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ORIGINAL ARTICLE

Antecedent use of fluoroquinolones is associated with resistance to moxifloxacin in *Clostridium difficile*

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Objective Moxifloxacin is characterized by high activity against Gram-positive cocci and some Gram-positive and -negative anaerobes, including *Clostridium difficile*. This study investigates the role of prior quinolone use in relation to patterns of susceptibility of *C. difficile* to moxifloxacin.

Methods Sixty-three clinical isolates of *C. difficile* were investigated for toxigenicity, susceptibility to moxifloxacin, and mutations in the DNA gyrase gene. The medical histories for 50 of these patients were available and used to identify previous fluoroquinolone use.

Results Thirty-three (52.4%) strains showed resistance to moxifloxacin (MICs $\geq$16 mg/L). All moxifloxacin-resistant strains harbored a mutation at amino acid codon Ser-83 of *gyrA*. Forty-five isolates (71.4%) were toxigenic; all moxifloxacin-resistant strains were in this group. Resistance to moxifloxacin was associated with prior use of fluoroquinolones (*P*-value 0.009, chi-square).

Conclusions Although the use of moxifloxacin to treat *C. difficile*-associated diarrhea is not likely to be common, these data show a relationship between antecedent fluoroquinolone use and resistance to moxifloxacin in *C. difficile* isolates, and raise questions regarding selection pressure for resistance placed on colonizing bacteria exposed to fluoroquinolones. Mutations in *gyrA* are involved in moxifloxacin resistance.

Keywords *Clostridium difficile*, moxifloxacin, resistance

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INTRODUCTION

Worldwide, physicians are confronted with significant increases in the prevalence of resistance to antibiotics. The broad acceptance of fluoroquinolones and the introduction of new highly active agents suggest that their use will increase. Recognizing the factors contributing to the emergence of resistance will be useful for the management and monitoring of antibiotic resistance in healthcare facilities.

Clostridium difficile-associated diarrhea (CDAD) remains the leading cause of nosocomial diarrhea, and constitutes a major financial burden, due to the high costs of prolonged hospital stays and increased morbidity [1]. Although all antibiotics are capable of causing CDAD, clindamycin and second- and third-generation cephalosporins are known to be major risk factors for *C. difficile* infection. Aminoglycosides and group I–III fluoroquinolones with poor activity against anaerobes are rarely associated with CDAD [2,3]. Whether these classes of antimicrobials have any effect on the anaerobe population of the intestinal tract is not clear. A study from 1997 reported a risk of acquisition of quinolone-resistant strains of

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Gram-negative bacilli due to selective contamination of the digestive tract with low-dose ciprofloxacin [4]. *C. difficile* is readily transmissible in hospital settings. The organism is prevalent in the hospital environment, and clonal transmission has been reported [5,6]. Careful strain identification and typing are important tools in epidemiologic and microbiological studies.

New-generation fluoroquinolones (group IV) are characterized by improved activity against Gram-positive cocci and some Gram-positive and -negative anaerobes, including *C. difficile* [3,7]. Some data exist on the susceptibility of *C. difficile* to newer fluoroquinolones [8–11]. Whether clinical use of fluoroquinolones for *C. difficile* infection has potential as a treatment option requires further study.

Fluoroquinolones have been broadly accepted for the treatment of patients with community-acquired and nosocomial infections. The convenience of newer derivatives in terms of dosing regimens (once or twice daily) and wide antimicrobial activity suggests that their use will increase. Resistance has already emerged in several microorganisms, and mechanisms of resistance have been extensively studied. The process whereby organisms develop resistance can lead to either cross-resistance or dichotomous resistance to other quinolones, depending on the quinolones and mechanisms involved. Newer fluoroquinolones, especially those with an 8-methoxy group, such as moxifloxacin, cause different patterns of resistance. Dichotomous resistance occured between older and newer quinolones in studies with Gram-positive bacteria [12]. The greater lethal effect of the newer compounds, and the fact that they target both DNA gyrase and topoisomerase IV, may contribute to less frequent selection of resistance [12].

The aim of this study was to determine whether antecedent fluoroquinolone use in patients with CDAD was associated with resistance to moxifloxacin of the *C. difficile* isolates.

MATERIALS AND METHODS

Patient population and bacterial strains

This study was done at two tertiary teaching hospitals (University of California, Davis, Medical Center, and University of Leipzig, University Hospital, Germany). *C. difficile* isolates recovered from 1994 to 2000 from the stools of 63 inpatients were included (four strains isolated in Germany, 59 in the USA). Only one isolate per patient was analyzed in the study (19 of the 63 isolates were also used in an earlier study) [9]. All patients had symptoms of CDAD. The medical records from 50 patients were available and searched for prior use of fluoroquinolones. We defined antecedent antimicrobial use as treatment with fluoroquinolones within three months before isolation of *C. difficile*.

Strain identification

The strains of *C. difficile* were grown anaerobically on the selective medium cycloserine–cefoxitin–fructose agar (CCFA). Isolates were identified as *C. difficile* by the latex agglutination test (Becton Dickinson, Cockeysville, MD, USA) and the Pro-Disc test (Remel, Norcross, GA, USA), and PCR was used to identify toxin A and toxin B genes. Strains were maintained in cooked meat broth (Hardy Diagnostics, Santa Maria, CA, USA). Toxin A and B genes were amplified as described elsewhere [13].

Antimicrobial susceptibility testing

MICs were determined with Etest strips (AB Biodisk, Solna, Sweden), used according to the manufacturer's instructions. *Bacteroides fragilis* ATCC 25285 and *Staphylococcus aureus* ATCC 29123 were used as reference strains.

Genotyping

Arbitrarily primed PCR (AP-PCR) was performed with the 19-mer oligonucleotide T-7 as the primer. The DNA banding patterns were compared by running PCR products on the same gel as described previously [14].

Amplification and sequencing of *gyrA*

A 247-bp fragment of *gyrA* was amplified using specific primers CdgaV (5′-TTTAAAGCCAGTTCATAG-3′) and CdgaR (5′-GAACCAAAGTTACCATG-3′) [9]. Thirty cycles of the following PCR profile were run: 30 s at 95 °C, 30 s at 48 °C, and 60 s at 72 °C. The resulting DNA fragments were purified with Amicon Microcon-PCR Centrifugal Filter Devices (Millipore Corporation, Bedford, MA, USA). Complementary strands were sequenced on

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an ABI310 sequencer (Perkin Elmer Applied Biosystems, Foster City, CA, USA), using either PCR primer.

Statistical analysis

Chi-square analysis was performed with Statview 5 (SAS Institute, Cary, NC, USA).

RESULTS

Susceptibility to moxifloxacin

Since the National Committee for Clinical Laboratory Standards (NCCLS) did not have approved breakpoints for moxifloxacin and anaerobes, in this study MIC values $\leq$2 mg/L were interpreted as susceptible, and $\geq$8 mg/L as resistant, according to breakpoints published for trovafloxacin and anaerobic bacteria [15].

Thirty-three strains (52.4%) were resistant to moxifloxacin, showing MIC values of $\geq$32 mg/L (Table 1). Thirty strains were susceptible to moxifloxacin, with MICs of $\leq$1.5 mg/L. No strains with 'intermediate' susceptibility were found.

DNA sequencing

Wild-type and quinolone-susceptible strains of *C. difficile* carry the amino acid threonine at position 83 within *gyrA* [9]. Mutations at amino acid position 83 (*Escherichia coli* coordinates) were found in *gyrA* of moxifloxacin-resistant strains but not in *gyrA* of moxifloxacin-susceptible strains, resulting in an amino acid change. The 33 isolates showed the same nucleotide transition (ACT $\rightarrow$ ATT), resulting in threonine $\rightarrow$ isoleucine. All moxifloxacin-susceptible strains had the same sequence as found in the moxifloxacin-susceptible *C. difficile* ATCC 43255 strain.

Toxigenicity

Forty-seven strains (71.4%) were toxigenic. Toxin A and B gene sequences were not detected in 19 isolates. All moxifloxacin-resistant strains were toxigenic.

AP-PCR genotyping

Among the 33 moxifloxacin-resistant strains, seven different groups were identified with the T-7 primer and the DICE similarity coefficient [14]. Most of the strains could be grouped into two fingerprinting types.

Previous use of fluoroquinolones

The medical records of 50 patients were available. Thirty patients had been treated with fluoroquinolones; 23 of these patients received treatment within three months before the isolation of *C. difficile* (time-related treatment). The medical history was searched for quinolone use for 27 patients with moxifloxacin-resistant isolates and for 23 patients with moxifloxacin-susceptible isolates (Table 2). Seventeen of the 27 patients with moxifloxacin-resistant strains had received fluoroquinolones prior to the isolation of *C. difficile*. Twelve patients from whom moxifloxacin-resistant isolates were recovered had been treated with ciprofloxacin, three with levofloxacin, one with trovafloxacin, and one with trovafloxacin after a course of ciprofloxacin (Table 3). Six patients with moxifloxacin-susceptible strains had received ciprofloxacin. None of the patients had received moxifloxacin.

Table 2 Results of several investigations for 63 strains recovered from 63 patients

MIC moxifloxacin $\geq$32 mg/L	33[a] (52.4%)
Toxin A^+/B^+	47 (71.4%)
gyrA mutation	33 (52.4%)
Fluoroquinolone treatment, total ($n = 50$)	30 (47.6%)
Fluoroquinolone treatment, time-related ($n = 50$)	23/50 (36.5%)

[a]All toxin A^+/B^+.

Table 1 MIC distribution of moxifloxacin for *C. difficile* strains isolated from 63 patients

	Number of strains inhibited at MIC (mg/L)							
	0.38	**0.5**	**0.75**	**1**	**1.5**	**2↔16**	**$\geq$32**	**$MIC_{50/90}$**
Moxifloxacin	1	5	4	15	5	0	33	$\geq$32/$\geq$32

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Table 3 Prior treatment with fluoroquinolones

	Moxifloxacin resistant	Moxifloxacin susceptible	E	*P*-value[a]
Quinolone treatment	17	6	23	0.0091
No quinolone treatment	10	17	27	
No data available	6	7	13	
Total	33	30	63	

[a]Chi-square, calculated for the two groups with available data.

DISCUSSION

The incorporation of newer fluoroquinolones into guidelines for treatment of respiratory tract infections will increase selection pressure for resistance to this already widely used group of antimicrobials. Therefore, a better understanding of mechanisms of resistance as well as measures to manage and monitor resistance are needed to preserve the potency and advantages of new compounds.

The impact on the intestinal flora of antimicrobials such as moxifloxacin is unclear. Compounds predominantly excreted via the gastrointestinal tract reach high fecal concentrations, exposing colonizing intestinal bacteria to many broad-spectrum antimicrobials. Organisms that are carrying single-step mutations due to exposure to older quinolones may undergo a second mutation and express resistance when a newer quinolone is introduced. The knowledge of which quinolones are more efficient in dealing with infections and less likely to select for resistance would help clinicians to choose the right treatment for the patient.

Only a few data exist on the susceptibility of *C. difficile* to newer fluoroquinolones [8–11]. Most of the studies do not address the issue of strain typing, bringing the significance of their results into question. Wilcox et al. found a more than seven-fold difference in susceptibility to new-generation fluoroquinolones between clonal and distinct strains [11]. A recent study from Spain reported the in vitro activity of new quinolones against 113 *C. difficile* isolates collected in a university hospital within 6 months. In this study, trovafloxacin was the most active agent, with an MIC_{90} of 8 mg/L [10].

The genotyping of the strains investigated in this study indicated that multiple strains were resistant to moxifloxacin, rather than a single clone.

In addition, the study reported here showed a relationship between antecedent fluoroquinolone use and resistance to moxifloxacin in *C. difficile* isolates (*P*-value 0.009, chi-square). The impact of older fluoroquinolones such as ciprofloxacin on the development of impaired susceptibility to newer compounds is not yet known. The selection of single-step mutants due to exposure to older compounds eases the way for the development of further mutations and for the appearance of phenotypically expressed resistance to new fluoroquinolones. The ability of some new compounds to act on dual targets (DNA gyrase and topoisomerase IV) requires mutations in both enzymes for the expression of resistance. This advantage could be nullified by already acquired single-step mutations due to earlier fluoroquinolone treatment. Additionally, the reduction of the intracellular accumulation of the drug, mediated by increased drug efflux, might be involved in the expression of fluoroquinolone resistance.

As in our previous report, the data presented here strongly suggest a relationship between resistance to moxifloxacin and a mutation in *gyrA* of *C. difficile* [9]. All moxifloxacin-resistant strains and none of the moxifloxacin-susceptible strains harbored a mutation at amino acid codon Ser-83 of *gyrA*. However, topoisomerase IV is described as the primary target for fluoroquinolones for most of the Gram-positive bacteria. Attempts in our laboratory to identify and sequence topoisomerase IV of *C. difficile* have not been successful. With the use of degenerated primers developed from a consensus sequence from the topoisomerase IV gene of closely related organisms, only PCR fragments containing the *gyrA* sequences were obtained. These findings suggest a very high homology between the nucleotide sequences of the two genes. Additionally, in order to identify the gene, sequences from published topoisomerase IV genes were aligned to the sequence of *C. difficile* published by the Sanger Center, UK (http://www.sanger.ac.uk/Projects/C_difficile/). Since the sequencing of the whole *C. difficile* genome (4.4 Mb) is not yet finished, it is possible that

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the sequence for topoisomerase IV of *C. difficile* is not yet published. However, it is also possible that C. *difficile* might lack the gene for topoisomerase IV, as is the case for *Mycobacterium tuberculosis*, *Helicobacter pylori*, and *Treponema pallidum* [16–18]. These organisms offer only one target for fluoroquinolones, which could lead to rapid development of resistance, since only a single mutation would be required.

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REFERENCES

1. Kelly CP, Pothoulakis C, LaMont JT. *Clostridium difficile* colitis. *N Engl J Med* 1994; 330: 257–62.
2. Freeman J, Wilcox MH. Antibiotics and *Clostridium difficile*. *Microb Infect* 1999; 1: 377–84.
3. Naber KG, Adam D, Expertengruppe der Paul-Ehrlich-Gesellschaft fuer Chemotherapie e. V. Einteilung der fluorchinolone. *Chemother J* 1998; 2: 66–8.
4. Van de Leur JJ, Vollard EJ, Janssen AJ *et al.* Influence of low dose ciprofloxacin on microbial colonization of the digestive tract in healthy volunteers during normal and during impaired colonization resistance. *Scand J Infect Dis* 1997; 29: 297–300.
5. Rafferty ME, Baltch AL, Smith RP *et al.* Comparison of restriction enzyme analysis, arbitrarily primed PCR, and protein profile analysis typing for epidemiologic investigation of an ongoing *Clostridium difficile* outbreak. *J Clin Microbiol* 1998; 36: 2957–63.
6. Cohen SH, Tang YJ, Rashmani D *et al.* Persistence of an endemic (toxigenic) isolate of *Clostridium difficile* in the environment of a general medicine ward. *Clin Infect Dis* 2000; 30: 952–4.
7. Blondeau JM. Expanded activity and utility of the new fluoroquinolones: a review. *Clin Ther* 1999; 21: 3–40.
8. Ackermann G, Tang YJ, Rodloff AC *et al.* In-vitro activity of sitafloxacin against *Clostridium difficile*. *J Antimicrob Chemother* 2001; 47: 722–3.
9. Ackermann G, Tang YT, Kueper R *et al.* Resistance to moxifloxacin in toxigenic *Clostridium difficile* isolates is associated with mutations in the *gyrA* gene. *Antimicrob Agents Chemother* 2001; 45: 2348–53.
10. Alonso R, Pelaez T, Gonzales-Abad MJ *et al.* In vitro activity of new quinolones against *Clostridium difficile*. *J Antimicrob Chemother* 2001; 47: 195–7.
11. Wilcox MH, Fawley W, Freeman J *et al. In vitro* activity of new generation fluoroquinolones against genotypically distinct and indistinguishable *Clostridium difficile* isolates. *J Antimicrob Chemother* 2000; 46: 551–5.
12. Sanders CC. Mechanisms responsible for cross-resistance and dichotomous resistance among the quinolones. *Clin Infect Dis* 2001; 32: S1–8.
13. Gumerlock PH, Tang YJ, Weiss JB *et al.* Specific detection of toxigenic strains of *Clostridium difficile* in stool specimens. *J Clin Microbiol* 1993; 31: 507–11.
14. Cohen SH, Tang YT, Silva J Jr. Molecular typing methods for the epidemiological identification of *Clostridium difficile* strains. *Exp Rev Mol Diagn* 2001; 1: 89–98.
15. National Committee for Clinical Laboratory Standards. *Methods for antimicrobial susceptibility testing of anaerobic bacteria*. Approved standard M11-A4. Wayne, PA: NCCLS, 1997.
16. Cole ST, Brosch R, Parkhill J *et al.* Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 1998; 393: 537–44.
17. Fraser CM, Norris SJ, Weinstocks GM *et al.* Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. *Science* 1998; 281: 375–88.
18. Tomb JF, White O, Kerlavage AR *et al*. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* 1997; 388: 539–47.

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