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Rifampin and Rifaximin Resistance in Clinical Isolates of *Clostridium difficile*▽†

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Rifaximin, a poorly absorbed rifamycin derivative, is a promising alternative for the treatment of *Clostridium difficile* infections. Resistance to this agent has been reported, but no commercial test for rifaximin resistance exists and the molecular basis of this resistance has not been previously studied in *C. difficile*. To evaluate whether the rifampin Etest would be a suitable substitute for rifaximin susceptibility testing in the clinical setting, we analyzed the in vitro rifaximin susceptibilities of 80 clinical isolates from our collection by agar dilution and compared these results to rifampin susceptibility results obtained by agar dilution and Etest. We found rifaximin susceptibility data to agree with rifampin susceptibility; the MICs of both antimicrobials for all isolates were either very low or very high. Fourteen rifaximin-resistant (MIC, ≥32 μg/ml) unique isolates from patients at diverse locations in three countries were identified. Molecular typing analysis showed that nine (64%) of these isolates belonged to the epidemic BI/NAP1/027 group that is responsible for multiple outbreaks and increased disease severity in the United Kingdom, Europe, and North America. The molecular basis of rifaximin and rifampin resistance in these isolates was investigated by sequence analysis of *rpoB*, which encodes the β subunit of RNA polymerase, the target of rifamycins. Resistance-associated *rpoB* sequence differences that resulted in specific amino acid substitutions in an otherwise conserved region of RpoB were found in all resistant isolates. Seven different RpoB amino acid substitutions were identified in the resistant isolates, which were divided into five distinct groups by restriction endonuclease analysis typing. These results suggest that the amino acid substitutions associated with rifamycin resistance were independently derived rather than disseminated from specific rifamycin-resistant clones. We propose that rifaximin resistance in *C. difficile* results from mutations in RpoB and that rifampin resistance predicts rifaximin resistance for this organism.

In recent years, the incidence and severity of *Clostridium difficile* infection (CDI) has increased significantly. Numerous *C. difficile* outbreaks in North America, the United Kingdom, and Europe have been caused by the BI/NAP1/027 epidemic group. This clonal group of isolates, which has been characterized by restriction endonuclease analysis (REA), pulsed-field gel electrophoresis, and PCR ribotyping, is described as hypervirulent due to the associated increase in disease severity compared to nonepidemic isolates (12, 13, 21, 24). Treatment failures and CDI recurrences appear to be more frequent as well, which highlights the need for the development of new therapeutic strategies (20). Rifaximin, a poorly absorbed rifamycin derivative with broad efficacy across the gram-positive and gram-negative spectra, has been approved in the United States for the treatment of traveler's diarrhea and is being evaluated for the treatment of CDI (8, 11). Several studies have investigated the efficacy of rifaximin against *C. difficile* in vitro and in vivo, and although rifaximin appears to have good activity against most *C. difficile* isolates, some strains for which the MICs are high have been identified (7, 11, 15).

Antimicrobials belonging to the rifamycin group, including rifampin and rifaximin, inhibit protein synthesis in bacteria by binding to RpoB, the β subunit of RNA polymerase (6). Studies of a variety of bacterial genera have shown that exposure to rifamycins in vitro and in vivo can lead to the selection of resistant organisms, which carry specific single amino acid mutations within RpoB (5, 10, 17, 23). These mutations have been systematically mapped in *Staphylococcus aureus* and *Mycobacterium tuberculosis* RpoB and occur within a defined region of the protein (17, 19). X-ray crystallography analysis of *Thermus aquaticus* RpoB complexed with rifampin provided an explanation for the locations of the specific mutations found in rifamycin-resistant organisms (2). In three-dimensional space, the RpoB amino acids that confer rifamycin resistance either directly interact with rifampin or are in close proximity to those that are involved in rifampin interactions (2). However, it is not known whether *C. difficile* strains for which the rifaximin MICs are high carry RpoB amino acid substitutions characteristic of other rifamycin-resistant bacteria.

We hypothesized that *C. difficile* isolates for which the MICs of rifaximin are high would be differentiated from isolates for which the MICs are low by specific RpoB amino acid substitutions. In addition, we hypothesized that the rifampin susceptibility and rifaximin susceptibility results for these isolates

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TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence (5′–3′)	Use
DGP22	AATATTGATGATATAGATCACCTAGGAAACA	Forward primer, amplification/sequencing of *rpoB*
DGP23	AAATTGTGCTTTACCACCTAGTGGCT	Reverse primer, amplification/sequencing of *rpoB*
DGP26	GATGCGTTAGAATGAACTATTG	Forward primer, amplification of *rpoB*
DGP27	AAGTGCATCATCTTTATTAAATGC	Forward primer, amplification/sequencing of *rpoB*
DGP28	GAAAGAGCTGGATTCGAAGTG	Forward primer, amplification/sequencing of *rpoB*
DGP29	GAAGGTTATAACTACGAGGATGCT	Forward primer, amplification/sequencing of *rpoB*
DGP30	GAATAACTGTTGGATATATGTATTACTTG	Forward primer, amplification/sequencing of *rpoB*
DGP31	TCAAACAAGGAGTTCTCTCC	Reverse primer, amplification/sequencing of *rpoB*
DGP32	ACTTCTAGAACCTGACCGATGT	Reverse primer, amplification/sequencing of *rpoB*
DGP33	TATATCTATCTCTGTTTCCATCTTCTC	Reverse primer, amplification/sequencing of *rpoB*
DGP34	GCTGAAACTGGTCTTATGTTAACT	Reverse primer, amplification/sequencing of *rpoB*
DGP35	CCTAAAAGGTCAATTATCTCTGCATC	Reverse primer, amplification/sequencing of *rpoB*
DGP36	TGGAAAATTGTACATAATACATAATATATGC	Forward primer for amplification/sequencing of *rpoB*
DGP37	ATGGAGCAGAAAGAGTTATAGTAAGTCA	Forward primer, amplification/sequencing of *rpoB* conserved region
DGP38	TCATCAGTAACTGTTGATGTTTCTTTATCA	Reverse primer, amplification/sequencing of *rpoB* conserved region

would be in agreement. Therefore, commercially available rifampin susceptibility test kits could be used to predict the rifaximin susceptibility of *C. difficile* isolates in the clinical setting.

MATERIALS AND METHODS

***C. difficile* isolates.** A total of 80 unique patient isolates were selected from our *C. difficile* collection as follows. Sixteen isolates (4 rifaximin resistant [MICs, ≥32 μg/ml] and 12 nonresistant [MICs, <32 μg/ml]) were selected from previous analyses in which rifaximin susceptibility testing was performed (7, 11). Two rifaximin-resistant isolates were obtained from the clinical practice of one us (S. Johnson, unpublished data).

The remaining 62 isolates were obtained from patients enrolled in a multicenter clinical treatment trial, which compared the efficacy of the toxin-binding polymer tolevamer to vancomycin and metronidazole for the treatment of CDI (14). The study was conducted in the United States and Canada with patients enrolled from 2005 to 2007 (14). This sample of 62 isolates had not previously been tested for susceptibility. If two or more isolates were obtained from the same patient, had the same susceptibility profile, and could not be differentiated at the molecular level, only one representative was included in our study. All of the *C. difficile* isolates in this study were characterized by HindIII REA as previously described (3).

Susceptibility testing. Agar dilution susceptibility testing was performed as previously described, by using the Clinical and Laboratory and Standards Institute-recommended reference agar dilution method for anaerobes (4, 7). Rifaximin (Salix Pharmaceuticals, Inc., Morrisville, NC) was dissolved in methanol and then diluted in 0.1 M phosphate buffer (pH 7.4) plus 0.45% sodium dodecyl sulfate. Rifampin (Sigma-Aldrich, St. Louis, MO) was dissolved in methanol and diluted in water. The range of rifaximin concentrations tested was 0.0009 to 256 μg/ml. The range of rifampin concentrations tested was 0.00001 to 256 μg/ml. The interpretation of endpoints was conducted according to Clinical and Laboratory and Standards Institute guideline M11-A7, and *C. difficile* ATCC 70005 was used as a control (4). Rifampin Etests were performed according to the manufacturer's instructions (AB Biodisk, Solna, Sweden), with brucella blood agar (Anaerobe Systems, San Jose, CA). The range of rifampin MICs detected by Etest was 0.002 to 32 μg/ml. Although no susceptibility or resistance breakpoints have been determined for rifaximin or rifampin against anaerobes, we designated isolates for which the MICs were ≥32 μg/ml resistant.

Identification of sequence differences in the *rpoB* gene. The *rpoB* gene of *C. difficile* QCD-32g58 (accession no. NZ_AAML04000015) was identified by a BLASTn search of the database for that sequence (http://www.ncbi.nlm.nih.gov/genomes/geblast.cgi?gi=5410) by using the *rpoB* gene from *C. difficile* strain 630 as the query sequence (accession no. NC_009089.1) (1). *C. difficile* QCD-32g58 *rpoB* was then used as the template for designing oligonucleotide primers. PCR amplification and sequencing of *rpoB* were carried out on 19 *C. difficile* isolates by using primers listed in Table 1 and the FailSafe PCR system with reaction buffer E, according to the manufacturer's recommendations (Epicenter Biotechnologies, Madison, WI). Amplicons were either directly purified from the PCR or gel purified, as required (Qiagen Inc., Valencia CA). The entire *rpoB* gene was sequenced from eight strains (four resistant and four nonresistant isolates). The genomic DNA templates for these experiments were prepared as previously described (18).

PCR amplification and sequencing of the genomic region corresponding to amino acids 136 to 550 of RpoB were conducted for 10 rifaximin-resistant isolates and 1 nonresistant isolate with the primers listed in Table 1. This amino acid sequence corresponds to the defined region of RpoB in *S. aureus* and *M. tuberculosis* where mutations leading to rifamycin resistance commonly occur (17). DNAs were extracted from overnight agar cultures of these 11 isolates by resuspending *C. difficile* cells in 50 μl sterile deionized water until the suspension was visibly turbid. The suspension was boiled for 10 min, incubated on ice for 5 min, and then centrifuged at 22,000 × *g* at 4°C for 10 min, and the supernatant was used in a PCR. Ten microliters of the crude DNA preparation was used in each reaction mixture, with a final volume of 50 μl. DNA sequencing was carried out at the DNA Services Facility, Research Resources Center, University of Illinois at Chicago.

RESULTS

Rifaximin and rifampin MICs for *C. difficile* isolates are comparable. Rifampin and rifaximin MICs determined by agar dilution and rifampin MICs obtained by Etest were consistent. The MICs of both rifamycin derivatives were either high (>32 μg/ml) or low MICs (≤0.002 μg/ml) by both susceptibility testing methods. None of the MICs fell within the range of 0.002 to 32 μg/ml. The rifampin and rifaximin MICs were >32 μg/ml for 14 of the 80 unique patient isolates.

Most rifaximin-resistant isolates were from epidemic REA group BI. Nine of the 14 isolates that were resistant to rifampin and rifaximin (MICs, >32 μg/ml) were typed as epidemic REA group BI. This REA group is responsible for the large North American and European outbreaks reported in previous studies (13, 16, 24). These 9 resistant BI isolates accounted for all of the resistant isolates identified within the group of 62 from the tolevamer study (14). The overall proportion of BI group isolates in this 62-isolate sample was 51.6%. The remaining five resistant isolates were typed as REA group R ($n = 2$), K ($n = 1$), N ($n = 1$), and CF ($n = 1$) isolates (Table 2).

Amino acid sequence substitutions in *C. difficile* RpoB are associated with rifamycin resistance. RpoB of *C. difficile* QCD-32g58 (accession no. NZ_AAML04000015) was identified and aligned with RpoB of *S. aureus* (accession no. CAG39568), *M. tuberculosis* (accession no. ABR05024), and *T. aquaticus* (accession no. CAB65465) (data not shown). Figure 1 depicts the conserved region of *C. difficile* RpoB aligned with that of *S. aureus*, which was the most similar protein of the

TABLE 2. Rifaximin and rifampin MICs for *C. difficile* clinical isolates[a]

Antimicrobial agent (method)	MIC (μg/ml)		
	Range	50% of strains	90% of strains
Rifaximin (AD)[b]	0.0019–>256	0.0078	>256
Rifampin (AD)	0.00006–>256	0.0009	>256
Rifampin (Etest)	<0.002–>32	<0.002	>32

[a] See Table S1 in the supplemental material.
[b] AD, agar dilution.

TABLE 3. RpoB sequence substitutions, REA groups, and geographic origins of 14 rifaximin-resistant isolates

RpoB sequence substitution(s)	REA group (no. of isolates)	Geographic origin(s) (no. of isolates)
S488T, R505K	BI (1)	Quebec
D492N, R505K	K (1)	Illinois location a[a]
H502N, R505K	BI (4)	Delaware (1), California locations c[a] (2) and d[a] (1)
H502N, R505K	R (2)	Argentina (2)
H502Y	CF (1)	Illinois location b[a]
H502R	N (1)	Illinois location b[a]
R505K, I548M	BI (1)	Vermont
R505K[b]	BI (3)	Maryland (1), Nova Scotia (2)

[a] Rifamycin-resistant isolates were obtained from two geographically separate hospitals or clinics in Illinois (locations a and b) and two in California (locations c and d).
[b] Agar dilution reproducibly yielded rifampin MICs of 128 μg/ml and rifaximin MICs of >256 μg/ml for the three resistant isolates with just the R505K amino acid substitution. The MICs of both rifampin and rifaximin for the other 11 resistant isolates were >256 μg/ml by agar dilution.

three comparison sequences (66% amino acid identity with *C. difficile* RpoB). Twenty-five of the 26 *S. aureus* RpoB single amino acid substitutions that are known to be associated with rifamycin resistance occur between residues 137 and 529, which are equivalent to amino acids 136 to 550 of *C. difficile* RpoB (Fig. 1) (17). This region was analyzed in all 14 rifamycin-resistant and 5 nonresistant *C. difficile* isolates.

The predicted amino acid sequence of the analyzed region of RpoB (amino acids 136 to 550) from 19 *C. difficile* isolates was conserved and could clearly be divided into two categories according to phenotype, i.e., rifamycin resistant and nonresistant. All 14 resistant isolates had sequence differences within this region compared to the 5 nonresistant isolates; a total of seven amino acid substitutions were identified at five locations (Table 3; Fig. 1). Four of these amino acids were equivalent to amino acids of *T. aquaticus* RpoB that interact with rifampin (Fig. 1) (2). The locations of all of the amino acid substitutions corresponded to locations where substitutions were associated with rifamycin resistance in *S. aureus* RpoB (Fig. 1) (17). All of the resistant *C. difficile* isolates were obtained from separate patients and originated from at least 10 different hospitals in Canada (Quebec and Nova Scotia), the United States (Illinois, Delaware, California, Vermont, and Maryland), and Argentina (Table 3).

One set of paired patient specimens came from a patient before and after the completion of two courses of rifaximin, which was used in an attempt to interrupt multiple recurrences of *C. difficile* disease (11). These two isolates had identical REA patterns (group CF strains, Fig. 2), but the rifaximin MICs for the pre- and posttreatment isolates were 0.0039 and >256 μg/ml, respectively. Remarkably, comparison of the entire *rpoB* sequences of these two isolates revealed that they differed by only a single nucleotide, which resulted in an amino acid substitution within the conserved region of RpoB of the resistant isolate (H502Y) (Table 3).

DISCUSSION

In this study, 80 *C. difficile* unique patient isolates were analyzed for susceptibility to rifaximin and rifampin. Comparison of the agar dilution rifaximin and rifampin MICs to rifampin Etest results showed that the rifampin Etest can reliably predict rifaximin resistance in *C. difficile* (Table 2). Rifamycin susceptibility testing of these isolates yielded a bimodal distribution pattern. The MICs for the isolates were either very high or very low, which agrees with previous findings (7, 15). In addition, rifamycin resistance was detected among the members of epidemic REA group BI. These isolates accounted for more than half of the 14 resistant unique patient isolates included in this study (Table 3). However, determination of the clinical implications of our findings will require the correlation of *C. difficile* rifaximin resistance with treatment response in patients infected with resistant strains.

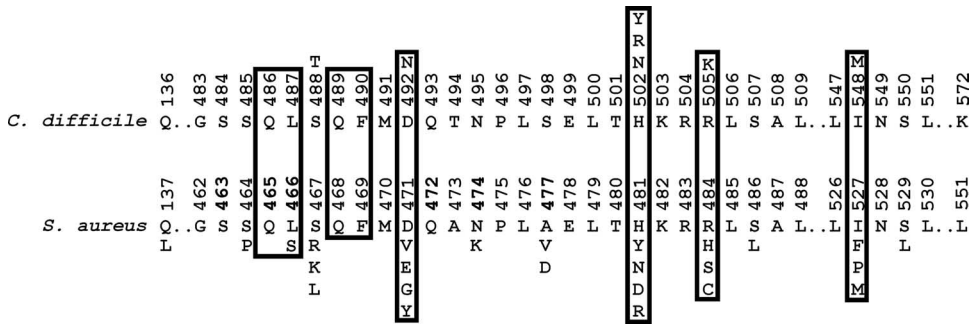

FIG. 1. Locations of amino acid substitutions within the conserved region of RpoB in rifamycin-resistant *C. difficile* relative to those which are associated with resistance in *S. aureus*. Amino acid numbering for *S. aureus* is based on strain MRSA252 (accession no. CAG39568), and for *C. difficile*, amino acid numbering is based on strain QCD-32g58 (accession no. NZ_AAML04000015). Amino acid substitutions associated with rifamycin resistance are shown above (*C. difficile*) or below (*S. aureus*) the amino acid sequence (17). Boxed residues are equivalent to *T. aquaticus* RpoB amino acids that directly interact with rifampin (2).

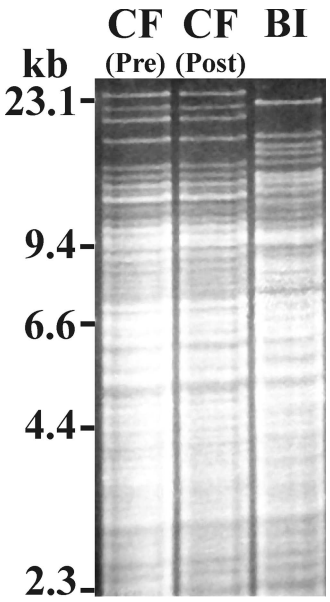

FIG. 2. REA patterns of HindIII-digested genomic DNAs purified from two group CF *C. difficile* isolates obtained from a patient prior to rifaximin therapy [CF (Pre)] and after treatment failure [CF (Post)] (11). The REA pattern of a group BI strain is shown for comparison.

Clinical correlation studies will be particularly important, considering that patients undergoing rifaximin therapy can have fecal rifaximin concentrations as high as 8,000 μg/g (9).

Fourteen of the 80 isolates we analyzed were resistant to rifaximin and rifampin (MICs, >32 μg/ml). Compared to nonresistant isolates, sequence differences in the *rpoB* gene of the resistant isolates resulted in amino acid substitutions that most likely explain this phenotype. No other rifamycin resistance mechanisms have been characterized in *C. difficile*. RpoB amino acid substitutions associated with rifamycin resistance were not clonal and were most likely selected for due to exposure to these antimicrobials (Table 3). Analysis of the REA grouping of the rifaximin-resistant isolates showed that the same sequence substitutions (H502N and R505K) could be detected in more than one REA group (Table 3). This result is not unexpected, since the equivalent amino acids of the *T. aquaticus* RpoB ortholog directly interact with rifampin (Fig. 1) (2). In addition, previous studies with *S. aureus* and *M. tuberculosis* have shown that independently derived mutations in these residues are frequently detected (17, 19, 22).

In our study, the nine rifamycin-resistant isolates within the BI epidemic group had four RpoB sequence differences compared to nonresistant isolates. The R505K amino acid substitution was found in all of the resistant BI group isolates, either as the only substitution or in addition to S488T, H502N, or I548M (Table 3). Each of these isolates was sampled from a separate patient and originated from four different states within the United States and two Canadian provinces (Table 2). These data suggest that the BI isolates were unlikely to have resulted from the clonal dissemination of a single resistant strain. This information further demonstrates that rifamycin therapy could be responsible for the selection of these resistant isolates in individual patients. This conclusion is also supported by the results from the paired patient isolates, which were sampled before rifaximin therapy and after treatment failure (Fig. 2) (11). The RpoB sequence of the rifaximin-resistant posttreatment isolate differed by only one amino acid from that of the nonresistant pretreatment strain (H502Y) (Table 3 and Fig. 1).

We have shown that the rifampin Etest is a suitable surrogate for rifaximin susceptibility testing by agar dilution. Rifaximin-resistant *C. difficile* isolates all carried sequence substitutions within a conserved RpoB region compared to nonresistant isolates. Molecular typing and analysis of geographic origins determined that these isolates were likely to be independently derived and that rifamycin therapy may have selected for them. We have shown that rifamycin resistance in clinical isolates of *C. difficile* may be more common than initially suspected, particularly among epidemic BI isolates (7). Further studies are warranted to address the clinical implications of these findings.

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