Alterations of the pilQ gene in Neisseria gonorrhoeae are unlikely contributors to decreased susceptibility to ceftriaxone and cefixime in clinical gonococcal strains

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Objectives: Gonorrhoea remains a global public health problem and the treatment options are diminishing through the emergence of gonococci resistant to most antimicrobials. Previous in vitro studies have indicated a role for Neisseria gonorrhoeae pilQ alterations in conferring resistance to antimicrobials, including penicillin. In this study, we investigated whether pilQ polymorphisms were associated with decreased susceptibility to extended-spectrum cephalosporins (ESCs) in clinical gonococcal strains.

Methods: Full-length pilQ nucleotide and PilQ amino acid sequences from geographically and temporally diverse gonococcal clinical isolates (n=63), including the 2008 WHO reference strains, representing a range of ceftriaxone and cefixime MICs (≤0.008–0.25 and ≤0.016–0.5 mg/L, respectively) and 38 N. gonorrhoeae multiantigen sequence types, were examined. Previously described alterations associated with decreased ESC susceptibility (mosaic penA, mtrR and penB alterations) were also examined.

Results: Fifteen different pilQ nucleotide sequence types and nine different PilQ amino acid sequence types were observed, with two PilQ types accounting for 53 (84%) of the isolates. Independent of other genetic resistance determinants (penA mosaic, mtrR promoter deletion and penB), only one pilQ alteration, a D526N substitution, provided a statistically significant association with ceftriaxone (P<0.01) and cefixime (P<0.05) MICs. However, the two isolates exhibiting D526N lacked all three previously described alterations associated with decreased ESC susceptibility, thereby providing an alternative basis for the low MICs (≤0.008 mg/L) observed for these strains. The previously described E666K (pilQ2) and F595L (pilQ1) mutations were absent in all 63 isolates.

Conclusions: pilQ polymorphisms are unlikely contributors to decreased susceptibility to ESCs in clinical gonococcal strains.

Keywords: gonorrhoea, antimicrobial resistance, penA, third generation, extended-spectrum cephalosporins

Introduction

Gonorrhoea remains a global public health problem. Effective treatment options have rapidly diminished through the emergence and spread of Neisseria gonorrhoeae harbouring resistance to multiple classes of antimicrobials, including penicillins, tetracyclines, macrolides and quinolones. Extended-spectrum cephalosporins (ESCs), including the oral cefixime and the injectable ceftriaxone, are the last remaining options and, currently, the mainstay of treatment in most settings. However, there are now renewed concerns regarding the control of gonorrhoea, arising from recent reports of gonococci harbouring decreased in vitro susceptibility to all ESCs, and documented treatment failures of urogenital gonorrhoea using oral ESCs in Japan and...
Hong Kong. Accordingly, gonorrhoea may become untreatable under certain circumstances.1

Decreased susceptibility to ESCs in *N. gonorrhoeae* is due to:

- the presence of mosaic *penA*, encoding an altered penicillin-binding protein (PBP) 2; alterations in the mtrR promoter, causing an overexpression of the MtrC-MtrD-MtrE efflux pump; and
- penB, encoding altered forms of the porin PorB1b, particularly G120K and A121D.2 However, it has become clear that the decreased susceptibility to ESCs in *N. gonorrhoeae* is more complex. For example, an A501V alteration in PBP2, together with mtrR promoter deletion and penB mutations, may significantly contribute to decreased ESC susceptibility in non-mosaic penA gonococci.3,4

Using transformation experiments, Zhao et al.5 showed that the gonococcal PilQ oligomer forms a pore in the outer membrane through which antimicrobials may diffuse into the periplasm, and that an E666K alteration (*pilQ2*) in PilQ, when present in combination with penA, mtrR and penB alterations, leads to increased resistance to penicillin and tetracycline. A previous study showed that an F595L mutation (*pilQ1*) in PilQ increased the susceptibility to several antimicrobials, including penicillin, providing additional evidence for the involvement of gonococcal PilQ as a route of antimicrobial entry.6 In this study, we examined full-length *pilQ* nucleotide and PilQ amino acid sequences from temporally, geographically and genetically diverse *N. gonorrhoeae* clinical isolates representing a wide range of ceftriaxone and cefixime MICs.

### Materials and methods

*N. gonorrhoeae* strains

A total of 63 gonococci were retrospectively selected on the basis of ceftriaxone MIC, and temporal and geographical diversity. Ceftriaxone MICs and cefixime MICs were in the range ≤0.008–0.25 and ≤0.016–0.5 mg/L (Table 1), respectively, as determined by using agar dilution (ceftriaxone) as previously described7 and Etest (cefixime) according to the manufacturer's instructions (AB bioMérieux, Solna, Sweden). The clinical isolates examined included 13 isolates from Australia, 7 from Asia (China, 1; Hong Kong, 1; Japan, 1; Korea, 1; Mongolia, 1; Thailand, 1; and the Philippines, 1), 25 from Sweden, 4 from the UK and 5 from the USA. The years

<table>
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<tr>
<th>Alleles</th>
<th>Ceftriaxone (cefixime) MIC (mg/L) for isolates</th>
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<tbody>
<tr>
<td><em>pilQ</em></td>
<td>mtrR&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>I</td>
<td>WT&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>II</td>
<td>A-del</td>
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<tr>
<td>III</td>
<td>A-del</td>
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<tr>
<td>III</td>
<td>WT&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>IV</td>
<td>WT&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>V</td>
<td>WT&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>VI</td>
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<td>A to C</td>
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<td>VII</td>
<td>WT&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>VII</td>
<td>WT&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>VIII</td>
<td>WT&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>VIII</td>
<td>A-del</td>
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<tr>
<td>IX</td>
<td>WT&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>Total</td>
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<sup>a</sup>Alterations in the inverted repeat in the mtrR promoter region causing an overexpression of the MtrC-MtrD-MtrE efflux pump. The most common alteration is a deletion of a single nucleotide (‘A-del’).

<sup>b</sup>Alterations of the outer membrane porin PorB1b resulting in decreased intake of antimicrobials. The most frequent penB alterations are G120K and A121D. K, lysine; D, aspartic acid; A, alanine; G, glycine; S, serine; N, asparagine.

<sup>c</sup>WT, wild-type.

<sup>d</sup>Possessed a PIA (PorB1a) porin.
of isolation were 1988 \( (n=1) \), 1999 \( (n=1) \), 2000 \( (n=2) \), 2001 \( (n=1) \), 2002 \( (n=6) \), 2003 \( (n=9) \), 2004 \( (n=7) \), 2005 \( (n=11) \), 2007 \( (n=1) \), 2008 \( (n=14) \) and 2009 \( (n=1) \). A further nine gonococci (WHO C, F, G, K, L, M, N, O and P) used as WHO reference strains were included in the study. The isolates were represented by types III (WHO C), IV (WHO L), VI (WHO K), VII (WHO G, M, N, O and P) and IX (WHO F).

The previously described E666K \( (\text{pilQ}^2) \) and F595L \( (\text{pilQ}^1) \) mutations were not observed in these isolates. Independent of other genetic resistance determinants \( (\text{penA} \text{mosaic, mtrR promoter deletion and penB}) \), a statistically significant association with ceftriaxone and cefixime MICs was observed for the D526N substitution only \( (P<0.01 \text{ and } P<0.05, \text{ respectively}) \). However, the two isolates exhibiting the D526N alteration of \( \text{pilQ} \) \( (\text{pilQ} \text{ type I}; \text{Table 1} \text{ and Figure 1}) \) lacked all the three previously described alterations associated with reduced ESC susceptibility, and displayed low ceftriaxone and cefixime MICs \( (\leq 0.008 \text{ and } <0.016 \text{ mg/L, respectively}) \). \text{Table 1}). Consistent with previous studies, significant associations with ceftriaxone and cefixime MICs were observed for mosaic \( \text{penA} \) \( (P<0.001) \), the adenine deletion in the \( \text{mtrR} \) promoter \( (P<0.01 \text{ and } P<0.001, \text{ respectively}) \) and the most common \( \text{penB} \) alterations, i.e. G120K and A121D \( (P<0.01) \) \( (\text{Table 1}) \).

### Discussion

The results of the present study showed that there were only eight different \( \text{pilQ} \) amino acid alterations (resulting in nine divergent sequence types) in 63 temporally and geographically diverse gonococci representing a wide range of ceftriaxone and cefixime MICs, with two \( \text{pilQ} \) types \( (\text{VI} \text{ and } \text{VII}) \) accounting for the majority (84%) of isolates. Accordingly, the gonococcal pore-forming secretin \( \text{PilQ} \) is highly conserved for an outer membrane protein implicated in antimicrobial resistance, particularly when compared with the highly variable \( \text{PorB} \) porin. The only \( \text{pilQ} \) alteration that was significantly associated with ceftriaxone and cefixime MICs was D526N; however, both isolates harbouring this particular mutation lacked mosaic \( \text{penA} \), as well as \( \text{mtrR} \) and \( \text{penB} \) alterations \( (\text{Table 1}) \). Thus, the low MICs observed for these two isolates could be explained by the absence of these key alterations. The previously described E666K \( (\text{pilQ}^2) \) and F595L \( (\text{pilQ}^1) \) alterations were absent in all isolates investigated in this study.

It is becoming increasingly clear that emerging chromosomally mediated resistance to ESCs, similar to penicillin, in \( N. \gonorrhoeae \) is exceedingly complex and multifaceted, and involves multiple alterations in various genes and that these act synergistically.\(^2\)\(^-\)\(^4\) In this respect, \( \text{pilQ} \) E666K \( (\text{pilQ}^2) \) was previously shown to have little to no impact in the absence of \( \text{penA} \), \( \text{mtrR} \) promoter and \( \text{penB} \) alterations.\(^5\) It is therefore conceivable that the lack of clear association between \( \text{pilQ} \) and ceftriaxone and cefixime susceptibility observed in our study arose from use of univariate analysis and that larger studies using multivariate analysis may be required to fully investigate the relative contributions of each mutation. However, based on our data there are no clear candidate \( \text{pilQ} \) alterations that may be implicated in decreased susceptibility to ESCs in clinical gonococcal isolates. Furthermore, the previously described \( \text{pilQ}^2 \) polymorphism is also unlikely to contribute to clinical resistance, because this mutation interferes with the formation of the \( \text{PilQ} \) secretin complex and disrupts proper piliation. Accordingly, this \( \text{PilQ} \) mutant is not functional for pilus assembly and the type IV pilis are essential in gonococcal mucosal pathogenesis, as they are involved in the adherence to the epithelium cells of the host.\(^10\)\(^,\)\(^11\)
Figure 1. Full-length PilQ amino acid sequences from 63 temporally, geographically and genetically diverse *N. gonorrhoeae* isolates. The sequences are classified into different amino acid types (I–IX) and are aligned with the *N. gonorrhoeae* FA1090 sequence (GenBank accession number AE004969). The numbers of isolates of each amino acid type are indicated in parentheses.
In conclusion, the present study showed that the *N. gonor-
 rhoae pilQ* nucleotide sequence and PilQ amino acid sequence
are highly conserved in temporally, geographically and geneti-
cally diverse gonococci. Furthermore, alterations of *pilQ* are unli-
kely contributors to decreased susceptibility to ESCs, such as
ceftriaxone and cefixime, in clinical gonococcal strains.

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**Transparency declarations**

None to declare.

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