gastrointestinal model of CDAD and in resolving symptoms in both a hamster model of CDAD and CDAD patients as noted in a preliminary report of a phase II clinical trial (8, 31).

Other antimicrobial agents tested in the present study demonstrated variability in activity against *C. difficile* isolates. Meropenem and doripenem both showed in vitro activity against all *C. difficile* strains within a narrow testing range (0.5 to 4 μg/ml), levels that may be achievable in the colon and could suggest a lower risk of developing of CDAD. However, distribution of this agent in the gut and its effect on other gut flora may also contribute to increase the risk of CDAD. Tigecycline in vitro activity was similar to that of metronidazole; however, there is little clinical data for this agent in regard to the risk of CDAD. The fluoroquinolones demonstrated higher MICs than that of nearly all other antimicrobial agents tested and were the least active in vitro. High fluoroquinolones MICs have been a shared characteristic for the epidemic NAP1/BI/027 *C. difficile* isolates and may, in part, explain why fluoroquinolone use has been implicated in recent CDAD outbreaks (16, 19, 24). Importantly, with the exception of decreased susceptibility to fluoroquinolones in recent years, especially among BI isolates, MICs were highly similar for all other agents, including metronidazole and vancomycin, over the 21-year span of isolates tested in the present study. The lack of change in susceptibility results to metronidazole and vancomycin, agents most commonly used to treat CDAD, is reassuring.

In summary, several newer agents, as well as established antimicrobials, have very good to excellent activity against a wide range of *C. difficile* isolates, and additional evaluation of their clinical efficacy in treatment of CDAD is warranted.

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