Increasing prevalence of Neisseria gonorrhoeae with decreased susceptibility to ceftriaxone and resistance to azithromycin in Hangzhou, China (2015–17)

Jing Yan1, Juan Xue1, Yan Chen2, Shi Chen3, Qiang Wang4, Chuanling Zhang5, Shenghai Wu6, Huoyang Lv7, Yunsong Yu2 and Stijn van der Veen1,8,9*

1Department of Microbiology and Parasitology, School of Medicine, Zhejiang University, Hangzhou, China; 2Department of Infectious Diseases, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China; 3Clinical Laboratory Department, Hangzhou Third Hospital, Hangzhou, China; 4Department of Clinical Laboratory, The Second Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, China; 5Clinical Laboratory, Zhejiang Xiaoshan Hospital, Hangzhou, China; 6Department of Laboratory, Affiliated Hangzhou First People’s Hospital, Zhejiang University School of Medicine, Hangzhou, China; 7Centre of Laboratory Medicine, Zhejiang Provincial People’s Hospital, People’s Hospital of Hangzhou Medical College, Hangzhou, China; 8Department of Dermatology, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China; 9State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China

*Corresponding author. Tel: +86-571-88206684; Fax: +86-571-88208022; E-mail: stijnvanderveen@zju.edu.cn

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Objectives: Development of resistance in Neisseria gonorrhoeae to ceftriaxone monotherapy or ceftriaxone plus azithromycin dual therapy is a global public health concern. The aim of this study was to analyse the trend in antimicrobial resistance in Hangzhou, China, over the period 2015–17.

Methods: In total, 379 clinical isolates were collected from seven hospitals and antimicrobial susceptibility was determined using the agar dilution method. Isolates showing resistance to ceftriaxone, azithromycin or cefixime were analysed for the presence of resistance determinants. STs were determined with the N. gonorrhoeae multi-antigen sequence typing (NG-MAST) method and phylogenetic analysis and strain clustering was determined using porB and tbpB sequences.

Results: Ceftriaxone resistance, decreased susceptibility to ceftriaxone and azithromycin resistance were observed in 3%, 17% and 21% of the isolates, respectively. This resulted in 5% of the isolates showing both decreased susceptibility to ceftriaxone and azithromycin resistance. Importantly, resistance levels to ceftriaxone and azithromycin increased over the study period, resulting in 5% ceftriaxone resistance, 27% decreased susceptibility to ceftriaxone and 35% azithromycin resistance in 2017 and 11% of the isolates showing both decreased susceptibility to ceftriaxone and azithromycin resistance. Phylogenetic and cluster analysis showed the emergence and expansion in 2017 of a clonally related cluster containing strains with high abundance of decreased susceptibility to ceftriaxone and/or cefixime, which was related to the presence of the mosaic penA allele X. Co-resistance to azithromycin was also observed in this cluster.

Conclusions: Our findings have major implications for the future reliability of ceftriaxone monotherapy and ceftriaxone plus azithromycin dual therapy in China.

Introduction

Gonorrhoea is one of the most common bacterial sexually transmitted diseases worldwide, with an estimated 78 million new cases annually.1 This bacterium typically colonizes the urogenital system, but can also be detected in the rectal and oropharyngeal mucosa.2 Gonorrhoea is usually symptomatic in men and typically manifests as urethritis, while in women gonococcal infections may lead to cervicitis, although they often remain asymptomatic. Importantly, untreated gonococcal infections can result in complications such as pelvic inflammatory disease, infertility or ectopic pregnancies and disseminated infections such as tenosynovitis or septic arthritis.3–5 In addition, pharyngeal and rectal infections are also often asymptomatic, but are an important factor for transmission.3,5

Future treatment of gonorrhoea is threatened by the rise of MDR strains. Specifically, resistance to the last available first-line
amoxicillin treatments, ceftriaxone monotherapy or ceftriaxone plus azithromycin dual therapy, is increasing globally. Ceftriaxone treatment failures have already been reported in a number of countries and several surveillance studies have shown that the incidence of ceftriaxone-resistant strains (MIC > 0.125 mg/L, EUCAST) or strains displaying decreased susceptibility to ceftriaxone (MIC > 0.125 mg/L) is increasing. Also, azithromycin-resistant strains (MIC > 0.5 mg/L, EUCAST) are increasingly common. Worryingly, high-level azithromycin-resistant Neisseria gonorrhoeae strains, with MICs ≥256 mg/L, have been isolated in many countries, including Ireland, Scotland, England, Argentina, Italy, the USA, Sweden, Australia and China. 

Fortunately, treatment failure after ceftriaxone plus azithromycin dual therapy has so far been exceedingly rare, although confirmed failures have already been reported due to strains displaying resistance to both antimicrobials. In addition, the occurrence of N. gonorrhoeae isolates displaying both azithromycin resistance or high-level azithromycin resistance and ceftriaxone resistance or decreased susceptibility to ceftriaxone has been reported in recent years in China, Japan, Canada and the USA state of Hawaii.

Resistance of N. gonorrhoeae to ceftriaxone and azithromycin is the result of both separate and overlapping resistance determinants. Ceftriaxone resistance arises from mutations in penA, encoding PBP2, which is the direct target of ceftriaxone. Multiple modifications are required for N. gonorrhoeae to obtain intermediate-level resistance and a ‘mosaic allele’ in which up to 60–70 amino acids are changed results in higher-level resistance. Furthermore, the outer membrane of N. gonorrhoeae is an important permeability barrier for antimicrobials. Several mutations in the outer-membrane porin, PorB, have been associated with reduced influx of ceftriaxone, while mutations in mtrR and its promoter region that induce expression of the MtrCDE multidrug efflux pump have been shown to increase efflux of ceftriaxone, thereby increasing the resistance of N. gonorrhoeae. Increased expression of the MtrCDE and MacAB efflux pumps also provides increased resistance to azithromycin. Further resistance to azithromycin is the result of mutations in loop V of 23S rRNA, which is the specific target of azithromycin. Azithromycin resistance can arise from a C2611T mutation (Escherichia coli numbering), while high-level azithromycin resistance is the result of an A2059G mutation.

In our previous retrospective analysis of N. gonorrhoeae clinical isolates from 2011–12 in Hangzhou, China, we reported a high prevalence of high-level azithromycin-resistant strains. The current follow-up study, 379 isolates obtained from outpatients of seven hospitals in Hangzhou between January 2015 and December 2017 were analysed for antimicrobial susceptibility and the presence of resistance determinants. Importantly, a high prevalence of strains displaying ceftriaxone resistance (3%), decreased susceptibility to ceftriaxone (17%), high-level azithromycin resistance (7%) or both azithromycin resistance and decreased susceptibility to ceftriaxone (5%) was observed.

**Materials and methods**

**N. gonorrhoeae isolates and species verification**

N. gonorrhoeae isolates were collected from clinical laboratories of seven hospitals in the Hangzhou area between January 2015 and December 2017. Clinical isolates were anonymized, so no ethical approval was required for this study. Clinical samples were cultured on gonococcal selective media and positive samples were verified by Gram staining and oxidase testing. Isolates were preserved in brain heart infusion broth (Oxoid) containing 15% glycerol (BioSharp) at −80°C. After collection, all isolates were reconfirmed by 16S rDNA sequencing using the primers NG-16S-F (AGACGTTACTTAAAGCAGGA) and NG-16S-R (GCCTCCGGCTTCGCTTCAA).

**Antimicrobial susceptibility testing**

All isolates were tested for their antimicrobial susceptibility to ceftriaxone, azithromycin, cefixime, ciprofloxacin, tetracycline, spectinomycin and penicillin using the WHO guidelines for the agar dilution method and N. gonorrhoeae strain ATCC 49226 was used for quality control. N. gonorrhoeae isolates were revived on GC agar (Oxoid) supplemented with 1% (v/v) Vitox (Oxoid) and grown overnight at 37°C in the presence of 5% CO₂. Approximately 10⁶ cfu was subsequently applied to plates containing a 2-fold dilution series of the respective antimicrobials and growth was determined after overnight incubation. The MIC was recorded as the lowest concentration of the antimicrobial agent for which no growth was observed. Antimicrobial susceptibility breakpoints were adopted from the EUCAST guidelines (http://www.eucast.org). Penicillinase-producing N. gonorrhoeae (PPNG) isolates were identified using a nitrocefin solution (Sigma) and high-level tetracycline-resistant N. gonorrhoeae (TRNG) isolates were defined by an MIC ≥16 mg/L.

**Identification of resistance determinants**

All ceftriaxone- and cefixime-resistant isolates were investigated for polymorphisms associated with resistance in penA, porB and mtrR and its promoter region by PCR and sequencing using the primers penA-F (TGTTGCAATCAGGAAAGCAGTA), penA-R (AACAATCTGGTAGATCG), penB-F (CCGGTGTCTAAATTTCTTCTA), penB-R (TATATTGATTGAGGCAGCAG). mtrR-F (GAGACAGTGCCACATGCAAC) and mtrR-R (TCAGGCGCTTGGTAGTGTATCCA). Alleles for penA were assigned based on the classic alleles described by Ohtsuki et al. and, when unavailable, by the NG-STAR database (https://ngstar.canada.ca). Similarly, all high-level azithromycin-resistant strains were investigated for resistance polymorphisms in 23S rDNA, mtrR and its promoter region, and the macA promoter region, and the macA promoter using the primers 23S-F (ACGGATGCGACATGCCGCA), 23S-R (TAAACGATCTACCATCATTCG), 23S-R(321)-R (GAATCCGGCGAGATAAGCG), 23S-R(357)-R (GCGCCCATCAAAACACCCACG), 23S-R(357)-R (TCAGAATCTCCACGATCTT), macA-F (TAGGATGAGCTTGGTCCG), macA-R (CCAGCTTGGCCTGATACTGG), mtrR-F and mtrR-R. Chromosomal DNA of N. gonorrhoeae strain ATCC 49226 and double distilled water were included in all PCR assays as positive and negative controls, respectively.

**Molecular epidemiology typing**

The ST was determined for all isolates using the N. gonorrhoeae multi-antigen sequence typing (NG-MAST) method. Genotypes were assigned to closely related STs when the porB or tpbB allele was identical to the most frequent ST of that genogroup and the other allele showed at least 99% similarity. A phylogenetic tree was constructed in MEGA7 with concatenated porB and tpbB alleles using the neighbour-joining tree topology based on distance estimates from the ‘number of nucleotide differences’ model. Clustering of strains was performed on concatenated porB and tpbB alleles using Bayesian Analysis of Population Structure (BAPS) software following the ‘clustering of linked loci’ format.
Increasing prevalence of resistant gonoccci in Hangzhou

Table 1. Antimicrobial susceptibility of 379 N. gonorrhoeae strains isolated between January 2015 and December 2017 in Hangzhou, China

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>MIC range (mg/L)</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (mg/L)</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (mg/L)</th>
<th>Susceptibility category&lt;sup&gt;a&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRO</td>
<td>0.001–0.25</td>
<td>0.03</td>
<td>0.125</td>
<td>S 97, I 67, R 3</td>
</tr>
<tr>
<td>AZM</td>
<td>0.008–2048</td>
<td>0.125</td>
<td>16</td>
<td>S 87, I 12, R 21</td>
</tr>
<tr>
<td>CFM</td>
<td>0.001–0.5</td>
<td>0.06</td>
<td>0.25</td>
<td>S 87, I NA, R 13</td>
</tr>
<tr>
<td>PEN</td>
<td>0.03–2048</td>
<td>2</td>
<td>128</td>
<td>S 1, I 35, R 64</td>
</tr>
<tr>
<td>CIP</td>
<td>0.03–128</td>
<td>16</td>
<td>32</td>
<td>S 0, I 0, R 100</td>
</tr>
<tr>
<td>SPT</td>
<td>1–64</td>
<td>32</td>
<td>100</td>
<td>S 100, I NA, R 0</td>
</tr>
<tr>
<td>TET</td>
<td>0.006–128</td>
<td>2</td>
<td>64</td>
<td>S 11, I 9, R 80</td>
</tr>
</tbody>
</table>

<sup>a</sup>According to the breakpoints defined by EUCAST (in mg/L): ceftriaxone (CRO; R > 0.125, S ≤ 0.125), azithromycin (AZM; R > 0.5, S ≤ 0.25), cefixime (CFM; R > 0.125, S ≤ 0.125), penicillin (PEN; R > 1, S ≤ 0.06), ciprofloxacin (CIP; R > 0.06, S ≤ 0.03), spectinomycin (SPT; R > 64, S ≤ 64) and tetracycline (TET; R > 1, S ≤ 0.5).

**Results**

**Antimicrobial susceptibility of N. gonorrhoeae isolates**

A total of 379 N. gonorrhoeae isolates were obtained from seven hospitals in Hangzhou during the study period, including 127 isolates in 2015, 145 isolates in 2016 and 107 isolates in 2017. Antimicrobial susceptibility testing showed that 12 isolates (3%) were resistant to ceftriaxone, 80 isolates (21%) to azithromycin and 48 isolates (13%) to cefixime (Table 1). Importantly, decreased susceptibility to ceftriaxone and cefixime (MIC ≥ 0.125 mg/L)<sup>16,17</sup> was observed in 17% and 30% of the isolates, respectively, while 7% of the isolates were high-level azithromycin resistant. Further analysis of N. gonorrhoeae susceptibility to antimicrobials that are currently no longer recommended showed that the prevalence of resistance to ciprofloxacin (100%), penicillin (64%, including 36% PPNG) and tetracycline (80%, including 37% TRNG) was still very high, while all isolates were susceptible to spectinomycin (Table 1). Since ceftriaxone plus azithromycin dual therapy is currently recommended in many countries and is currently under consideration in China, the distribution of ceftriaxone and cefixime susceptibility in azithromycin-resistant strains was analysed further. While only 1 isolate showed resistance to both ceftriaxone and azithromycin, a total of 19 isolates (5%) showed both azithromycin resistance and decreased susceptibility to ceftriaxone and 6 of these isolates were high-level azithromycin resistant. Similarly, of the azithromycin-resistant strains, 10 (3%) were also cefixime resistant and 26 (7%) showed decreased susceptibility to cefixime, including 8 high-level azithromycin-resistant strains.

To investigate the development of resistance to ceftriaxone, cefixime and azithromycin over the 3 year study period, the yearly resistance levels were analysed (Figure 1). The ceftriaxone-resistant strains accounted for 2% of the isolates in 2015, 3% in 2016 and 5% in 2017, while the percentage of isolates with decreased susceptibility to ceftriaxone increased from 10% in 2015 to 15% in 2016 and 27% in 2017 (Figure 1a). Similarly, the percentage of cefixime-resistant strains increased from 8% in 2015 to 12% in 2016 and 20% in 2017, while the percentage of strains with decreased susceptibility to cefixime increased over this period from 20% in 2015 to 28% in 2016 and 46% in 2017 (Figure 1b). Finally, the percentage of azithromycin-resistant strains also showed an upward trend over the study period and increased from 14% in 2015 to 17% in 2016 and 35% in 2017 (Figure 1c), while simultaneously the percentage of high-level azithromycin-resistant strains increased from 5% in 2015 to 7% in 2016 and 11% in 2017. Therefore, for all three antimicrobials an upward trend in resistance levels was observed over the study period. This was also reflected in the increase in azithromycin-resistant strains showing decreased susceptibility to ceftriaxone, which increased from 2% in 2015 to 3% in 2016 and 11% in 2017.

**Characterization of resistance determinants**

The 12 ceftriaxone-resistant isolates were analysed for the presence of resistance determinants (Table 2). The mosaic penA allele X was identified in five strains, while the non-mosaic alleles VII, XII, XIII, XXVII and 43 (NG-STAR) were identified in the other seven ceftriaxone-resistant isolates. In addition, only two of the ceftriaxone-resistant strains did not show the mtrR G45D mutation or the adenine deletion in the 13 bp inverted repeat of its promoter, while the other 10 strains showed either one or both of these mutations. Also, seven of the ceftriaxone-resistant strains showed the porB G120K mutation in combination with A121D or A121N. Similarly, the mosaic penA allele X was present in 24 of the 48 cefixime-resistant isolates, while the non-mosaic alleles XIII (7 isolates), VII (6 isolates), XVII (4 isolates), II (1 isolate), IX (1 isolate), IV (1 isolate), 43 (NG-STAR; 3 isolates) and 54 (NG-STAR; 1 isolate) were identified in the remainder of the cefixime-resistant isolates. In addition, the G45D polymorphism of mtrR was present in 22 isolates, while the adenine deletion in the mtrR promoter was identified in 29 isolates. Also, 29 strains showed mutations in porB, namely G120K (26 isolates), G120R (2 isolates), A121D (16 isolates), A121N (10 isolates) and A121G (2 isolates) and 1 isolate showed a G120D mutation in combination with a deletion of A121. The 28 high-level azithromycin-resistant isolates were also analysed for resistance determinants and the A2059G mutation was present in all four alleles of 23S rRNA in 27 isolates and in 2B.
Figure 1. Development of antimicrobial susceptibility for ceftriaxone, cefixime and azithromycin over the period 2015–17. The graphs show the distribution of MICs as the percentage of the total isolates for each year (2015, n = 127; 2016, n = 145; 2017, n = 107). (a) Annual distribution of ceftriaxone MICs. (b) Annual distribution of cefixime MICs. (c) Annual distribution of azithromycin MICs.

Table 2. Characteristics of the 12 ceftriaxone-resistant N. gonorrhoeae isolates from Hangzhou in 2015–17

<table>
<thead>
<tr>
<th>Year</th>
<th>ST</th>
<th>mtrR promoter</th>
<th>mtrR G45</th>
<th>porB G120</th>
<th>porB A121</th>
<th>penA allele</th>
<th>MIC (mg/L)</th>
<th>CRO</th>
<th>CFM</th>
<th>AZM</th>
<th>SPT</th>
<th>PEN</th>
<th>CIP</th>
<th>TET</th>
<th>PPNG</th>
<th>TRNG</th>
</tr>
</thead>
<tbody>
<tr>
<td>2015 ST4823</td>
<td>ΔA</td>
<td>WT</td>
<td>G201K</td>
<td>A121D</td>
<td>XXVII</td>
<td>0.25</td>
<td>0.25</td>
<td>0.06</td>
<td>32</td>
<td>8</td>
<td>32</td>
<td>128</td>
<td>no</td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2015 ST12866</td>
<td>WT</td>
<td>G45D</td>
<td>WT</td>
<td>WT</td>
<td>VII</td>
<td>0.25</td>
<td>0.125</td>
<td>0.5</td>
<td>16</td>
<td>0.5</td>
<td>16</td>
<td>2</td>
<td>no</td>
<td>no</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2016 ST15144</td>
<td>ΔA</td>
<td>G45D</td>
<td>WT</td>
<td>WT</td>
<td>43b</td>
<td>0.25</td>
<td>0.25</td>
<td>0.008</td>
<td>16</td>
<td>1</td>
<td>64</td>
<td>64</td>
<td>no</td>
<td>yes</td>
<td></td>
<td></td>
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<tr>
<td>2016 ST5308</td>
<td>WT</td>
<td>WT</td>
<td>G201K</td>
<td>A121D</td>
<td>X</td>
<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
<td>32</td>
<td>32</td>
<td>64</td>
<td>4</td>
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<td></td>
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<tr>
<td>2016 ST12049</td>
<td>ΔA</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>VII</td>
<td>0.25</td>
<td>0.25</td>
<td>0.125</td>
<td>32</td>
<td>0.5</td>
<td>16</td>
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<tr>
<td>2016 ST14590</td>
<td>ΔA</td>
<td>G45D</td>
<td>WT</td>
<td>WT</td>
<td>XII</td>
<td>0.25</td>
<td>0.25</td>
<td>0.03</td>
<td>16</td>
<td>512</td>
<td>4</td>
<td>128</td>
<td>yes</td>
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<tr>
<td>2017 ST4734</td>
<td>ΔA</td>
<td>G45D</td>
<td>G201K</td>
<td>A121D</td>
<td>X</td>
<td>0.25</td>
<td>0.25</td>
<td>1</td>
<td>16</td>
<td>2</td>
<td>32</td>
<td>32</td>
<td>no</td>
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<tr>
<td>2017 ST3741</td>
<td>WT</td>
<td>G45D</td>
<td>G120K</td>
<td>A121N</td>
<td>VII</td>
<td>0.25</td>
<td>0.03</td>
<td>0.5</td>
<td>32</td>
<td>1</td>
<td>16</td>
<td>32</td>
<td>no</td>
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<tr>
<td>2017 ST7553</td>
<td>WT</td>
<td>G45D</td>
<td>G120K</td>
<td>A121N</td>
<td>X</td>
<td>0.25</td>
<td>0.25</td>
<td>0.03</td>
<td>32</td>
<td>4</td>
<td>64</td>
<td>16</td>
<td>no</td>
<td>yes</td>
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<tr>
<td>2017 ST12866</td>
<td>ΔA</td>
<td>WT</td>
<td>G120K</td>
<td>A121N</td>
<td>XIII</td>
<td>0.25</td>
<td>0.25</td>
<td>0.03</td>
<td>32</td>
<td>4</td>
<td>64</td>
<td>32</td>
<td>no</td>
<td>yes</td>
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<tr>
<td>2017 ST16447</td>
<td>ΔA</td>
<td>G45D</td>
<td>G201K</td>
<td>A121D</td>
<td>X</td>
<td>0.25</td>
<td>0.25</td>
<td>0.06</td>
<td>32</td>
<td>4</td>
<td>64</td>
<td>16</td>
<td>no</td>
<td>yes</td>
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</tr>
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</table>

CRO, ceftriaxone; CFM, cefixime; AZM, azithromycin; SPT, spectinomycin; CIP, ciprofloxacin; TET, tetracycline; PEN, penicillin.

aAccording to the NG-MAST method.

bAccording to the NG-STAR database.
three alleles for 1 isolate. In addition, all high-level azithromycin-resistant strains contained the adenine deletion in the promoter of mtrR and 14 strains contained the mtrR G45SD mutation.

**NG-MAST analysis of N. gonorrhoeae isolates**

Based on the NG-MAST method, the 379 N. gonorrhoeae isolates were assigned to 248 different STs and included 187 new STs. The most prevalent STs were ST4539 (15 isolates), ST3741 (10 isolates), ST1766 (7 isolates), ST1866 (6 isolates), ST5308 (6 isolates), ST2268 (6 isolates), ST2318 (6 isolates) and ST5061 (6 isolates). Furthermore, analysis of the ceftriaxone-, cefixime- and azithromycin-resistant isolates showed that the 12 ceftriaxone-resistant isolates belonged to 10 different STs and included 2 isolates of ST3741 and ST12866. The 48 cefixime-resistant isolates belonged to 43 different STs and only ST3038 (5 isolates) and ST4539 (2 isolates) were found multiple times. The 80 azithromycin-resistant isolates belonged to 67 different STs; STs containing multiple isolates were ST1866 (6 isolates), ST5304 (4 isolates), ST4539 (3 isolates), ST5309 (2 isolates), ST1766 (2 isolates) and ST7554 (2 isolates). Finally, the 28 high-level azithromycin-resistant isolates belonged to 22 different STs and included 5 isolates of ST1866, 2 isolates of ST309 and 2 isolates of ST4539.

To obtain further insight into the genetic relationship between antimicrobial-resistant and -susceptible isolates and to investigate possible clonal expansion of resistance, a phylogenetic tree was constructed and clusters of strains were identified following the BAPS clustering method. The strains were grouped into 11 different BAPS clusters that largely followed the structure of the phylogenetic tree. BAPS clusters and antimicrobial susceptibility categories for ceftriaxone, cefixime and azithromycin are indicated by colour coding of the phylogenetic tree (Figure 2a). All BAPS clusters contained strains displaying decreased susceptibility to ceftriaxone or cefixime and strains displaying azithromycin resistance, except for cluster 1, which only contains two strains. The relative abundance of strains showing decreased susceptibility to ceftriaxone or cefixime for each BAPS cluster in comparison with the overall population was analysed and particularly BAPS cluster 4 (Figure 2b) contained a significantly higher population of strains showing decreased susceptibility to ceftriaxone or cefixime (BAPS 4: 30 of 45 strains versus BAPS 1–3 and 5–11: 103 of 334 strains, \( P < 0.0001 \), Fisher’s exact test). Importantly, BAPS cluster 4 also appeared to have expanded recently and showed a significantly higher proportion of strains from 2017 compared with the other BAPS clusters (BAPS 4: 26 of 45 strains versus BAPS 1–3 and 5–11: 81 of 334 strains, \( P < 0.0001 \), Fisher’s exact test). The strains in this emerging BAPS cluster all contain \( \text{tbpB} \) allele 10, except for two strains with \( \text{tbpB} \) allele 907, which differs by one SNP. Also, for the majority of these strains the \( \text{porB} \) alleles are closely related and 24 of the 45 strains of this BAPS cluster belonged to a single genogroup (G5308). Of the 22 ceftriaxone- or cefixime-resistant isolates of BAPS cluster 4, 19 isolates contained the mosaic \( \text{penA} \) allele X, while this \( \text{penA} \) allele is encountered in only 8 ceftriaxone- or cefixime-resistant isolates belonging to other BAPS clusters. Although BAPS cluster 4 did not contain more azithromycin-resistant isolates compared with other BAPS clusters, it did contain 12 azithromycin-resistant strains, including 4 high-level azithromycin-resistant strains, of which 2 strains also displayed decreased susceptibility to ceftriaxone. Therefore, this emerging cluster of largely clonally related strains might pose a particular threat to our ceftriaxone monotherapy and ceftriaxone plus azithromycin dual-therapy regimens.

**Discussion**

Current gonococcal treatment guidelines generally recommend ceftriaxone monotherapy or ceftriaxone plus azithromycin dual therapy. Ceftriaxone monotherapy (single dose of 250 mg intramuscularly) is still the recommended treatment in China; however, due to rising resistance levels, shifting to dual therapy is under consideration.26,51 Previously, we reported on the antimicrobial susceptibility of 118 N. gonorrhoeae isolates from 2011–12 in Hangzhou and showed that all isolates were susceptible to ceftriaxone and cefixime, with only 1 isolate showing decreased susceptibility to ceftriaxone and 2 isolates showing decreased susceptibility to cefixime.50 However, azithromycin resistance was already observed for 21% of the isolates and 18% of the isolates showed high-level azithromycin resistance, raising the question of whether azithromycin should be included in dual therapy in China. In the current follow-up study, we analysed the antimicrobial susceptibility of 379 isolates from Hangzhou over the period 2015–17. We showed that resistance levels to ceftriaxone, cefixime and azithromycin increased over this period, resulting in 2017, in a high number of strains (11%) showing both decreased susceptibility to ceftriaxone and azithromycin resistance. These strains pose a major threat to the possible implementation of ceftriaxone plus azithromycin dual therapy.

The 2017 antimicrobial resistance levels for ceftriaxone and azithromycin in our study are higher than data presented by other studies on gonococcal antimicrobial resistance in China, although all these studies represent clinical isolates from 2016 and earlier. For instance, data from the China Gonococcal Resistance Surveillance Program (China-GRSP) for seven Chinese provinces over the period 2013–16 showed that azithromycin resistance fluctuated between 17% and 21%, while decreased susceptibility to ceftriaxone ranged between 10% and 12%.20 Furthermore, the proportion of strains showing both decreased susceptibility to ceftriaxone and azithromycin resistance reached 3% in 2016, which is similar to what we reported for 2015–16. Similarly, analysis of 128 clinical isolates from Changsha from 2015–16 showed that 2% of the strains were ceftriaxone resistant and 11% had decreased susceptibility to ceftriaxone,60 while analysis of 126 isolates from Hefei from 2014–15 showed that 10% of the strains had decreased susceptibility to ceftriaxone and 29% were azithromycin resistant, including 10% that were high-level azithromycin resistant.53 However, only two strains from Hefei showed both decreased susceptibility to ceftriaxone and azithromycin resistance. A higher incidence of strains displaying both decreased susceptibility to ceftriaxone and azithromycin resistance was already reported for an earlier study from Guangzhou.61 Analysis of 485 isolates from 2009–13 showed that decreased susceptibility to ceftriaxone was observed for 22% of the isolates and azithromycin resistance in 16%, resulting in 5% of the isolates displaying both decreased susceptibility to ceftriaxone and azithromycin resistance. A similar incidence of 5% of the strains displaying both decreased susceptibility to ceftriaxone and azithromycin resistance was also reported for 384 isolates from
Importantly, this study also reported that 32% of the isolates displayed azithromycin resistance and 10% showed high-level azithromycin resistance. Lower levels of resistance to ceftriaxone and azithromycin were observed in earlier studies and studies from other regions in China.\(^6^2,6^3\) In comparison with other countries, high incidences of gonococcal isolates showing ceftriaxone or azithromycin resistance have been found in Japan for over a decade. A recent study from Japan covering the period 2000–15 showed that the incidence of ceftriaxone resistance (MIC \(\geq 0.125\) mg/L) fluctuated between 5% and 20%, and this coincided with a high incidence of strains displaying both ceftriaxone and azithromycin resistance (up to 8% in 2015).\(^3^8\) In most other countries, including Europe, the USA and Australia, the incidence of strains displaying ceftriaxone resistance or both decreased susceptibility to ceftriaxone and azithromycin resistance is still relatively low.\(^6^4–6^7\) However, clusters of related strains displaying both decreased susceptibility to ceftriaxone and azithromycin resistance or high-level azithromycin resistance have been identified in Hawaii (USA)\(^3^7,6^8\) and Ontario (Canada).\(^3^6\)

In our study, phylogenetic analysis and BAPS clustering showed that decreased susceptibility to ceftriaxone and cefixime was encountered in variety of clonally unrelated strains; however, there seemed to be a specific increase in 2017 of clonally related strains displaying decreased susceptibility to ceftriaxone and/or cefixime. This cluster of strains contained mostly \(tbpB\) allele 10 and a variety of \(porB\) alleles, with the majority of strains being closely related and belonging to genogroup G5308. This recently emerging cluster also contained numerous strains displaying azithromycin resistance or high-level azithromycin resistance. Furthermore, the majority of ceftriaxone- and/or cefixime-resistant strains from this cluster contained the mosaic \(penA\) allele X. This mosaic \(penA\) allele is thus far not common in China and ceftriaxone resistance, decreased susceptibility to ceftriaxone or cefixime resistance has

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**Figure 2.** Genetic relationship between ceftriaxone-, cefixime- and azithromycin-resistant and -susceptible isolates. (a) Concatenated \(porB\) and \(tbpB\) alleles were used for all 379 isolates to construct the phylogenetic tree in MEGA7 following the neighbour-joining tree topology and to perform BAPS clustering following the linked loci format. BAPS clusters and antimicrobial susceptibility categories for ceftriaxone (CRO), cefixime (CFM) and azithromycin (AZM) are indicated by colour coding; STs and year of isolation are shown for each isolate. Susceptibility categories (R, resistant; DS, decreased susceptibility; I, intermediate; S, susceptible; HLR, high-level resistant) are defined as: CRO-R > 0.125 mg/L, CRO-DS = 0.125 mg/L, CRO-S < 0.125 mg/L, CFM-R > 0.125 mg/L, CFM-DS = 0.125 mg/L, CFM-S < 0.125 mg/L, AZM-HLR \(\geq 256\) mg/L, AZM-R > 0.5 mg/L, AZM-I = 0.5 mg/L and AZM-S \(\leq 0.25\) mg/L. (b) Expansion of BAPS cluster 4 from the phylogenetic tree depicted in (a), showing the antimicrobial susceptibility category, ST and year of isolation for each isolate.
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generally been encountered in strains containing the penA alleles V, XIII, XVIII or XXI. However, penA allele X was commonly encountered in strains displaying cefixime resistance and decreased susceptibility to cefixime in Japan over a decade ago. These strains generally belonged to MLST ST7365 and generally contain tbpB allele 10. Importantly, in recent years, ceftriaxone-resistant strains displaying high-level resistance have been identified in Japan, Australia, Denmark, Spain, and Canada, and the strains H041 (Japan), OU140106 (Japan) and A8806 (Australia) all contain tbpB allele 10 and porB alleles closely related to the porB alleles found in our emerging cluster. Specifically, the porB alleles of strains OU140106 and A8806 differ only by eight and seven SNPs from ST5308, respectively. Therefore, these strains might be derived from a common ancestor, although the penA alleles related to high-level ceftriaxone resistance have diverged afterwards.

In conclusion, in this study we analysed the susceptibility of 379 N. gonorrhoeae isolates from Hangzhou in 2015–17 and found that 3% of the isolates were ceftriaxone resistant, 21% were azithromycin resistant and 5% showed both decreased susceptibility to ceftriaxone and azithromycin resistance. Importantly, resistance levels appeared to increase over the study period, resulting in 2017 in 5% ceftriaxone resistance, 35% azithromycin resistance and 11% decreased susceptibility to ceftriaxone plus azithromycin resistance. In addition, 2017 witnessed the emergence of a largely clonally related cluster of strains with a high abundance of isolates displaying decreased susceptibility to ceftriaxone and/or cefixime, including some cross-resistance to azithromycin. The emergence of this cluster poses a major threat to the current ceftriaxone monotherapy and the possible implementation of ceftriaxone plus azithromycin dual therapy in Hangzhou and the rest of China.

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Transparency declarations

None to declare.

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