2006

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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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Received 16 June 2006; returned 24 July 2006; revised 22 August 2006; accepted 11 September 2006

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 (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.² 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 were associated with the emergence of this strain.⁷

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were more likely to induce CDAD. Furthermore, Muto et al., 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 trovafloxacin (≤2 mg/L, susceptible; 4 mg/L, intermediate; ≥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.

Table 1. Characterization of representative isolates in this study

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Toxigenic status</th>
<th>Ribotype</th>
<th>Ciprofloxacin (mg/L)</th>
<th>Ofloxacin (mg/L)</th>
<th>Levofloxacin (mg/L)</th>
<th>Gatifloxacin (mg/L)</th>
<th>Moxifloxacin (mg/L)</th>
<th>Amino acid substitution in GyrB</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3⁷</td>
<td>A’B⁺</td>
<td>A</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>Asp-426—Val</td>
</tr>
<tr>
<td>M7⁷</td>
<td>A’B⁺</td>
<td>A</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>Asp-426—Val</td>
</tr>
<tr>
<td>J11⁷</td>
<td>A’B⁺</td>
<td>A</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>Asp-426—Val</td>
</tr>
<tr>
<td>V21ᵃ</td>
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<td>A</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>Asp-426—Val</td>
</tr>
<tr>
<td>V64ᵃ</td>
<td>A’B⁺</td>
<td>A</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>Asp-426—Val</td>
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<td>A’B⁺</td>
<td>B</td>
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<td>0.38</td>
<td>&gt;32</td>
<td>Asp-426</td>
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</tr>
<tr>
<td>M52ᵇ</td>
<td>A’B⁺</td>
<td>C</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>0.38</td>
<td>&gt;32</td>
<td>Asp-426</td>
<td></td>
</tr>
<tr>
<td>R10567ᵈ</td>
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<td>D</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>0.38</td>
<td>0.25</td>
<td>Asp-426</td>
<td></td>
</tr>
<tr>
<td>1470ᵈ</td>
<td>A’B⁻</td>
<td>A</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>0.38</td>
<td>0.25</td>
<td>Asp-426</td>
<td></td>
</tr>
<tr>
<td>VPI10463ᵈ</td>
<td>A’B⁻</td>
<td>E</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>0.38</td>
<td>0.25</td>
<td>Asp-426</td>
<td></td>
</tr>
</tbody>
</table>

ᵃClinical isolates representing the 60 A’B⁺ C. difficile in this study.
ᵇClinical isolate representing four of the ten A’B⁺ clinical isolates.
ᶜClinical isolate representing six of the ten A’B⁺ clinical isolates.
ᵈControl isolates and type strains.
Fluoroquinolone resistance associated with chromosome mutations in \( \text{gyrA} \) and \( \text{gyrB} \) has been previously described in \( C. \text{difficile} \). Ackermann et al.\(^7\) described two substitutions in \( \text{gyrA} \) corresponding to codon 83 in \( E. \text{coli} \). Thirteen of 18 isolates studied had a substitution corresponding to Thr-83\(\rightarrow\)Ile while one strain had a Thr-83\(\rightarrow\)Val substitution. Dridi et al.\(^8\) described mutations in \( \text{gyrA} \) and \( \text{gyrB} \) associated with fluoroquinolone resistance in several serogroups of \( C. \text{difficile} \). The substitutions in \( \text{gyrA} \) included Thr-83\(\rightarrow\)Ile in six strains (serogroups H1, A9 and 1C), an Asp-71\(\rightarrow\)Val substitution in one strain (serogroup H) and an Ala-118\(\rightarrow\)Thr substitution in one serogroup D strain. Two substitutions in \( \text{gyrB} \), including an Arg-447\(\rightarrow\)Leu substitution in a non-typeable isolate and an Asp-426\(\rightarrow\)Asn substitution in five isolates (4 serogroup C and 1 serogroup K), were described.

Coden Asp-426 has been highlighted as a critical region in \( \text{gyrB} \) and mutations corresponding to this codon have been associated with fluoroquinolone resistance in \( E. \text{coli} \), \( S. \text{aureus} \) and \( S. \text{pneumoniae} \). In \( C. \text{difficile} \), Dridi et al.\(^9\) described an Asp-426\(\rightarrow\)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 \( A^+ B^+ \) \( C. \text{difficile} \) 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 \( A^+ B^+ \) \( C. \text{difficile} \) 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 \( C. \text{difficile} \) 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 \( C. \text{difficile} \) 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 \( \text{gyrB} \) associated with high-level fluoroquinolone resistance in \( C. \text{difficile} \) PCR-017. Efflux pump activity does not appear to contribute to the resistance phenotype. Emergence of resistance to fluoroquinolones in \( C. \text{difficile} \) 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 \( C. \text{difficile} \) disease.

Acknowledgements

The cooperation of our colleagues in the Medical Microbiology Departments of the Mater Misericordiae University Hospital, St Vincents’ University Hospital and St James University Hospital with regard to sample collection is gratefully acknowledged. We acknowledge the financial support provided by The Health Research Board, Ireland (RP/2005/72), The Mater Foundation and The Newman Scholarship Programme—University College Dublin.

Transparency declarations

None to declare.
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