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High-level resistance to moxifloxacin and gatifloxacin associated with a novel mutation in *gyrB* in toxin-A-negative, toxin-B-positive *Clostridium difficile*

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***Objectives*: To determine the mechanism of high-level resistance to fluoroquinolone antimicrobials in toxin-A-negative, toxin-B-positive (A^-B^+) *Clostridium difficile* isolates.**

***Methods*: Following culture 16–23S PCR ribotyping was used to determine genomic relationships between A^-B^+ *C. difficile* isolates. Antimicrobial susceptibilities were determined using Etests in the presence and absence of the efflux pump inhibitors reserpine (20 μg/mL), L-phenylalanine-L-arginine-β-naphthylamide (PAβN; 20 μg/mL) and verapamil (100 μg/mL). Genomic regions including the quinolone-resistance-determining-region (QRDR) of *gyrA* and *gyrB* were amplified and characterized.**

***Results*: PCR ribotyping profiles identified one major cluster of A^-B^+ *C. difficile*, universally resistant to the fluoroquinolones tested (ofloxacin, ciprofloxacin, levofloxacin, moxifloxacin and gatifloxacin; MICs > 32 mg/L). All isolates with high-level resistance had a transversion mutation (A→T) resulting in the amino acid substitution Asp-426→Val in *gyrB*. Non-clonal isolates were susceptible to moxifloxacin and gatifloxacin (MICs 0.3 and 0.4 mg/L, respectively) with reduced susceptibility to levofloxacin (MIC 3 mg/L) consistent with the wild-type genotype. The MICs for resistant isolates were not significantly affected by the addition of any of the efflux pump inhibitors. No amino acid substitutions were identified in the QRDR of *gyrA*.**

***Conclusions*: High-level resistance to fluoroquinolones in A^-B^+ *C. difficile* is associated with a novel transversion mutation in *gyrB*. The emergence of universal resistance in different *C. difficile* strain types may be a factor promoting outbreaks in hospitals.**

Keywords: fluoroquinolone resistance, A^-B^+ *C. difficile*, transversion mutation

Introduction

Clostridium difficile is a major cause of bacterial diarrhoea in the developed world.[1] Patients in hospital receiving antibiotics are the most at risk. Certain classes of antimicrobials have been associated with a high risk of *C. difficile*-associated disease (CDAD) including clindamycin, cephalosporins and ampicillin/amoxicillin.[1] Historically, fluoroquinolone antimicrobials were considered low risk for CDAD, although a number of case reports associated with ciprofloxacin exposure have been published.[2] However, recent studies indicate a shift in the risk associated with the use of fluoroquinolone antimicrobials.[3–5]

The risk of antibiotic-associated CDAD appears to increase if *C. difficile* is resistant to the administered antibiotic. A number of outbreaks in different states in the United States were associated with a clonal clindamycin-resistant strain, wherein this drug was identified as a specific risk factor.[6] More recently, an investigation into an outbreak caused by the hyper-virulent fluoroquinolone-resistant strain (PCR-027/NAP1) in the Quebec region of Canada indicated that fluoroquinolone antimicrobials

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were more likely to induce CDAD.[4] Furthermore, Muto *et al*.[5] reported that exposure to levofloxacin was an independent risk factor for CDAD in a large outbreak in Pittsburgh.

A recent outbreak at one Dublin hospital identified the emergence of a clonal toxin-A-negative, toxin-B-positive (A^-B^+) *C. difficile* strain type demonstrating high-level resistance to several fluoroquinolone antibiotics. A second investigation identified these strains in a number of additional healthcare settings in Dublin. The purpose of this study was to determine the contribution of target gene mutations and active efflux to fluoroquinolone resistance in these isolates.

Materials and methods

Bacterial strains and culture

Seventy *C. difficile* strains were studied. These included 30 representative *C. difficile* strains that were collected during surveillance of a hospital outbreak in 2003, 10 isolates collected from that institution prior to the outbreak and 30 isolates collected from six healthcare settings in Dublin in 2004. *C. difficile* strains were cultured on cycloserine-cefoxitin-fructose agar (CCFA). Type strains VPI10463 and 1470 along with a non-toxigenic strain (R10567) were included as controls.

Antibiotic susceptibility of C. difficile

Fluoroquinolone susceptibility was determined using Etests (AB Biodisk, Solna, Sweden). The MIC was interpreted by the Clinical and Laboratory Standards Institute (CLSI) breakpoints for trovofloxacin ($\leq$2 mg/L, susceptible; 4 mg/L, intermediate; $\geq$8 mg/L, resistant).

Molecular analysis of C. difficile strains

Genomic DNA was extracted from broth cultures of *C. difficile* and PCR ribotyping and PCR–RFLP (toxinotyping) were performed as previously described.

PCR amplification and sequencing of the quinolone-resistance-determining-region (QRDR) of gyrA and gyrB

Primers GyrAF (TTG AAA TAG CGG AAG AAA TGA), GyrAR (TTG CAG CTG TAG GGA AAT C), GyrBF (GAA GGT CAA ACT AAA ACA AA) and GyrBR (GGG CTC CAT CTA CAT CAG) were designed from the sequence of the *C. difficile* 630 genome and were used to amplify 633 and 514 bp amplicons from *gyrA* and *gyrB*, respectively (http://www.sanger.ac.uk/Projects/C_difficile/blast_server.shtml). Amplicons were purified using a QIAquick PCR purification kit (Qiagen, GmbH, Germany) and sequenced commercially by Qiagen. Clustal W amino acid sequence alignments were produced for comparison.

Nucleotide sequence accession numbers

The nucleotide sequence data for partial sequences of the *gyrB* gene were submitted to GenBank and were assigned the accession numbers DQ642011, DQ642012 and DQ642013, respectively.

Efflux contribution

Ten representative isolates (Table 1) were chosen for efflux studies. The MICs of all five antibiotics were determined using Etest strips, in the presence and absence of the following efflux pump inhibitors: reserpine (20 μg/mL); L-phenylalanine-L-arginine-β-naphthylamide (20 μg/mL); and verapamil (100 μg/mL). Concentrations of PAβN and reserpine up to 80 μg/mL, and verapamil up to 800 μg/mL, were used to determine the effect (if any) of these inhibitors on bacterial growth.

Results

Antimicrobial susceptibility patterns and efflux contribution

Sixty *C. difficile* were universally resistant to all five fluoroquinolones tested (MICs > 32 mg/L). Control isolates VPI10463, 1470 and R10567 and 10 clinical isolates were susceptible

Table 1. Characterization of representative isolates in this study

Isolate	Toxigenic status	Ribotype	Fluoroquinolone MIC (mg/L)					Amino acid substitution in GyrB
			ciprofloxacin	ofloxacin	levofloxacin	gatifloxacin	moxifloxacin	
M3[a]	A^-B^+	A	>32	>32	>32	>32	>32	Asp-426→Val
M7[a]	A^-B^+	A	>32	>32	>32	>32	>32	Asp-426→Val
J11[a]	A^-B^+	A	>32	>32	>32	>32	>32	Asp-426→Val
V21[a]	A^-B^+	A	>32	>32	>32	>32	>32	Asp-426→Val
V64[a]	A^-B^+	A	>32	>32	>32	>32	>32	Asp-426→Val
M47[b]	A^+B^+	B	>32	>32	3	0.38	0.25	Asp-426
M52[c]	A^+B^+	C	>32	>32	3	0.38	0.25	Asp-426
R10567[d]	A^-B^-	D	>32	>32	3	0.38	0.25	Asp-426
1470[d]	A^-B^+	A	>32	>32	3	0.38	0.25	Asp-426
VPI10463[d]	A^+B^-	E	>32	>32	3	0.38	0.25	Asp-426

[a]Clinical isolates representing the 60 A^-B^+ *C. difficile* in this study.
[b]Clinical isolate representing four of the ten A^+B^+ clinical isolates.
[c]Clinical isolate representing six of the ten A^+B^+clinical isolates.
[d]Control isolates and type strains.

to moxifloxacin and gatifloxacin (MICs of 0.3 and 0.4 mg/L, respectively) and showed reduced susceptibility to levofloxacin (MIC 3 mg/L). These strains were resistant to ciprofloxacin and ofloxacin. The MICs for representative isolates are shown in Table 1. The addition of efflux pump inhibitors had no effect on MICs or on bacterial growth of any of the isolates investigated (data not shown).

16–23S PCR ribotyping

16–23S ribotyping identified one major cluster containing 60 A^-B^+ isolates. Toxinotyping confirmed that these isolates were toxinotype VIII (PCR-017) (data not shown). The remaining ten clinical isolates belonged to two different ribotype groups (data not shown).

Amplification and sequence analysis of gyrA and gyrB

Amplicons from *gyrA* and *gyrB* were sequenced for 10 representative isolates (Table 1). Sequence analysis of the QRDR of *gyrA* revealed a number of silent mutations. Deduced sequence analysis of the QRDR of *gyrB* revealed an Asp-426→Val amino acid substitution at codon 426 in all resistant isolates. A Ser-366→Ala substitution was found in all A^-B^+ *C. difficile* (data not shown). As this substitution was found in clinical strains resistant to moxifloxacin, gatifloxacin and levofloxacin and in the 1470 type strain that was susceptible to moxifloxacin and gatifloxacin, it is unlikely to contribute to the fluoroquinolone-resistant phenotype. No amino acid substitutions were identified in any of the susceptible A^+B^+ clinical isolates or control strains R10567 and VPI10463.

Discussion

This study identified an A^-B^+ clone of *C. difficile* resistant to five fluoroquinolone antimicrobials. The β-subunit of DNA gyrase is the primary target wherein a single nucleotide transversion resulted in a novel Asp→Val substitution at codon 426. This is the first report of *gyrB* mutations in this strain type.

Fluoroquinolone resistance associated with chromosomal mutations in *gyrA* and *gyrB* has been previously described in *C. difficile*. Ackermann *et al.*[7] described two substitutions in *gyrA* corresponding to codon 83 in *Escherichia coli*. Thirteen of 18 isolates studied had a substitution corresponding to Thr-83→Ile while one strain had a Thr-83→Val substitution. Dridi *et al.*[8] described mutations in *gyrA* and *gyrB* associated with fluoroquinolone resistance in several serogroups of *C. difficile*. The substitutions in *gyrA* included Thr-83→Ile in six strains (serogroups H1, A9 and 1C), an Asp-71→Val substitution in one strain (serogroup H) and an Ala-118→Thr substitution in one serogroup D strain. Two substitutions in *gyrB,* including an Arg-447→Leu substitution in a single non-typeable isolate and an Asp-426→Asn substitution in five isolates (4 serogroup C and 1 serogroup K), were described.

Codon Asp-426 has been highlighted as a critical region in *gyrB* and mutations corresponding to this codon have been associated with fluoroquinolone resistance in *E. coli*, *Staphylococcus aureus* and *Streptococcus pneumoniae*.[9] In *C. difficile*, Dridi *et al.*[8] described an Asp-426→Asn substitution in five isolates with MICs of moxifloxacin of 8 or 16 mg/L. Substitution of Asp with Asn results in the replacement of a negatively charged polar hydrophilic amino acid with an uncharged polar residue. In this study, Asp is substituted with valine at position 426 in fluoroquinolone-resistant A^-B^+ *C. difficile* isolates. This results in the introduction of a non-polar side chain and the loss of negative charge. Moreover, as valine is a branched chain amino acid it can add bulk to the protein backbone thereby restricting conformational flexibility. It is tempting to speculate that Val-426 may alter the shape of the drug-binding pocket more significantly than Asn thereby producing a more extreme phenotype, as measured by MIC. This may explain the high-level resistance encountered in the A^-B^+ *C. difficile* strains in this study.

Increased fluoroquinolone resistance associated with newer groups of fluoroquinolones with increased anti-anaerobic activity has been described.[9] Ackerman *et al.*[10,11] reported *C. difficile* moxifloxacin resistance rates of 12% and 50% in two studies where the majority of resistant isolates clustered into two clonal groups. None of the patients with moxifloxacin-resistant *C. difficile* strains had received this antibiotic. Similarly, in this study neither moxifloxacin nor gatifloxacin was used clinically. This highlights the possibility that resistance to the newer antimicrobials may result from mechanisms that were acquired or evolved following exposure to older fluoroquinolone antimicrobials.

Isolates demonstrating high-level resistance to fluoroquinolones in this study were clonal. These A^-B^+ strains were isolated from patients from three different university hospitals along with three community-acquired specimens indicating the emergence and dissemination of this strain type throughout the greater Dublin area. Wilcox *et al.*[12] have previously highlighted the importance of typing isolates when describing rates of resistance. It is possible that acquired antimicrobial resistance may be at least one mechanism that contributed to the selection and proliferation of this strain type in an environment where fluoroquinolones are frequently used.

In conclusion, we report a novel transversion mutation in *gyrB* associated with high-level fluoroquinolone resistance in *C. difficile* PCR-017. Efflux pump activity does not appear to contribute to the resistance phenotype. Emergence of resistance to fluoroquinolones in *C. difficile* strains may be a factor that contributes to the persistence and dissemination of strain types in the hospital environment. Fluoroquinolone use is now a recognized high risk of CDAD; therefore careful use of this antibiotic class needs to be encouraged as part of infection control policies to reduce *C. difficile* disease.

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Transparency declarations

None to declare.

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