Shanghai Neisseria gonorrhoeae Isolates Exhibit Resistance to Extended-Spectrum Cephalosporins and Clonal Distribution

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The emergence of Neisseria gonorrhoeae strains with resistance (R) to extended-spectrum cephalosporins (ESCsR) represents a public health threat of untreatable gonococcal infections. This study was designed to determine the prevalence and molecular mechanisms of ESCR of Shanghai N. gonorrhoeae isolates. A total of 366 N. gonorrhoeae isolates were collected in 2017 in Shanghai. Susceptibility to ceftriaxone (CRO), cefixime (CFM), azithromycin (AZM), ciprofloxacin (CIP), spectinomycin, penicillin, and tetracycline was determined using the agar dilution method. A subset of 124 isolates was subjected to phylogenetic analysis for nine antimicrobial resistance-associated genes, i.e., penA, porB, ponA, mtrR, 23S rRNA, gyrA, parC, 16S rRNA, and rpsE. Approximately 20.0% of the isolates exhibited CFM R [minimum inhibitory concentration (MIC) >0.125 mg/L], and 5.5% were CRO R (MIC > 0.125 mg/L). In total, 72.7% of ESCR isolates were clonal and associated with mosaic penA10 and 60 alleles. Non-mosaic penA18 allele and substitutions of PenA A501T, G542S, and PorB1b G213S/Y were observed in non-clonal ESCR. Approximately 6.8% of the isolates showed AZM MIC above the epidemiological cutoff (ECOFF, 1 mg/L), were associated with 23S rRNA A2059G mutation, and did not exhibit clonal distribution. Almost all isolates were CIP R (resistance to ciprofloxacin) and associated with GyrA-91/92 and ParC-85/87/88/89/91 alterations. Isolates with ParC S88P substitution were clustered into the ESCR clade. The Shanghai isolates exhibited a high level of ESCR and distinct resistant patterns.

Keywords: Neisseria gonorrhoeae, extended-spectrum cephalosporins, multidrug resistance, resistance determinants, phylogenetic analysis

INTRODUCTION

Neisseria gonorrhoeae is the causative agent of gonorrhea. The World Health Organization (WHO) estimated that N. gonorrhoeae causes more than 86.9 million new infections worldwide annually (World Health Organization [WHO], 2018). Meanwhile, gonococcal antimicrobial resistance (AMR) continues to spread worldwide and could lead to a pandemic of extensively drug-resistant gonococci (World Health Organization [WHO], 2018). Of particular concern is the fact that ESCR
one-third of the participating countries reported that ≥5% of isolates are resistant to ESCs (CRO and/or CFM), and half reported ≥5% resistance to azithromycin (AZM\textsuperscript{R}). Of the 59 countries reporting ciprofloxacin resistance (CIP\textsuperscript{R}), 95% reported ≥5% resistance and 17% reported ≥90% resistance (Wi et al., 2017; World Health Organization [WHO], 2018). In China, from 2013 to 2016, a high prevalence of decreased susceptibility to CRO (CRO\textsuperscript{D} (9.7–12.2%, MIC ≥ 0.125 mg/L) and AZM\textsuperscript{R} (18.6%, MIC ≥ 1.0 mg/L) has been reported (Yin et al., 2018). In Shanghai, the proportion of CRO\textsuperscript{R} (MIC ≥ 0.125 mg/L) ranged from 7 to 13% during 1988–2013 (Gu et al., 2014).

Drug-resistant _N. gonorrhoeae_ has been attributed to several molecular mechanisms. The primary mechanism for ESC\textsuperscript{R} (resistance to ESC) is mutations of the penA gene (encodes penicillin (PEN)-binding protein 2, PBP2, PenA), including a recombinant mosaic allele from commensal _Neisseria_ (Ameyama et al., 2002; Lee et al., 2010). Mutations in the Mtr repressor genes _mtrR_ and _porB_ have been shown to contribute to ESC\textsuperscript{R} (Barry and Klauser, 2009; Unemo and Shafer, 2014). Loci involved in other AMR include mutations of 23S rRNA (Ng et al., 2002) and _mtrR_ (Zarantonelli et al., 1999) for AZM\textsuperscript{R}, mutations in _gyrA_ and _parC_ for CIP\textsuperscript{R} (Yang et al., 2006), and mutations in 16S rRNA and _rpsE_ for spectinomycin (SPT) (Galmand et al., 2000; Unemo et al., 2013). Currently, the identified resistance determinants do not fully account for the observed drug resistance, and thus, other factors may be involved (Unemo and Shafer, 2014).

Genetic analysis has provided insight into outbreaks and transmission networks for several pathogens with greater resolution than traditional methods (Diep, 2013). Using genetic methods, researchers found that ESC\textsuperscript{R} in Canada first emerged from a group of diverse isolates in the 1990s with non-mosaic _penA_ alleles, followed in 2000/2001 with the mosaic _penA_ 10 allele and then in 2007 with the mosaic _penA_ 34 allele (Demczuk et al., 2015). ESC\textsuperscript{R} strains in the United States are mainly clonal and associated with the mosaic _penA_ 34 allele and derivatives, whereas AZM\textsuperscript{R} strains have arisen through multiple mechanisms and show limited clonal spread (Grad et al., 2014, 2016). To date, reported cases of CRO\textsuperscript{R} are sporadic, except for the FC428 strain, which was first identified in Japan in 2015 and has since then been observed in other countries (Lahra et al., 2018; Lee et al., 2019).

Genetic analysis has been used to study strain distribution along with multi-locus sequence typing (MLST) (Unemo and Dillon, 2011), _N. gonorrhoeae_ multi-antigen sequence typing (NG-MAST) (Unemo and Dillon, 2011), _N. gonorrhoeae_ sequence typing for AMR (NG-STAR) (Demczuk et al., 2017), and whole-genome sequencing (De Silva et al., 2016; Harris et al., 2018; Lee et al., 2018). We have reported NG-STAR analysis of seven loci in 124 _N. gonorrhoeae_ isolates (Yang et al., 2020); specific NG-STAR genotypes are found to be associated with ESC\textsuperscript{R} and AZM\textsuperscript{R}.

The objectives of this study were to assess whether nine loci can increase the resolution of genetic analysis and to determine the association of genetic characterization and ESC\textsuperscript{R} phenotypes in _N. gonorrhoeae_ isolates in Shanghai. This is the first in-depth genomic analysis based on nine AMR-associated loci in _N. gonorrhoeae_ in a high-level AMR setting. This study provides solid information on the molecular mechanisms and genetic characteristics of AMR in _N. gonorrhoeae_ in Shanghai.

### MATERIALS AND METHODS

**Neisseria gonorrhoeae Isolate Collection and Antimicrobial Susceptibility Testing**

*Neisseria gonorrhoeae* isolates were collected from male patients with uncomplicated urogenital gonorrhea (symptoms may include pain or a burning sensation when urinating, a greater frequency or urgency of urination, and abnormal urinary discharge) at the Shanghai Skin Disease Hospital in conjunction with the China GASP. Patient consent was obtained, and ethics approval was received from the Shanghai Skin Disease Hospital. The first 30 _N. gonorrhoeae_ isolates of each month in 2017 (except for 36 isolates collected in July to avoid recovery failure, making a total of 366 isolates) were used in this study. Basic demographic data of all patients were collected. The median age was 34 years (range: 18–69). Of the 366 subjects, 363 (99.2%) were ethnic Han. All patients were heterosexual. A majority of the patients had abnormal urinary discharge (98.9%). Approximately 16.4% of the patients had previous history of gonorrhea, and 12.8% received antibiotic treatment in the past month. One isolate was collected from one patient. Briefly, one urogenital specimen was collected using sterile Dacron swab and streaked on Thayer–Martin (T–M) medium (Oxoid; GuangZhou LOSO Science, Ltd., supplemented with 1% IsoVitaleX (Oxoid; GuangZhou LOSO Science, Ltd.). _N. gonorrhoeae_ was identified using criteria that included an oxidase test, Gram staining, and glucose utilization test (WHO Western Pacific Gonococcal Antimicrobial Surveillance Programme, 2008). One identified _N. gonorrhoeae_ isolate for each patient was collected and stored at −70°C. Minimum inhibitory concentrations (MICs) for seven antimicrobials, including PEN, tetracycline (TET), ciprofloxacin (CIP), azithromycin (AZM), CFM, CRO, and SPT, were determined using the agar dilution method. Antimicrobial agents were purchased from Shanghai ANPEL Scientific Instrument, Co., Ltd. (Shanghai, China; distributors of Sigma-Aldrich, United States). Each MIC determination was performed in duplicate, and _N. gonorrhoeae_ ATCC 49226 strain was used as a reference strain. Antimicrobial susceptibility testing results were interpreted using the EUCAST (2020) breakpoints.

**DNA Sequencing and Analysis**

As previously reported, a total of 124 _N. gonorrhoeae_ isolates (first 10 isolates of each month, one CIP susceptible isolate, and three isolates with CRO MICs ≥ 1.0 mg/L) were subjected to genetic analysis (Yang et al., 2020). Genomic DNA from each isolate was extracted using the Genomic DNA Purification Kit (Shanghai Promega Biological Products, Ltd., Shanghai, China). Seven loci (penA, _mtrR_, _porB_, _ponA_, _gyrA_, _parC_, and 23S rRNA) were PCR amplified as described previously (Yang et al., 2020). 16S rRNA was amplified by PCR (Perkin Elmer...
Among the 366 N. gonorrhoeae isolates, 5.5% of the isolates were CRO\(\text{R}\) (MICs > 0.125 mg/L), and 18.6% of isolates had CRO MICs ≥ 0.125 mg/L (Table 1 and Supplementary Table S1). About 19.4% of the 366 isolates were CFM\(\text{R}\) (MICs > 0.125 mg/L). About 6.8% of the isolates showed AZM MIC above the epidemiological cutoff (ECOFF, 1 mg/L), and 99.5% of the isolates were CIP\(\text{R}\). The percentages of PEN\(\text{R}\) and TET\(\text{R}\) were 82.5% and 60.9%, respectively. One isolate was SPT\(\text{R}\). Demographic/clinical information including age, ethnicity, abnormal urinary discharge, previous history of gonorrhea, and antibiotic use in the past month was not associated with resistance to CFM, CRO, and AZM (Supplementary Table S2).

Supplementary Table S3 shows that 24.0% (88/366) of the sequenced isolates exhibited multidrug-resistant (MDR) phenotypes (resistance to ESC or AZM plus resistance to at least two other antimicrobials) (Martin et al., 2019). Among these phenotypes, ESC-associated phenotypes accounted for 17.7%, and AZM-associated phenotype accounted for 6.3%. Extensively drug-resistant phenotypes (resistance to ESC and AZM plus resistance to at least two other antimicrobials) (Martin et al., 2019) were noted in two isolates, namely, CFM\(\text{R}\) (MIC = 0.25 mg/L)–AZM\(\text{R}\) (MIC = 2 mg/L)–CIP\(\text{R}\) (MIC ≥ 16 mg/L)–PEN\(\text{R}\) (MIC = 4 mg/L)–TET\(\text{R}\) (MIC = 4 mg/L) and CRO\(\text{R}\) (MIC = 0.25 mg/L)–CFM\(\text{R}\) (MIC = 0.25 mg/L)–AZM\(\text{R}\) (MIC ≥ 8 mg/L)–CIP\(\text{R}\) (MIC ≥ 16 mg/L)–PEN\(\text{R}\) (MIC ≥ 16 mg/L)–TET\(\text{R}\) (MIC = 2 mg/L).

### Genotyping of N. gonorrhoeae ESC\(\text{R}\) Isolates

**Mosaic penA and Substitutions in penA and Association With NG-STAR Types**

Approximately 76.2% of the CFM\(\text{R}\) isolates (16/21) had mosaic penA alleles, and only 2.9% of the CFM\(\text{S}\) isolates (3/103) possessed mosaic penA alleles (Table 2). Mosaic penA 10 and 60 alleles and substitutions in the mosaic penA coding region such as D101E and A549T were significantly associated with CFM\(\text{R}\). Specifically, 10 out of 11 (90.9%) penA-10.001 isolates were CFM\(\text{R}\), and four out of four (100%) penA-60.001 isolates were CFM\(\text{R}\). Substitutions of F374V, H541N, P552V, K1555QV, I566V, and A574V were also statistically associated with CFM\(\text{R}\).

**Mosaic penA alleles were detected in 37.5% of CRO\(\text{R}\) isolates and in 13.8% of CRO\(\text{S}\) isolates. Only the mosaic penA 60 allele was significantly associated with CRO\(\text{R}\) (Table 2). PenA substitutions F374V and A501T showed significantly higher frequencies in CRO\(\text{R}\) isolates than in CRO\(\text{S}\) isolates.**

NG-STAR ST-233 (penA 60) was associated with CFM\(\text{R}\) and CRO\(\text{R}\) (Table 2), while ST-348 (penA 10, exhibited by NG-MAST ST3308, ST7554, and ST12784) was associated with CFM\(\text{R}\) and ST-428 (penA 18) was associated with CRO\(\text{R}\).

The metadata of four mosaic penA 60 isolates are listed in Table 3. Demographic and clinical information revealed that four patients were young (age range: 18–44), all of them had abnormal urinary discharge, and none reported previous history of gonorrhea or any antibiotic use in the past month. Three of four mosaic penA 60 isolates were NG-STAR genotype 233, whereas one was NG-STAR genotype 1143. Four penA 60 isolates had the same pattern of PenA substitutions, which contained A311V and T483S alterations, the key CRO\(\text{R}\) substitution.
**TABLE 1 |** MIC distribution of seven antimicrobial agents for 366 *N. gonorrhoeae* isolates from Shanghai.

| Antimicrobials          | 0.002 | 0.004 | 0.008 | 0.016 | 0.03  | 0.06  | 0.125 | 0.25  | 0.5   | 1     | 2     | 4     | 8     | 16    | 32    | 64    | ≥128  | ≥256  |
|------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|------|------|
| Ceftriaxone             |       |       |       |       | 1     | 1     | 6     | 1     | 1     | 2     | 1     | 2     | 4     | 8     | 16    | 32    | 64    |      |      |
| Cum%                   | 0.2%  | 1.4%  | 4.2%  | 4.4%  | 5.7%  | 4.4%  | 15.9% | 51.9% | 82    | 94.5% | 98.9% | 99.2% | 100%  |       |       |      |      |      |      |
| Cefixime               |       |       |       |       | 0.5   | 1.9   | 7.9   | 25.1% | 59.3% | 80.6% | 90.7% | 97%   | 98.9% | 99.2% | 100%  |       |      |      |      |
| Azithromycin           |       |       |       |       | 0.3   | 0.8   | 4.6   | 14.5% | 42.1% | 73.8% | 91%   | 93.2% | 94.3% | 94.8% | 97.3% | 98.1% | 100%  |      |      |
| Ciprofloxacin          |       |       |       |       |       |       | 0.5   | 0.5   | 0.5   | 0.5   | 0.5   | 2.2   | 7.7   | 22.1% | 44.3% | 91.5% |      |      |      |      |
| Penicillin             |       |       |       |       | 0     | 0     | 0     | 0     | 0     | 5     | 4     | 53    | 95    | 63    | 17    | 75    | 52    |      |      |
| Tetracycline           |       |       |       |       | 0     | 2     | 0     | 0     | 0     | 2     | 12    | 27    | 102   | 107   | 17    | 1     | 29    | 69    |      |      |
| Spectinomycin          |       |       |       |       | 0     | 1     | 0     | 0     | 0     | 0     | 0     | 3.8   | 11.2% | 39.1% | 68.3% | 73%   | 73.2% | 81.1% | 100%  |      |      |

Vertical lines indicate the breakpoint concentrations for each antimicrobial. Breakpoints: MICs > 0.125 mg/L for ceftriaxone or cefixime, ECOFF value is 1 mg/L for azithromycin, MICs > 0.06 mg/L for ciprofloxacin, MICs > 1 mg/L for penicillin or tetracycline, and MICs > 64 mg/L for spectinomycin (EUCAST, 2020).

aNumber of isolates.
bCumulative percentage.

All penA 60 isolates have identical ponA, mtrR, 23S rRNA, gyrA, parC, 16S rRNA, and rpsE patterns and different PorB substitutions. Additional MICs and the molecular profiles of four isolates are summarized in Table 3.

Among the non-mosaic penA allele isolates, the penA 18 allele and substitutions of PenA A501T and G542S were associated with ESCR (Table 2). The proportion of non-mosaic penA ESCR isolates harboring the PenA A501T substitution (for CFM, 60%, 3/5; for CRO, 80%, 4/5) was significantly higher than the proportion of non-mosaic penA ESCR isolates harboring that substitution (for CFM, 15%, 15/100; for CRO, 14%, 14/100). Eighty percent (4/5) of non-mosaic penA CRO R isolates had the G542S substitution, which was significantly higher than non-mosaic penA CRO S isolates (26%, 26/100). Interestingly, all ESCR with the PenA double substitutions of A501T and G542S (three CFM R and four CRO R) exhibited a penA 18 allele.

**mtrR Gene and Promoter**

MtrR G45D and mtrR promoter -35A were significantly lower in CFM R isolates than in CFM S isolates. MtrR A40D and T86A were significantly associated with CFM S. No mtrR mutations was found to be associated with CRO R.

**Characteristic Genotypes of *N. gonorrhoeae* Isolates With MICs Above AZM ECOFF**

In isolates with MICs above the AZM ECOFF value (1 mg/L), 87.5% (7/8, AZM MICs ≥ 256 mg/L) harbored the A2059G mutation in 23S rRNA, which is significantly higher than in isolates with MICs below AZM ECOFF (0/116, AZM MIC range: ≤0.03–1 mg/L). MtrR G45D and NG-STAR ST-202 (NG-MAST ST1866) were significantly higher in isolates above AZM ECOFF than in isolates below AZM ECOFF (p = 0.007 for MtrR G45D, p < 0.0001 for NG-STAR ST-202).

**Characteristic Genotypes in *N. gonorrhoeae* SPT R Isolates**

There was only one SPT R isolate identified. This SPT R isolate was the only strain that harbored 16S rRNA C1192U mutation in our dataset (Figure 1), which has earlier been reported to be associated with SPT R. The K26E substitution in RpsE was not detected.
### TABLE 2 | Molecular profiles associated with resistance to cefixime, ceftriaxone, and azithromycin in N. gonorrhoeae.

| Molecular markers | All isolates | Non-mosaic penA isolates | All isolates |
|------------------|-------------|-------------------------|-------------|
|                  | CFM<sup>S</sup> n (%) | CFM<sup>R</sup> n (%) | p | CFM<sup>S</sup> n (%) | CFM<sup>R</sup> n (%) | p | CFM<sup>S</sup> n (%) | CFM<sup>R</sup> n (%) | p |
| **NG-STAR genotype<sup>a</sup> (penA type)** | | | | | | | | | | |
| ST-202 (penA 2) | 4 (3.9) | 0 (0) | 1 | 4 (3.4) | 0 (0) | 1 | 0 (0) | 4 (50.0) | <0.0001 |
| ST-233 (penA 60) | 0 (0) | 3 (14.3) | 0.004 | 0 (0) | 3 (37.5) | <0.001 | 3 (2.6) | 0 (0) | 1 |
| ST-34B (penA 10) | 1 (1.0) | 4 (19.0) | 0.003 | 5 (4.3) | 0 (0) | 1 | 5 (4.3) | 0 (0) | 1 |
| ST-428 (penA 18) | 3 (2.9) | 2 (9.5) | 0.199 | 3 (2.6) | 2 (25.0) | 0.033 | 3 (3.0) | 2 (40.0) | 0.017 |
| **PenA type** | | | | | | | | | | |
| Mosaic | 3 (2.9) | 16 (76.2) | <0.0001 | 16 (13.8) | 3 (37.5) | 0.196 | |
| penA 10 | 1 (1.0) | 10 (47.6) | <0.0001 | 11 (9.5) | 0 (0) | 1 | |
| penA 34 | 2 (1.9) | 1 (4.8) | 0.430 | 3 (2.6) | 0 (0) | 1 | |
| penA 60 | 0 (0) | 4 (19.0) | <0.001 | 1 (0.9) | 3 (37.5) | <0.001 | |
| penA 71 | 0 (0) | 1 (4.8) | 0.169 | 1 (0.9) | 0 (0) | 1 | |
| **Non-mosaic** | 100 (87.1) | 5 (23.8) | <0.0001 | 100 (86.2) | 5 (62.5) | 0.196 | |
| penA 18 | 15 (14.6) | 3 (14.3) | 0.759 | 14 (12.1) | 4 (50.0) | 0.015 | 15 (15.0) | 3 (60.0) | 0.034 |
| **Substitutions in mosaic penA** | | | | | | | | | | |
| D101E plus<sup>b</sup> | 3 (2.9) | 11 (52.4) | <0.0001 | 14 (12.1) | 0 (0) | 0.599 | |
| YGED201HAGE, Q214E | 4 (3.9) | 12 (57.1) | <0.0001 | 16 (13.8) | 0 (0) | 0.562 | |
| A311V, T483S, T485I | 0 (0) | 4 (19.0) | <0.001 | 1 (0.9) | 3 (37.5) | <0.001 | |
| I312M, V316T, N612Y plus<sup>c</sup> | 3 (2.9) | 16 (76.2) | <0.0001 | 16 (13.8) | 3 (37.5) | 0.196 | |
| P341S | 3 (2.9) | 12 (57.1) | <0.0001 | 15 (12.9) | 0 (0) | 0.594 | |
| GA375TP | 3 (2.9) | 13 (61.9) | <0.0001 | 16 (13.8) | 0 (0) | 0.562 | |
| A549T | 1 (1.0) | 15 (71.4) | <0.001 | 13 (11.2) | 3 (37.5) | 0.061 | |
| **PenA Substitutions** | | | | | | | | | | |
| F374V | 0 (0) | 4 (19.0) | <0.001 | 1 (0.9) | 3 (37.5) | <0.001 | |
| A501T<sup>d</sup> | 15 (14.6) | 3 (14.3) | 0.759 | 14 (12.1) | 4 (50.0) | 0.015 | 15 (15.0) | 3 (60.0) | 0.034 |
| A501V | 58 (56.3) | 1 (4.8) | <0.0001 | 58 (50.0) | 1 (12.5) | 0.091 | |
| A516G | 100 (87.1) | 5 (23.8) | <0.0001 | 100 (86.2) | 5 (62.5) | 0.119 | |
| H541N | 23 (22.3) | 16 (76.2) | <0.0001 | 36 (31.0) | 3 (37.5) | 0.990 | |
| G542S | 27 (26.2) | 3 (14.3) | 0.245 | 26 (22.4) | 4 (50.0) | 0.182 | 27<sup>b</sup> (27.0) | 3<sup>f</sup> (60.0) | 0.277 |
| P552V, KI555QV | 17 (16.5) | 15 (71.4) | <0.0001 | 29 (25.0) | 3 (37.5) | 0.716 | |
| i566V, A574IV | 47 (45.6) | 18 (85.7) | <0.001 | 58 (50) | 7 (87.5) | 0.091 | |
| **PorB genotype** | | | | | | | | | | |
| porB1a | 7 (6.8) | 1 (4.8) | 1 | 8 (6.9) | 0 (0) | 1 | |
| porB1b | 96 (93.2) | 20 (95.2) | 1 | 108 (93.1) | 8 (100) | 1 | |
| **PorB1b Substitutions** | | | | | | | | | | |
| T87A | 8 (8.3) | 2 (10.0) | 0.844 | 7 (6.5) | 3 (37.5) | 0.021 | |
| T89S | 10 (10.4) | 3 (15.0) | 0.816 | 10 (9.3) | 3 (37.5) | 0.045 | |
| GA120KD | 52 (54.2) | 15 (75.0) | 0.086 | 60 (55.6) | 7 (87.5) | 0.163 | |
| G213S<sup>f</sup> | 11 (11.5) | 4 (20.0) | 0.503 | 10 (9.3) | 5 (62.5) | <0.001 | 11 (11.8) | 2 (40.0) | 0.1294 |
| G214L | 7 (7.3) | 2 (10.0) | 0.962 | 6 (5.6) | 3 (37.5) | 0.015 | |
| G259A | 61 (63.5) | 17 (85.0) | 0.083 | 70 (64.8) | 8 (100) | 0.041 | |
| **PonA Substitutions** | | | | | | | | | | |
| T375A | 100 (87.1) | 21 (100) | 1 | 113 (97.4) | 8 (100) | 1 | |
| L421P | 99 (96.1) | 21 (100) | 1 | 112 (96.8) | 8 (100) | 1 | |
| **MtrR Substitutions** | | | | | | | | | | |
| A39T | 10 (9.7) | 3 (14.3) | 0.816 | 13 (11.2) | 0 (0) | 1 | 13 (11.20) | 0 (0) | 1 |
| A40D | 4 (3.9) | 8 (38.1) | <0.0001 | 12 (10.3) | 0 (0) | 1 | 12 (10.3) | 0 (0) | 1 |

(Continued)
23S rRNA Mutations

*G45D, T86A, H105Y, promotor -35A, and G545S.*

With the 23S rRNA A2059G mutation (*Table 2*), across the phylogenetic tree (*Figure 1*), Isolates with MICs above AZM ECOFF appeared sporadically.

Isolates with MICs above AZM ECOFF

Approximately 76% (16 of 21) of CFM ≥ 0.125 mg/L isolates were classified into clade A. NG-STAR genotypes were reported previously (Yang et al., 2020). All isolates were penA 18 allele.

**DISCUSSION**

A large proportion of *N. gonorrhoeae* isolates in Shanghai in 2017 exhibited resistance to ESCs. Approximately 19.4% of 366 *N. gonorrhoeae* isolates were CFM≥ 0.125 mg/L, and 40.7% of the isolates had CFM MICs > 0.125 mg/L. About 5.5% of the isolates were CRO≥ 0.125 mg/L, and 18.0% of isolates had CRO MICs ≥ 0.125 mg/L. About 6.8% of the isolates had MICs above AZM ECOFF. One isolate was SPTR. *N. gonorrhoeae* CFM≥ isolates exhibited clonal distribution of one cluster containing mosaic penA alleles, whereas CRO≥ isolates appeared sporadically across the phylogeny.

The resistant percentages of *N. gonorrhoeae* isolates to CRO, CFM, or AZM in Shanghai exceeded the WHO cutoff of 5%, indicating a need to review recommended treatments (World Health Organization [WHO], 2012). Over 18.0% of *N. gonorrhoeae* isolates had CRO MICs ≥ 0.125 mg/L, higher than that reported in previous years in Shanghai and other places in China (Gu et al., 2014; Yin et al., 2018) as well as several other countries such as 0.1% in the United States in

### Clonal Distribution of *N. gonorrhoeae* ESCR Isolates by Phylogenetic Analysis

Phylogenetic analysis of nine genes (*Supplementary Figure 1*), the inclusion of two SPTR genes (16S rRNA and rpsE) did not change the structure or resolution of the phylogeny. Cluster analysis results showed that a nine-gene phylogeny had 14 clusters, while a seven-gene phylogeny had 16 clusters. Most ESCR strains were classified as one clade that had the mosaic penA alleles (*Figure 1*, clade A). NG-STAR genotypes were reported previously (Yang et al., 2020). All isolates were penA 18 allele.

Multiple linear regression analysis revealed that the mosaic penA allele harbored a penA 18 allele and was classified into clade C (*Figure 2*). The penA 18 allele consisted of the A501T and G542S double substitutions.

### Phylogenetic Analysis of *N. gonorrhoeae* Isolates With MICs Above AZM ECOFF

Isolates with MICs above AZM ECOFF appeared sporadically across the phylogenetic tree (*Figure 1*) and were highly associated with the 23S rRNA A2059G mutation (*Table 2* and *Figure 1*). All NG-STAR ST-202 isolates harbored the 23S rRNA A2059G mutation. Multiple linear regression analysis indicated that the 23S rRNA A2059G mutation was strongly associated with increased AZM MICs (*Supplementary Table S5*).

### Analysis of *N. gonorrhoeae* CIPR Isolates

The 124 isolates included 123 CIPR and 1 CIPR. Substitutions at GyrA-91 and GyrA-95 were highly predictive of the resistant phenotype (*Figure 3*). CIPR did not harbor GyrA or ParC substitutions. CIPR with CIP MICs > 0.06 mg/L had both GyrA-91 (S91F) and GyrA-95 (D95A/G/Y/N) substitutions. *N. gonorrhoeae* isolates with triple GyrA substitutions at positions 91, 92, and 95 exhibited a high level of quinolone resistance (CIP MICs ≥ 16 mg/L). Phylogenetic analysis (*Figure 1*) revealed that GyrA A92P and ParC S88P substitutions could be clustered into two clades, whereas other GyrA and ParC substitutions were distributed across the phylogeny. Specifically, eight out of nine (88.9%) isolates with GyrA A92P substitution were clustered into clade B, whereas 9 out of 14 (63.4%) isolates with ParC S88P substitution were clustered into clade A. Multiple linear regression analysis indicated that ParC-85/86/87/88/89/91 and GyrA-91/92 substitutions heavily contributed to CIP MIC increments (*Supplementary Table S6*).
TABLE 3 | Metadata of four mosaic penA 60.001 isolates in Shanghai.

| Isolate id | Age | Gender | Ethnicity | Transmission | Infection site | Abnormal urinary discharge | Previous history of gonorrhea | Antibiotic use in the past month | Date of clinic visit | MICs (mg/L) |
|------------|-----|--------|-----------|--------------|----------------|--------------------------|-----------------------------|-----------------------------|-------------------|-------------|
| 17–256     | 44  | Male   | Han       | Hetero       | Urethra        | Yes                       | No                          | No                          | 02/12/2017        | 0.125       |
| SH-40      | 18  | Male   | Han       | Hetero       | Urethra        | Yes                       | No                          | No                          | 25/11/2017        | ≥4          |
| SH-41      | 35  | Male   | Han       | Hetero       | Urethra        | Yes                       | No                          | No                          | 27/11/2017        | 1           |
| SH-48      | 28  | Male   | Han       | Hetero       | Urethra        | Yes                       | No                          | No                          | 06/12/2017        | 1           |

Molecular profiles

| Isolate ID | NG-STAR genotype | PPNG   | penA       | PorB1b                      | PonA             | MtrR | 23S rRNA | GyrA       | ParC         | 16S rRNA | rpsE |
|------------|------------------|--------|------------|-----------------------------|------------------|------|----------|------------|--------------|----------|------|
| 17–256     | PPNG 60.001      | 1143   | G120K, A121G, Q143K, T215A, I218M, M257R, S258R, G259A | T375A, L421P     | Promoter −35A, H105Y | WT   | S91F, D95A | S87R       | T1458C       | WT        |
| SH-40      | Non-PPNG 60.001  | 233    | G120K, A121D, V151A, I209M, YD211GY, G213Y, Q214L, T215, S217N, V242A, A256T, M257S, G259A, A272V | T375A, L421P     | Promoter −35A, H105Y | WT   | S91F, D95A | S87R       | T1458C       | WT        |
| SH-41      | Non-PPNG 60.001  | 233    | G120K, A121D, Q143K, T215V, M257R, S258R, G259A | T375A, L421P     | Promoter −35A, H105Y | WT   | S91F, D95A | S87R       | T1458C       | WT        |
| SH-48      | Non-PPNG 60.001  | 233    | G120K, A121D, V151A, I209M, YD211GY, G213Y, Q214L, T215, S217N, V242A, A256T, M257S, G259A, A272V | T375A, L421P     | Promoter −35A, H105Y | WT   | S91F, D95A | S87R       | T1458C       | WT        |
FIGURE 1 | Phylogenetic reconstruction of nine genes, patterns of antimicrobial resistance, and genetic polymorphisms in 124 \textit{N. gonorrhoeae} isolates. Left: Phylogenetic reconstruction of 124 isolates based on maximum likelihood. The red arrow indicates the reference strain FA1090. Heatmap. Columns 1 and 2: MIC values of CRO and CFM. Columns 3–5: Susceptible/resistant categories according to the EUCAST MIC breakpoints of CRO, CFM, and ESCs. Column 6: NG-STAR genotype (white band indicates NG-STAR genotypes new to the NG-STAR database). Columns 7–10: A specific NG-STAR genotype. Column 11: Any mosaic \textit{penA} allele. Columns 12–15: A specific mosaic \textit{penA} allele. Columns 16–21: Non-synonymous amino acid changes from wild type in PorB1b. Columns 22 and 23: Non-synonymous amino acid changes from wild type in Pona. Column 24: MIC values of AZM. Column 25: Susceptible/resistant categories according to the EUCAST MIC breakpoints of AZM. Column 26: The –35A deletion in the \textit{mtrR} promoter. Column 33: MIC values of SPT. Column 34: Susceptible/resistant categories according to the EUCAST MIC breakpoints of SPT. Column 35: C1192U mutation in 16S rRNA. Column 36: MIC values of CIP. Column 37: Susceptible/resistant categories according to the EUCAST MIC breakpoint of CIP. Columns 38–40: Non-synonymous amino acid changes from wild type in GyrA. Columns 41–46: Non-synonymous amino acid changes from wild type in ParC. The purple and orange rectangles indicate clades A and B described in the text, respectively. Sequence names are colored by cluster.

2013 and 2014 (Kirkcaldy et al., 2016), 1.8% in Canada in 2016 (Martin et al., 2019), and 10.7% in Japan in 2012–2013 (Hamasuna et al., 2015). The proportion of \textit{N. gonorrhoeae} CFMR isolates (MICs > 0.125 mg/L, 19.4%) in Shanghai in 2017 was much higher than that in the United States (0.4–0.8% in 2013–2014) (Kirkcaldy et al., 2016), Europe (1.7–2.0% in 2014–2015) (Cole et al., 2017), and Canada (0.3% in 2016) (Martin et al., 2019). These findings indicate that unlike those in the United States, European countries, Japan, and Canada, CRO and CFM may need to be reviewed as a treatment for gonorrhea in Shanghai. \textit{N. gonorrhoeae} isolates in Shanghai remain susceptible to SPT (Yang et al., 2006), suggesting that SPT may have potential as a first-line therapy for the treatment of uncomplicated urogenital gonorrhea in Shanghai. SPT is available in China. However, SPT is not suitable for the treatment of pharyngeal gonorrhea, as its efficacy rate is approximately 80% (Moran and Levine, 1995). Furthermore, SPTR isolates have been reported in several countries such as the Netherlands, the Philippines, South Korea, and the United Kingdom (Unemo and Shafer, 2014). There is concern that drug resistance would be rapidly selected when SPT is introduced as a first-line monotherapy. Therefore, SPT should be considered as a first-line treatment in combination with CRO or AZM in Shanghai.

\textit{Neisseria gonorrhoeae} ESCR Isolates Tend to Be Clonal

Previous studies have shown that \textit{N. gonorrhoeae} CFMR isolates in Japan (Yahara et al., 2018) and ESCRS isolates in the United States (Grad et al., 2014, 2016), Europe (Chisholm et al., 2013), and Canada (Demczuk et al., 2015) are predominantly clonal and associated with the mosaic \textit{penA} allele. We also found that \textit{N. gonorrhoeae} ESCR isolates were predominantly clonal in this study. In addition, we observed that ESCR isolates without the \textit{penA} mosaic alleles were distributed sporadically across the phylogenetic tree, which is also concordant with a previous report (Grad et al., 2016). In our study, of the six ESCR isolates that did not possess the mosaic \textit{penA} allele, four contained the \textit{penA} 18 allele that included the PenA A501T and G542S double substitutions. However, ESCR lineages in Canada were associated with non-mosaic \textit{penA} 12 and 13 alleles (Demczuk et al., 2015), while ESCR isolates with non-mosaic \textit{penA} reported in the United States have sporadically emerged.
Mosaic penA Alleles Are Associated With N. gonorrhoeae ESC\(^R\) and NG-STAR Clusters

Mosaic penA alleles have been associated with N. gonorrhoeae ESC\(^R\) (Ameyama et al., 2002; Lee et al., 2010). We observed that ESC\(^R\) is highly associated with mosaic penA 10, whereas reports in the United States and Canada indicated that N. gonorrhoeae ESC\(^R\) is highly associated with mosaic penA 34 (Grad et al., 2014, 2016; Demczuk et al., 2015). All of the N. gonorrhoeae isolates with an NG-STAR ST-348 genotype (\(n = 5\)) contained the mosaic penA 10 allele. Several mosaic penA alleles (penA 60, penA 71, and penA 34) are associated with ESC\(^R\) in this study.

penA 60 is significantly associated with both CRO\(^R\) and CFM\(^R\) and occurs in a single cluster; thus, it is of great concern when it spreads. None of the carriers of the penA 60 isolates reported a previous history of gonorrhea or any antibiotic use in the past month, which indicates that they were recently infected with penA 60 ESC\(^R\) strains. It is important to monitor the clonal expansion of penA 60 ESC\(^R\) strains to contain its spread. The reported CRO-resistant cluster FC428 has a mosaic penA 60 genotype with a NG-STAR sequence type ST-233 (Lee et al., 2019). Three of the four penA 60 N. gonorrhoeae isolates also have an NG-STAR ST-233. Links between the penA 60 isolates in this study and FC428 strains remain to be elucidated.

**Novel PorB1b Substitutions Associated With N. gonorrhoeae ESC\(^R\)**

In this study, we found that in contrast to CFM\(^R\), CRO\(^R\) is apparently associated with PorB1b substitutions other than mosaic penA or PenA substitutions. This is concordant with the results of a previous study that CRO is more severely affected by PorB1b than CFM (Unemo and Shafer, 2014), suggesting that either CFM does not readily diffuse into the periplasm through PorB1b or such diffusion is not altered by the porB determinant (Unemo and Shafer, 2014). To our knowledge, our study is the first to report that PorB1b substitutions T87A, T89S, S213S/Y, Q214L, and G259A are associated with CRO\(^R\). Similar to a previous report, although certain mutations in porB can contribute to N. gonorrhoeae resistance, most mutations in porB do not (Goire et al., 2014).

In vitro selection by introducing porB mutations into CRO\(^S\) isolates should be considered in the future to confirm the role of these mutations in the formation of CRO\(^R\) (Johnson et al., 2014). Previous studies have reported substitutions at amino acid positions 120 and 121 in putative loop 3 of PorB1b, which reduce the permeability of ESCs (Olesky et al., 2002). Interestingly, none of the substitutions detected in the present study are situated in any loops of PorB1b, and whether these substitutions could perturb protein structure remains unknown. Electrophysiological and biochemical studies...
FIGURE 3 | Heat map visualization of CIP MICs with ParC and GyrA substitutions in *N. gonorrhoeae*. MICs of CIP are indicated on the left panel. ParC and GyrA substitutions are indicated on the middle and right panels, respectively.

of PorB1b proteins to reveal the mechanism of CRO<sup>R</sup> conferred by these substitutions are warranted.

**ParC S88P Substitution Clustered in ESC<sup>R</sup> Clade**

We noticed that among the 14 isolates with ParC S88P substitution, nine were clustered in the ESC<sup>R</sup> clade (clade A), suggesting that this substitution may be associated with ESC<sup>R</sup>. Intriguingly, it was reported that various MDR bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae*, and ESBL-producing *Escherichia coli* were demonstrated to have been selected by favorable fitness balance associated with high-level resistance to fluoroquinolones, principally attained by the mutations of some serine residues in *gyrA* and *parC/grlA* (Fuzi et al., 2017, 2020). The association of the ParC S88P substitution and that of other QRDR serine replacements with fitness gain, the
promotion of particular clades, and the acquisition of the MDR phenotype warrant further investigation.

Limitations
This study only investigated a small percentage of N. gonorrhoeae isolates in Shanghai, with a total of 5,711 reported cases in this city in 2017. However, it is representative of the institution where the isolates were collected. A study with a larger sample size is required to extrapolate a broader strain distribution and to provide convincing evidence for the clonal distribution and to provide convincing evidence for the clonal distribution of ESCR with mosaic penA alleles and the sporadic distribution of ESCR with non-mosaic penA alleles. Specimens were obtained only from male patients, which may cause a higher proportion of CRO-resistant isolates, as reported by a Chinese national surveillance (Yin et al., 2018). Transmission of gonorrhea via different behaviors may result in infection of other mucosal sites, and isolates from other sites may exhibit different AMR phenotypes and genotypes. In the future, whole-genome sequencing should be considered to examine the population structure in Shanghai N. gonorrhoeae isolates, which would provide a significantly higher resolution for phylogenetic reconstruction.

CONCLUSION
This study observed a high percentage of N. gonorrhoeae isolates with reduced susceptibility to ESCs in Shanghai in 2017. Phylogenetic analysis of resistance determinants revealed that CFMRR isolates tend to be clonal. Mosaic penA alleles and certain substitutions in PenA and PorB1b are associated with N. gonorrhoeae ESCR. CRO and CFM may need to be reviewed as treatment for gonorrhea in Shanghai. Monitoring clonal expansion and development of novel antimicrobials for gonorrhea treatment are urgently needed.

DATA AVAILABILITY STATEMENT
The sequence data generated for this study has been submitted to GenBank and accession numbers can be found in the article.

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ETHICS STATEMENT
The studies involving human participants were reviewed and approved by Shanghai Skin Disease Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS
YD analyzed the data and wrote the manuscript. YY performed the experiments and collected the data. YW, IM, and WD revised the manuscript. WG designed experiments, performed the experiments, collected the data, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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