Dissemination and genome analysis of high-level ceftriaxone-resistant penA 60.001 *Neisseria gonorrhoeae* strains from the Guangdong Gonococcal antibiotics susceptibility Programme (GD-GASP), 2016–2019

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**ABSTRACT**

Background: After *Neisseria gonorrhoeae* FC428 was first found in Japan, ceftriaxone-resistant strains disseminated globally, and the gonococcal resistance rate increased remarkably. Epidemiological investigations are greatly significant for the analysis of antimicrobial resistance (AMR) trends, molecular features and evolution.

Objectives: To clarify the AMR trend from 2016–2019 and reveal the molecular characteristics and evolution of ceftriaxone-resistant penA 60.001 isolates.

Methods: The minimum inhibitory concentrations (MICs) of antibiotics against 4113 isolates were detected by the agar dilution method. *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), multilocus sequence typing (MLST) and *N.gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR) were used to identify the sequence types. Genome analysis was conducted to analyze resistance genes, virulence factors, and evolutionary sources.

Results: Isolates with decreased ceftriaxone susceptibility have increased from 2.05% (2016) to 16.18% (2019). Six ceftriaxone-resistant isolates possessing penA 60.001 appeared in Guangdong Province, and were resistant to ceftriaxone, penicillin, tetracycline, ciprofloxacin and cefixime, but susceptible to azithromycin and spectinomycin. Single-nucleotide polymorphisms (SNPs) in the *porB* gene were the major cause of different NG-MAST types. ST1903 was the main NG-STAR genotype and only strain-ZHS45 was ST7365, with molecular features consistent with the MICs. Furthermore, different MLSTs suggested diverse evolutionary sources. Genome analysis revealed a set of virulence factors along with the resistance genes “penA” and “blaTEM-1B”. Half of penA 60.001 strains were fully mixed with global FC428-related strains.

Conclusions: Global FC428-related clones have disseminated across Guangdong, possibly causing decreased ceftriaxone susceptibility. Enhanced gonococcal surveillance will help elucidate the trajectory of transmission and curb further dissemination.

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**KEYWORDS** *N. gonorrhoeae*; ceftriaxone-resistant; penA 60.001; epidemiological investigation

**Introduction**

Gonorrhea is a sexually transmitted disease caused by *Neisseria gonorrhoeae*, and more than 87 million cases are currently infected globally [1]. Currently, the World Health Organization (WHO) has included *N. gonorrhoeae* as a high priority pathogen because of therapy failures and dramatic case number increases [2]. The pathogen has developed antimicrobial resistance (AMR) to every antibiotic currently approved for therapy [3], and the effectiveness of the last remaining option for first-line antimicrobial monotherapy, ceftriaxone, has come into question due to the evolving resistance and, especially, spread of the FC428 clone [4].

The presence of FC428, which is a globally disseminated ceftriaxone-resistant clone characterized by mosaic penA 60.001, has been widely documented, including in Japan, Denmark, South Korea, Ireland, Canada, Australia, Singapore, France and the UK, since 2015 [4], raising concerns about the long-term effectiveness of ceftriaxone therapy. In a study analyzing FC428 isolates from 2016 to 2019, the first case was identified in northern China (July 2016, Beijing) [5] and then FC428 disseminated to the central (2017 June to September, Hunan) [6], western (2018, Sichuan) [7] and eastern (May to October 2019, Zhejiang) [8] region, showing that highly resistant to ceftriaxone FC428 related clones had already appeared in China. The wide spread of FC428 might have a considerable impact on the molecular characteristics and composition of local strains. The further dissemination of resistant/
virulence factors, without new antibiotics/vaccines for gonorrhea will be a major challenge in the future.

In China, the epidemic of gonococcal infections and AMR trends has been the most severe in Guangdong for nearly ten years. As an important part of the WHO Western Pacific Regional (WPR) Resistance Surveillance Program, southern China (Guangdong Province) reported a dramatic increase in N. gonorrhoeae in 2017, including 29,945 new isolates (accounting for 21.6% of notifiable cases in China), which is consistent with the situation in other countries, such as the United States, Japan and South Africa [9]. However, no research has been conducted to explore whether this phenomenon is related to the spread of FC428. Since this is a region undergoing rapid economic development with high personnel flow and close interactions with other countries, gonococcal AMR surveillance must be conducted to identify emerging AMR, monitor AMR trends and provide evidence for the revision of global, regional and national gonorrhea management guidelines and public health strategies and policies.

To date, research on penA 60.001 strains in this area is lacking, and detailed resistance and virulence features have rarely been presented.

In our study, we performed ceftriaxone susceptibility testing of 4113 N. gonorrhoeae isolates from 2016 to 2019 and subsequently found that the rate of decreased ceftriaxone susceptibility (minimum inhibitory concentration (MIC) ≥0.125 mg/L) increased sharply from 2.05% in 2016–16.18% in 2019. Through penA gene sequence typing, six resistant strains with MIC=0.25–0.5 mg/L harbouring penA 60.001 were found. The emergence of such highly ceftriaxone resistant isolates raised grave concerns because no FC428 clone or high-level ceftriaxone-resistant penA 60.001 strain had previously been identified in Guangdong Province. Further genome analysis revealed the sequence types (STs), resistance genes and virulence factors of penA 60.001 strain. Finally, evolutionary analysis showed that half of isolates were closely related to the FC428-related clones detected overseas, and remaining penA 60.001 strains have a similar evolutionary origin to the strain 18DG342. Overall, FC428-related clones have disseminated across Guangdong, and the need to strengthen antibiotic and molecular resistance monitoring should be emphasized.

**Materials and methods**

**Strain collection, cultivation, preservation**

A total of 4113 strains were collected from 2016 to 2019 in Guangdong, China (including Guangzhou, Shenzhen, Jiangmen, Dongguan, Zuhai, Shantou, Maoming, Shaoguan, Zhongshan, Zhanjiang and Foshan cities). Specifically, we collected 634, 758, 1633 and 1088 strains in 2016, 2017, 2018, and 2019 respectively. Then, the isolates were identified by gram staining and oxidase, catalase and sugar fermentation tests recommended by the WHO. Furthermore, the strains were cultured in Thayer-Martin (TM) medium and gonococcal agar supplemented with 10% defibrinated sheep blood for 18 h in a 37 incubator at 5% CO2. All strains were stored in fetal bovine serum containing 10% DMSO at −80°C.

**Antimicrobial susceptibility testing**

The MICs of ceftriaxone, penicillin, ciprofloxacin, tetracycline, spectinomycin, azithromycin and cefixime were determined by the agar dilution method according to the WHO Western Pacific N. gonorrhoeae Monitoring Program. WHO reference strains D, G, J, L, K and P were used for quality control.

**Whole genome sequencing**

The genomic DNA of ceftriaxone-resistant strains harbouring penA 60.001 was extracted for sequencing, assembly and analysis by Wentao Chen.

**Genotyping**

N. gonorrhoeae sequence typing for antimicrobial resistance (NG-STAR), N. gonorrhoeae multiantigen sequence typing (NG-MAST), and multilocus sequence typing (MLST) were performed to define the molecular epidemiological characteristics (https://pubmlst.org/).

**Resistance, virulence and phyllogenetic analysis**

Raw reads were downloaded from the Sequence Read Archive (SRA) database, except for a subset of strains for which complete genomes were unavailable. Sequencing reads were preprocessed with fastp (v0.20.1) to remove adaptors and low-quality reads. The assembled contigs were generated using SPAdes (v3.13) and then utilized to analyze the acquired resistance genes and virulence factors using ABRicate (v1.0.0). Phylogenetic analysis was conducted according to the pipeline described previously [10]. Variant calling and full-length genome alignment were performed using Snippy (v4.6) (https://github.com/tseemann/snippy) with FC428 (GenBank accession NZ_AP018377 set as the reference). The full-length alignment was used as an input into Gubbins (v2.3.1) with default parameters to predict and filter regions of homologous recombination, which was followed by re-construction of the filtered alignment [11]. The following default parameters of Gubbins were used: min SNPs to identify a recombination block (default: 3); minimum window size (default: 100); and maximum window size (default: 10000).
Results and discussion

Antimicrobial susceptibility

Between 2016 and 2019, a total of 4113 isolates were collected for ceftriaxone MIC testing, and the results showed that the prevalence rate of decreased susceptibility to ceftriaxone (ceftriaxone $\geq 0.25$ mg/L) increased from 2.05% (13 of 634) in 2016–16.18% (176 of 1088) in 2019 (Figure 1). As reported previously, the FC428 epidemic is one of the reasons for the increase in the rate of ceftriaxone resistance [12]. Thus, to evaluate the penA allele of ceftriaxone-resistant strains (MIC $\geq 0.25$ mg/L) and determine the prevalence of dominant ceftriaxone-resistant strains, we collected a total of 50 ceftriaxone-resistant N. gonorrhoeae samples from 2016 to 2019 for penA allele identification. Then, six clones were first identified as penA 60.001 strains. Furthermore, the antimicrobial susceptibility of such penA 60.001 clones to seven drugs was similar to that of the original FC428 strain. As shown, six isolates all displayed resistance to ceftriaxone (MIC $\geq 0.25$ mg/L), cefotaxime (MIC $\geq 1$ mg/L), ciprofloxacin (MIC $\geq 8$ mg/L), tetracycline (MIC $= 2-4$ mg/L) and penicillin (MIC $\geq 2$ mg/L), but were susceptible to spectinomycin (MIC=$8-16$ mg/L) and azithromycin (MIC=$0.06-0.5$ mg/L) (Table 1A).

Molecular surveillance of AMR in penA 60.001 N. gonorrhoeae

To clarify the molecular characteristics of penA 60.001 isolates, NG-MAST, NG-STAR and MLST were performed for genotyping. As shown in Table 1B, NG-MAST divided the six isolates into five STs, and differences were mainly identified in the SNPs in the porB gene, which were clearly different from those in the original FC428 (porB-1053, tlpB-21, ST3435) [13]. ST233, which is identical to FC428, was the main genotype determined by NG-STAR. Strains ZH545, DG18193 and SS74 were characterized as having different STs because of their differences in the porB and gyr genes. In conclusion, all molecular features were consistent with the antibiotic phenotype. For MLST, ST1903 was the most prevalent sequence type (66.7%, n=6), which is also a typical feature of FC428 found in Japan. However, ZH545 strain possessing ST7365 and ST13943 from strain DG19112 suggested that the origin of the six penA 60.001 isolates might have included different evolutionary trajectories.

To optimize the analysis of possible resistance genes, we used ABRicate for further analysis. As shown in Table 2A, the resistance gene "penA" was indeed the most important factor affecting cephalosporin efficacy, and one of the isolates also carried “bla-TEM-1B”, which was consistent with the antibiotic phenotype. In addition, the genomes of these six penA 60.001 strains were fully sequenced and analyzed to obtain a complete picture of virulence gene repertoires. The data revealed that all examined strains harboured the antigen 85 proteins (fbpB and fbpC), high-affinity ABC importer pathway for Mn(II) (mntB and mntC), mtrC-mtrD-mtrE efflux pump, Pil (msrA/B (pilB), pilF, pilG, pilH, pilK, pilM, pilN, pilO, pilP, pilT, pilT2, pilU, pilV, pilW, pilT and pilJ), porin porB, ABC transporter farB, DNA repair gene (recN), lactoferrin import receptor (lpbA), hemoglobin receptor gene (hmbR) and surface protein A (nsP). The coverage of the virulence factors pilD, pilX, pilZ, lpbB, fbpA, DldH catalase (katA), transferrin receptor (tpB) and outer membrane proteins (hpuA and hpuB) in the genome reached more than 80%, which might also play a key role in gonococcal infection and AMR. Only the mntA virulence factor was not detected in some strains (DG19112, SS74 and ZH545); thus, the importance of this virulence factor remains to be discussed at a later stage (Table 2B).

Phylogenomic analysis

Finally, phylogenomic analysis was performed to track evolution. The results in Figure 2 show that the six strains were scattered in different evolutionary branches. ZH545 was the closest strain to the classic FC428 clone found in Japan in 2015, SS74 alone constituted an evolutionary branch near A7536 (2017, Australia), and MM14, MM08 and DG18193 were classified into another subclade close to 18DG342.
from Singapore in 2018. DG19112 was not mixed with the globally abundant FC428-related strains. In addition, the purple-labelled strains in the figure are all FC428-related clones identified in China, but they were alienated from the six isolates in Guangdong. Moreover, it was reported that FC428-related clones found in Australia have a history of dissemination via sexual intercourse in China, and strains of similar evolutionary origin “SS74” were locally detected among FC428-related strains, indicating that foreign strains have already affected the composition of local N. gonorrhoeae strains.

**Discussion**

*N. gonorrhoeae* is a major public health threat worldwide, and is the second most common cause of bacterial STI after *Chlamydia trachomatis* [15]. Penicillin, tetracycline, ciprofloxacin, and cefixime were all first-line drugs used to treat gonorrhea until the resistance rate exceeded the WHO threshold of 5% [15]. Currently, the state of gonorrhea treatment is extremely serious because only ceftriaxone remains as a last-line effective monotherapy [16]. As reported, the prevalence of decreased ceftriaxone susceptibility in many countries, including China, has reached 10% [17]. According to clinical guidelines in the United States [18], UK [19] and China [17], one gram of ceftriaxone is recommended as the typical does. However, given the irregular use of antibiotics and spread of FC428, ceftriaxone resistance has become more severe. Overall, antibiotic resistance in gonorrhea is extremely severe, and the clinical use of ceftriaxone might need to be re-standardized.

Currently, whole-genome sequencing is an emerging drug resistance monitoring method, and the MICs of antibiotics can be reflected by gene features to an extent. As reported, 23S rRNA-100 both possessed wild-type genes, which was consistent with the azithromycin-sensitive phenotype. *PonA-1*, which include the AMR marker L421P, can significantly reduce the rate of penicillin acylation, leading to treatment failure [20]. *ParC-3*, containing S87R, was first found in Canada and is correlated with ciprofloxacin resistance [20]. Mutations in *gyrA* and *porB* reduce drug intake and determine resistance [21,22]. *PenA* 60.001, which a characteristic of FC428 and possesses A311V and T483S, was confirmed to be related to cephalosporin and penicillin resistance [23]. Moreover, the widespread distribution of FC428 has become a major threat to ceftriaxone-based therapy [24]; thus, it is essential to enhance penA sequencing to identify FC428 clone. Obviously, molecular features were consistent with the antibiotic phenotype. In summary, molecular epidemiological investigations are of great significance for gonorrhea prevention and control.

Since 1996, the Guangdong Gonococcal Antibiotic Resistance Monitoring Network has been part of the Gonococcal Antibiotic Susceptibility Programme.
(GASP). By 2020, we established a monitoring network covering 44 medical institutions across Guangdong Province and integrated antibiotic susceptibility testing and sequence genotyping, including NG-MAST, NG-STAR and MLST, into sentinel hospitals. The strategy of enhanced AMR surveillance in China was addressed at the 2017 International Forum on Gonococcal Infections and Resistance [25]. Expanding this network and collecting more isolates are essential to improving representative surveillance. Along with the previous study in supplemental Table 1, our study is the first report of penA 60.001 under large-sample screening in Guangdong Province. The data illustrated that ceftriaxone susceptibility has decreased, and penA 60.001 strains are mostly scattered in common global FC428-related clones. It is necessary to monitor the genetic characteristics of this strain and its subsequent dissemination. In addition to the major resistance gene penA, we revealed for the first time that penA 60.001 strains harboured a set of virulence factors related to the Mn(II) uptake system, ferric ion (Fe(III))-binding protein transport, drug efflux, adhesion, and detoxification, which are related to infection and AMR [26–34].

In conclusion, the ancestral FC428 lineage has disseminated into Guangdong, China. Enhanced epidemiological surveillance will help shed light on the molecular properties, transmission trajectory and antibiotic resistance rate, which will curb the dissemination of gonorrhea.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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Table 2. Resistance genes and virulence factor analysis.
(A) Resistance genes identified among penA 60.001 isolates

| Gene  | DG18193 | DG19112 | MM08 | MM14 | SS74 | ZH545 |
|-------|---------|---------|------|------|------|-------|
| penA  | 100     | 100     | 100  | 100  | 100  | 100   |
| blaTEM-18 | 0       | 0       | 0    | 0    | 0    | 0     |

(B) Virulence factors analysis of penA 60.001 isolates

| Factor | DG18193 | DG19112 | MM08 | MM14 | SS74 | ZH545 |
|--------|---------|---------|------|------|------|-------|
| farB   | 100     | 100     | 100  | 100  | 100  | 100   |
| fbpA   | 100     | 100     | 100  | 100  | 100  | 100   |
| fbpC   | 100     | 100     | 100  | 100  | 100  | 100   |
| hmbR   | 100     | 100     | 100  | 100  | 100  | 100   |
| mntB   | 100     | 100     | 100  | 100  | 100  | 100   |
| mntC   | 100     | 100     | 100  | 100  | 100  | 100   |
| msrA/B | 100     | 100     | 100  | 100  | 100  | 100   |
| mtrC   | 100     | 100     | 100  | 100  | 100  | 100   |
| lbpA   | 100     | 100     | 100  | 100  | 100  | 100   |
| mtrD   | 100     | 100     | 100  | 100  | 100  | 100   |
| mtrE   | 100     | 100     | 100  | 100  | 100  | 100   |
| nspA   | 100     | 100     | 100  | 100  | 100  | 100   |
| pilF   | 100     | 100     | 100  | 100  | 100  | 100   |
| pilG   | 100     | 100     | 100  | 100  | 100  | 100   |
| pilH   | 100     | 100     | 100  | 100  | 100  | 100   |
| pilK   | 100     | 100     | 100  | 100  | 100  | 100   |
| pilM   | 100     | 100     | 100  | 100  | 100  | 100   |
| pilN   | 100     | 100     | 100  | 100  | 100  | 100   |
| pilO   | 100     | 100     | 100  | 100  | 100  | 100   |
| pilP   | 100     | 100     | 100  | 100  | 100  | 100   |
| pilT   | 100     | 100     | 100  | 100  | 100  | 100   |
| pilT2  | 100     | 100     | 100  | 100  | 100  | 100   |
| pilU   | 100     | 100     | 100  | 100  | 100  | 100   |
| pilV   | 100     | 100     | 100  | 100  | 100  | 100   |
| pilW   | 100     | 100     | 100  | 100  | 100  | 100   |
| pilZ   | 100     | 100     | 100  | 100  | 100  | 100   |
| porB   | 100     | 100     | 100  | 100  | 100  | 100   |
| recN   | 100     | 100     | 100  | 100  | 100  | 100   |
| lbpB   | 100     | 81.43   | 100  | 100  | 81.43 | 81.43 |
| katA   | 99.88   | 99.88   | 99.88 | 99.88 | 99.88 | 99.88 |
| pilX   | 99.8    | 99.8    | 99.8  | 99.8  | 99.8  | 99.8  |
| fbpA   | 99.8    | 99.8    | 99.8  | 99.8  | 99.8  | 99.8  |
| hpuA   | 99.83   | 99.83   | 99.83 | 99.83 | 99.83 | 99.83 |
| pilJ   | 99.68   | 99.68   | 99.68 | 99.68 | 99.68 | 99.68 |
| tbpA   | 99.67   | 99.67   | 99.67 | 99.67 | 99.67 | 99.67 |
| pilL   | 99.51   | 99.51   | 99.51 | 99.51 | 99.51 | 99.51 |
| hpuB   | 98.32   | 98.32   | 98.32 | 98.32 | 98.32 | 98.32 |
| pilD   | 99.3    | 99.3    | 99.3  | 99.3  | 99.3  | 99.3  |
| mtrA   | 97.43   | 97.43   | 97.43 | 0     | 0     | 0     |

An absent gene is denoted "0" and a present gene is represented by its % COVERAGE.
Author contributions
Xiaomian Lin designed the study. Xiaomian Lin, Wentao Chen, Qinghui Xie, Yuqi Yu, Yiwen Liao, Xiaolin Qin, Zhanjin Feng, Xingzhong Wu, Sanmei Tang, and Heping Zheng conducted the experiments, analyzed the results and wrote the manuscript. All authors reviewed the final version of the manuscript.

Others
Raw reads of high-level ceftriaxone-resistant penA 60.001 Neisseria gonorrhoeae strains could be downloaded from the Sequence Read Archive (SRA) database (PRJNA778600).

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