

# Antimicrobial Resistance And Molecular Characteristics Among *Neisseria gonorrhoeae* Clinical Isolates In A Chinese Tertiary Hospital

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Zhou Zheng<sup>1</sup>  
Li Liu<sup>1</sup>  
Xiaofei Shen<sup>1</sup>  
Jingyi Yu<sup>1</sup>  
Lijiang Chen<sup>1</sup>  
Lingling Zhan<sup>1</sup>  
Han Chen<sup>1</sup>  
Chunchan Lin<sup>1</sup>  
Ye Jiang<sup>1</sup>  
Hong Xia<sup>1</sup>  
Liangxing Wang<sup>2</sup>  
Fangyou Yu<sup>1,3</sup>

<sup>1</sup>Department of Clinical Laboratory, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, People's Republic of China; <sup>2</sup>Department of Respiratory Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, People's Republic of China; <sup>3</sup>Department of Laboratory Medicine, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai 200443, People's Republic of China

Correspondence: Fangyou Yu  
Department of Clinical Laboratory, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, People's Republic of China  
Email wzjxyf@163.com

Liangxing Wang  
Department of Respiratory Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, People's Republic of China  
Email 38805@163.com

**Purpose:** The resistance of *N. gonorrhoeae* to antimicrobial agents has been increasing year by year due to the overuse of antibiotics. The primary aims of the present study were to investigate the molecular characteristics of the clinical isolates of *Neisseria gonorrhoeae* and the resistance to azithromycin in a Chinese tertiary hospital.

**Methods:** From January 2014 to May 2017, a total of 55 clinical isolates of *N. gonorrhoeae* were collected. Genes associated with azithromycin resistance (AZM-R), including mutations in 23S rRNA alleles, the *mtrR* promoter and coding regions, and *rplD* and *rplV* were evaluated by PCR and DNA sequencing. All clinical isolates were subjected to *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), while the AZM-R isolates were further characterized by multilocus sequence typing (MLST).

**Results:** The AZM-R rate in this study was 23.64% (13/55), and a single (A)-nucleotide deletion mutation in the *mtrR* promoter region, a G45D mutation in the *mtrR* coding region, a point mutation in *rplD*, and an A2047G mutation in 23S rRNA alleles were detected in 13, 4, 3 and 4 isolates, respectively; no mutations were found in *rplV*. There was no significant difference in the *mtrR* coding region mutation rate between the azithromycin-sensitive and AZM-R groups ( $P > 0.05$ ); however, there was a significant difference in the mutation rate of the *mtrR* promoter region ( $P < 0.05$ ). Among the 55 isolates studied, 43 distinct NG-MAST were determined, while the AZM-R isolates were allocated into 10 distinct MLST/NG-MAST combinations. All three isolates with high-level AZM-R belonged to the sequence types (STs) NG-MAST ST1866 and MLST ST10899.

**Conclusion:** *N. gonorrhoeae* clinical isolates from Wenzhou, eastern China, showed considerable genetic diversity. Measures should be implemented to monitor the spread of the NG-MAST ST1866 and MLST ST10899 *N. gonorrhoeae* clones, which exhibit high-level AZM-R in eastern China.

**Keywords:** *Neisseria gonorrhoeae*, azithromycin, antimicrobial resistance, NG-MAST, MLST

## Introduction

Gonorrhea is the second most common sexually transmitted bacterial infection in the world. According to a recent estimation of WHO, the number of gonococcal infections occurred worldwide reached 78.3 million cases in the year 2012.<sup>1</sup> Antimicrobial resistance (AMR) of *N. gonorrhoeae* has become a major public health problem worldwide. In recent years, azithromycin has been increasingly used for the treatment of gonorrhea, and the resistance rate of *N. gonorrhoeae* to azithromycin has risen alongside this elevated use.<sup>2,3</sup> In many countries, the sporadic development of

high-level azithromycin resistance (AZM-R) and numerous clinical failures have led to great concern and threaten the long-term efficacy of the currently recommended approach for treatment of gonorrhea.<sup>4-6</sup>

Multidrug resistance of *N. gonorrhoeae* is related to the overexpression of efflux pumps. The most important efflux mechanism is the MtrC-MtrD-MtrE system, encoded by the *mtr* operon, in which *mtrR* is a regulatory gene and *mtrCDE* are the structural genes.<sup>7</sup> Another efflux pump, encoded by the *mef* gene, was originally identified in some of the gram-positive organisms and has since also been discovered in clinical strains of *N. gonorrhoeae*.<sup>8</sup>

The ribosomal proteins, L4 (encoded by *rplD*) and L22 (encoded by *rplV*), bind to domain I of 23S rRNA and act as channels, through which macrolide antibiotics enter the ribosome. Point mutations in *rplD* and *rplV*, conferring resistance to macrolides, have been described in *Escherichia coli*,<sup>9</sup> and in *rplD* for *Streptococcus pneumoniae*;<sup>10</sup> however, such mutations are rarely detected in *N. gonorrhoeae*.<sup>11</sup> In contrast, 23S rRNA point mutations have been described, including C2611T (numbering refers to the *E. coli* genome), conferring low-to-moderate levels of AZM-R (minimum inhibitory concentrations (MICs) = 2 to 32 µg/mL) or A2059G (*E. coli* numbering), conferring high-level AZM-R (MICs ≥ 256 µg/mL).<sup>12</sup>

As a commonly used molecular typing method, *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) detects nucleotide sequence variations in both *porB* and *tbpB* and determines the sequence types (STs) of isolates.<sup>13</sup> A single *N. gonorrhoeae* can rapidly diversify into multiple NG-MAST STs by the introduction of mutations or re-combinational replacement events.<sup>14</sup> Thus, the evaluation of NG-MAST STs is useful for short-term epidemiology. Over longer periods of time, rapid diversification can conceal the fact that several NG-MAST STs may be minor variants of the same *N. gonorrhoeae* strain.<sup>15</sup>

Multilocus sequence typing (MLST) is used to characterize isolates by amplifying sequences from seven house-keeping loci and is commonly used to track the spread of *N. gonorrhoeae* strains, where indexed genetic variation accumulates over longer periods of time.<sup>13</sup>

Little is known for types of AZM-R gonococci currently circulating in China until 2013; hence, levels of AZM-R and the molecular characteristics of AZM-R *N. gonorrhoeae* remain unclear.<sup>6,11,16,17</sup> In the absence of new antimicrobial agents for the treatment of gonorrhea, the classification and evaluation of the dynamics of AZM-R *N. gonorrhoeae* strains on a regional and national basis are important for

successful review of treatment recommendations. The resistance levels of *N. gonorrhoeae* to azithromycin in Wenzhou, eastern China, are unclear. The purpose of this study was to investigate the prevalence and genetic characteristics of *N. gonorrhoeae* from a teaching hospital in Wenzhou.

## Materials And Methods

### Bacterial Strains

From January 2014 to May 2017, a total of 55 nonrepetitive *N. gonorrhoeae* isolates were selected from 189 strains by simple random sampling in the First Affiliated Hospital of Wenzhou Medical University, of which 11 were from 2014, 10 from 2015, 17 from 2016, and 17 from 2017, where more than one sample was obtained from the same patient and only the first isolate was retained. All strains were identified by Gram-staining, oxidase reaction, and sugar fermentation tests and further confirmed as *N. gonorrhoeae* using a fully automatic rapid microbial mass spectrometry detection system (VITEK MS from bioMérieux, SA, France).

### Antimicrobial Susceptibility Testing

Susceptibility of *N. gonorrhoeae* to antimicrobials, including penicillin, tetracycline, ciprofloxacin, spectinomycin, ceftriaxone, and azithromycin, was determined using the E-test method (Wenzhou Kangtai Biological Technology Co., Ltd.). E-test results were interpreted based on the Clinical and Laboratory Standards Institute (CLSI 2019) guidelines criteria for all antibiotics.<sup>18</sup> A single *N. gonorrhoeae* isolate was streaked onto Thayer-Martin (T-M) selective medium for separation and purification. Inoculated plates were incubated at 36°C in 5% CO<sub>2</sub> for 24–48 hrs. Then, bacteria at 0.5 M turbidity were smeared on six 9-cm diameter GC plates three times uniformly and densely and incubated for 24 to 48 hrs at 35°C in a 5% CO<sub>2</sub> atmosphere with six E-test strips to determine MIC values. All strains were preserved in glycerin broth and stored at –80°C until used. The quality control strain of the antibiotic susceptibility test is *N. gonorrhoeae* ATCC 49226, which is provided by the Clinical Laboratory of the Ministry of Health of the People's Republic of China and kept by the laboratory. The strain is stored in the glycerol broth liquid medium.

### Extraction Of Bacterial Genomic DNA

Genomic DNA was extracted by boiling. Briefly, 3–5 *N. gonorrhoeae* colonies were suspended in 200 µL of sterile

distilled water and boiled for 10 mins at 100°C, then centrifuged at 13,800 g for 15 mins at 4°C. Finally, supernatants were removed and stored at -20°C for further use.

## PCR And DNA Sequencing Of Genes Associated With Azithromycin Resistance

In an attempt to identify point mutations, sequences of genes related to AZM-R were determined, including 23S rRNA alleles, *rplD* and *rplV* (encoding ribosomal proteins L4 and L22, respectively),<sup>19</sup> and the *mtrR* promoter and coding regions. Primers (listed in Table 1) for *mtrR* were designed using primer premier 5.0, while those for *rplD* and *rplV* have been published previously.<sup>20</sup>

PCR conditions for the *mtrR* promoter region were 10 mins at 94°C for predenaturation, followed by 30 cycles of 1 min at 94°C for denaturation, 1 min at 50°C for annealing, and extension at 72°C for 1 min. PCR conditions for the *mtrR* coding region were 10 mins at 94°C, then 30 cycles of 1 min at 94°C, 45 s at 48°C, and 1 min at 72°C. PCR conditions for *rplD* were 4 mins at 94°C, then 30 cycles of 30 s at 94°C, 30 s at 60°C, and 45 s at 72°C. Conditions for amplification of *rplV* were 4 mins at 94°C, then 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C.

A two-step PCR method was developed to identify mutations within domain V of each of the four copies of the 23S rRNA gene. The first step used the Upstream-F primer, paired with a primer specific for each of the 23S

rRNA alleles, as previously described (Table 1).<sup>21</sup> PCR conditions were 30 cycles of 1 min at 94°C, 1.5 mins at 68°C (for alleles 1 and 4) or 66°C (for alleles 2 and 3), and 2.5 mins at 72°C. The products obtained were used as a template in a second PCR using the Upstream-F and Downstream-R2 primers to amplify the four copies of the 23S rRNA gene for sequence determination. For the second PCR step, the conditions were 35 cycles of: 1 min at 94°C, 1 min at 59°C, and 1 min at 72°C.<sup>21</sup>

DNA samples amplified by PCR were subjected to electrophoresis at 110 V for 30 mins. Then, all amplified products were photographed using a gel imager and confirmed to be single bands. Next, PCR products were purified using magnetic beads (by Beijing Qingke New Industry Biotechnology Co., Ltd. Hangzhou Branch) and directly sequenced using first-generation sequencing technology. DNA sequences were aligned using BLAST and GenBank programs (<http://www.ncbi.nlm.nih.gov/blast/>) to identify gene mutations.

## *N. gonorrhoeae* Multiantigen Sequence Typing (NG-MAST) And Multi-Locus Sequence Typing (MLST)

All *N. gonorrhoeae* isolates were subjected to molecular epidemiologic analysis using NG-MAST, which assigns sequence types (STs) based on a combination of two highly variable alleles (*porB* and *tbpB*). Alignments of the *porB*

**Table 1** The Resistance Gene Primer Sequences And Target Fragment Sizes

Designation		PCR Primer Sequence (5' → 3')	Product Size (bp)	Annealing Temperature (°C)
<i>mtrR</i> Promoter Region	F R	GCCAATCAACAGGCATTCTTA GTTGGAACAACGCGTCAAAC	367	50
<i>mtrR</i> coding region	F R	GCACGCATCGCCTTAG AGTTGTCCATCATTATCCC	842	48
<i>rplD</i>	F R	CAGCGATGTTGTAGTTCGT ACTCAAGTAATCTTGGCGC	482	60
<i>rplV</i>	F R	TCAGCGACAATATGGTTGGT AGCCCAGTCTTTAGTTACC	468	55
allele 1	R	TCAGAATGCCACAGCTTACAACT	2054	68
allele 2	R	GCGACCATACCAAACACCCACAGG	2240	66
allele 3	R	GATCCCGTTGCAGTGAAGAAAGTC	2217	66
allele 4	R	AACAGACTTACTATCCCATTTCAGC	1847	68
Downstream	R2	TTCGTCCACTCCGGTCCTCTCGTA	712	59
Upstream	F	ACGAATGGCGTAACGATGGCCACA		

and *thpB* nucleotide sequences were modified using EditSeq software (LaserGene software version 7.1; DNASTar Corp) before submission to the NG-MAST website ([www.ng-mast.net](http://www.ng-mast.net)) for NG-MAST STs. MEGA 7.0 software, with the maximum-likelihood method, was used to create *N. gonorrhoeae* phylogenetic trees by concatenating *porB* and *thpB* alleles.<sup>11</sup> AZM-R were further typed using MLST, for which seven housekeeping genes (*abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdh*, and *pgm*) were analyzed and the sequencing results submitted to the database (<http://pubmlst.org/neisseria/>) to obtain allele numbers. Strain sequences were then obtained based on allele numbers (STs).

## Results

### Patient Data

Among 55 patients with gonorrhea, there were 52 males and 3 females whose age distribution range was 16–78 years (mean  $\pm$  SD, 32.0  $\pm$  11.2 years); 65.5% (36/55) were married and the main diagnosis is urethritis.

### Antimicrobial Susceptibility

Among the 55 *N. gonorrhoeae* strains tested, 52 (94.5%), 19 (34.5%), 49 (90.1%), 0 (0.0%), 0 (0.0%), and 13 (23.6%) were resistant to penicillin, tetracycline, ciprofloxacin, spectinomycin, ceftriaxone, and azithromycin, respectively. MIC values for azithromycin ranged from 0.064 to >256  $\mu$ g/mL, with an MIC<sub>50</sub> of 0.5  $\mu$ g/mL and an MIC<sub>90</sub> of 1.0  $\mu$ g/mL. The rates of resistance to azithromycin for the years from 2014 to 2017 were 18.2% (2/11), 10.0% (1/10), 29.4% (5/17), and 29.4% (5/17), respectively. There were 21 multidrug resistant (MDR) strains (strains with resistance to three or more types of antimicrobial agent) among the 55 strains of *N. gonorrhoeae*, and all 13 AZM-R strains were multidrug resistant. Of the 13 AZM-R strains, all were resistant to penicillin and ciprofloxacin, five were resistant to tetracycline, and the remaining eight exhibited intermediate resistance to tetracycline. Among the 13 AZM-R isolates, eight isolates with MIC values of 1  $\mu$ g/mL were classified as exhibiting low-level resistance, two with MIC values of 4  $\mu$ g/mL showed moderate-level resistance, and three with MIC values of >256  $\mu$ g/mL exhibited high-level resistance.

### Detection Of Mutations In Genes Associated With Azithromycin Resistance

The following mutations were detected in 13, 4, and 4 of the 13 AZM-R isolates, respectively: a single-nucleotide

(A) deletion in the *mtrR* promoter region, G45D in the *mtrR* coding region, and A2047G (*N. gonorrhoeae* numbering, GenBank accession number: X67293.1) in 23S rRNA alleles (Table 2). Four allelic mutations in 23S rRNA were detected in two of three (66.7%) *N. gonorrhoeae* isolates exhibiting high-level AZM-R. Point mutations in *rplD* were identified in three isolates (G68D, G70D, and a combination of T69I and G70S). No mutations were detected in *rplV*.

Thirteen strains were randomly selected from the 42 azithromycin-sensitive (AZM-S) strains as a control group. Mutations detected in these controls included: a single-nucleotide (A) deletion in the *mtrR* promoter region and G45D in the *mtrR* coding region in eight and three isolates, respectively (Table 2). No mutations were detected in the *rplD* and *rplV* genes or the 23S rRNA alleles in the AZM-S group.

Comparison between the AZM-R and AZM-S groups indicated that the mutation rate in the *mtrR* coding region was not significantly different between them ( $P > 0.05$ ), whereas there was a significant difference in the rate of mutation of the *mtrR* promoter region between the two groups ( $P < 0.05$ ).

### Molecular Epidemiologic Typing

The 55 *N. gonorrhoeae* clinical isolates were typed by NG-MAST analysis and 43 different sequence types (STs) were identified, with 21 (48.8%, 21/43) represented by only a single isolate. ST14781 was the most prevalent ST, accounting for four isolates, followed by ST1766 (three isolates), ST1866 (three isolates), ST5061 (three isolates), ST1407 (two isolates), ST4007 (two isolates), and ST4846 (two isolates). Fifteen of the STs (34.9%, 15/43) identified in the present study have not been reported previously in the NG-MAST database. Among the 13 AZM-R isolates, ST1866 was the most prevalent (23.1%, 3/13), followed by ST1407 (15.4%, 2/13). All three NG-MAST ST1866 isolates (which were not epidemiologically linked to one another) exhibited high-level AZM-R.

A total of 10 different STs, including four novel STs determined by MLST, were identified among the 13 AZM-R isolates (Table 2), of which ST10,899 accounted for four isolates (30.8%, 4/13). Three NG-MAST ST1866 isolates with high-level AZM-R belonged to MLST ST10899, indicating that they originated from the same clone.

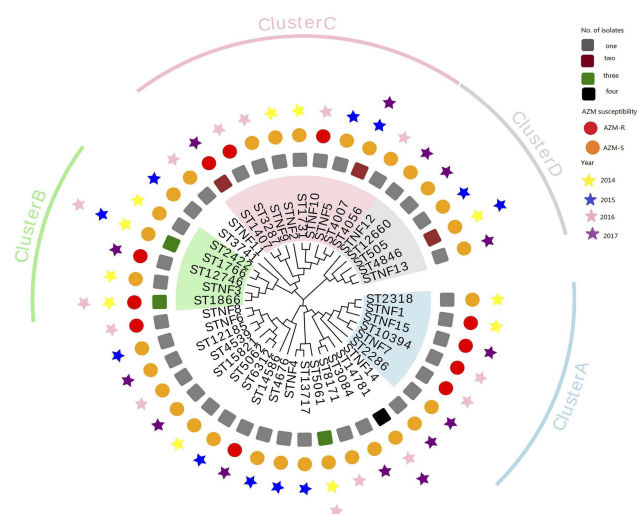
Based on phylogenetic analysis, four large clusters (including  $\geq 6$  isolates) were identified, which excluded 15 new-found types (Figure 1). Cluster A isolates included

Table 2 Characterization And Molecular Typing Results Of Clinical Isolates Of *Neisseria gonorrhoeae*

Group	Strain no. (Years)	CRO MIC (µg/mL) <sup>c</sup>	AZM MIC (µg/mL) <sup>c</sup>	MDR <sup>a</sup>	mtr <sup>b</sup> Promoter	mtr <sup>c</sup> Coding Region	rpID Mutation (L4 Protein) <sup>d</sup>	rpIV Mutation (L22 Protein) <sup>e</sup>	23S rRNA Mutation At Position 2047 <sup>f</sup>	MLST ST	NG- MAST ST	porB	tbpB
AZM-R (n=13)	10 (2014)	0.064	1	YES	A deletion	No mutation	No mutation	No mutation	No mutation	NF	NF1	866	135
	15 (2014)	0.064	>256	YES	A deletion	No mutation	No mutation	No mutation	A→G, alleles 1, 2, 3, and 4	10,899	1866	581	33
	16 (2014)	0.064	>256	YES	A deletion	G45D	No mutation	No mutation	A→G, alleles 1, and 2	10,899	1866	581	33
	49 (2016)	0.064	4	YES	A deletion	G45D	No mutation	No mutation	No mutation	12,039	1407	908	110
	55 (2016)	0.064	4	YES	A deletion	No mutation	No mutation	No mutation	A→G, alleles 1, and 4	10,899	3287	1042	110
	60 (2016)	0.064	1	YES	A deletion	G45D	No mutation	No mutation	No mutation	NF	NF8	2742	21
	71 (2016)	0.064	1	YES	A deletion	G45D	G70D	No mutation	No mutation	NF	10,394	609	75
	80 (2016)	0.064	1	YES	A deletion	No mutation	T691G70S	No mutation	No mutation	7367	NF10	3723	777
	93 (2017)	0.064	>256	YES	A deletion	No mutation	No mutation	No mutation	A→G, alleles 1, 2, 3, and 4	10,899	1866	581	33
	96 (2017)	0.064	1	YES	A deletion	No mutation	No mutation	No mutation	No mutation	8123	14,586	1135	566
	102 (2017)	0.125	1	YES	A deletion	No mutation	G72D	No mutation	No mutation	11,173	12,746	184	33
	105 (2017)	0.064	1	YES	A deletion	No mutation	No mutation	No mutation	No mutation	1901	1407	908	110
	109 (2017)	0.064	1	YES	A deletion	No mutation	No mutation	No mutation	No mutation	NF	NF15	809	520
AZM-S (n=13)	4 (2014)	0.064	0.25	YES	A deletion	No mutation	No mutation	No mutation	No mutation	NT	1731	4	186
	6 (2014)	0.064	0.25	NO	A deletion	No mutation	No mutation	No mutation	No mutation	NT	5061	2978	1058
	12 (2014)	0.064	0.125	YES	A deletion	No mutation	No mutation	No mutation	No mutation	NT	1766	1132	33
	18 (2014)	0.064	0.064	NO	A deletion	G45D	No mutation	No mutation	No mutation	NT	2318	1053	4
	25 (2015)	0.064	0.125	NO	A deletion	No mutation	No mutation	No mutation	No mutation	NT	4007	833	186
	34 (2015)	0.064	0.064	NO	A deletion	No mutation	No mutation	No mutation	No mutation	NT	6312	3723	1036
	39 (2015)	0.064	0.25	NO	A deletion	No mutation	No mutation	No mutation	No mutation	NT	13,717	7909	156
	72 (2016)	0.064	0.125	YES	No mutation	No mutation	No mutation	No mutation	No mutation	NT	14,781	6993	907
	83 (2016)	0.064	0.25	YES	No mutation	No mutation	No mutation	No mutation	No mutation	NT	NF11	3531	1482
	88 (2017)	0.064	0.064	NO	No mutation	No mutation	No mutation	No mutation	No mutation	NT	1407	908	110
	90 (2017)	0.064	0.064	NO	No mutation	G45D	No mutation	No mutation	No mutation	NT	12,660	1448	752
	100 (2017)	0.064	0.25	NO	No mutation	G45D	No mutation	No mutation	No mutation	NT	2286	1132	75
	107 (2017)	0.064	0.064	YES	A deletion	No mutation	No mutation	No mutation	No mutation	NT	14,781	6993	907
pg				0.002	0.039	1							

**Notes:** <sup>a</sup>Multidrug resistant strain: strains resistant to three or more classes of antimicrobial agents. <sup>b</sup>The mtr promoter mutation is the single-base-pair (A) deletion in the 13-bp inverted repeat in the promoter region. <sup>c</sup>GenBank accession number: Z25797.1. <sup>d</sup>GenBank accession number: YP-208871.1. <sup>e</sup>GenBank accession number: YP-208867.1. <sup>f</sup>Fisher test result.

**Abbreviations:** CRO, ceftriaxone; AZM, azithromycin; NF, new found; NT, not test.



**Figure 1** Phylogenetic tree constructed using MEGA7.0 for NG-MAST STs of 55 *N. gonorrhoeae* isolates from Wenzhou, China, 2014 to 2017.

six different STs, half of them with *tbpB* 75 (ST2286, ST10394, and STNF7 isolates). Cluster B isolates included five different STs with *tbpB* 186 (ST2422) or *tbpB* 33 (ST1766, ST1866, ST12746, and STNF3). Cluster C isolates were represented by nine different STs (ST1407 and ST3287 isolates with *tbpB* 110 and ST1731, ST4007, ST4056, and STNF2 isolates with *tbpB* 186). Finally, AZM-S cluster D contained five different STs, including ST505 and STNF12, both with *porB* 90; ST1660, ST4846, and STNF13 with *porB* 1448.

## Discussion

Gonorrhea is the second most common sexually transmitted infection (STI) in the world and remains a major public health problem in China. From 2015 to 2016, the number of gonorrhea cases reported in China increased by 14.7% (100,245 to 115,024).<sup>38</sup> In recent years, due to the overuse of antimicrobial agents (in China, it is common for doctors in small clinics to use antibiotics at will) and the evolution of bacteria, the resistance of *N. gonorrhoeae* to antibiotics has increased annually,<sup>21</sup> bringing enormous challenges for the clinical treatment of *N. gonorrhoeae* infections. The resistance rates of *N. gonorrhoeae* to penicillin and ciprofloxacin in the present study (>90%) were similar to those reported previously,<sup>11</sup> indicating that these two antibacterial drugs should not be used for the treatment of gonorrhea. Resistance to tetracycline and azithromycin was 34.5% and 23.6% in our study; therefore, their use is not at all advisable (based on the WHO recommendations: “in general; an antibiotic should not be used when

more than 5% of strains are resistant to it”; Tapsal 2001; WHO/CDS/CSR/DRS/2001.3). All strains were sensitive to spectinomycin and ceftriaxone in our study, which are widely used in China for the treatment of gonorrhea; hence these can still be used as first-line drugs to treat gonorrhea (in China, the extended-spectrum cephalosporin ceftriaxone has been recommended as a monotherapy for gonorrhea since 2007<sup>39</sup>). However, ceftriaxone-resistant *N. gonorrhoeae* has been detected in several studies;<sup>22–24</sup> therefore, it is necessary to strengthen surveillance for ceftriaxone resistance.

In this study, the rate of AZM-R was 29.41% in both 2016 and 2017, similar to other recent reports from China. For example, analysis of 126 isolates from Hefei from 2014 to 2015 showed that 29% were AZM-R, including 10% exhibiting high-level

AZM-R.<sup>17</sup> Furthermore, a similar incidence was reported among 384 isolates from Nanjing during 2013–2014, with 32% of isolates displaying AZM-R and 10% showing high-level resistance.<sup>11</sup> Notably, the levels of resistance to azithromycin observed in earlier studies from other regions in China were lower.<sup>25,26</sup> As two neighboring countries in Asia, the resistance rates of *N. gonorrhoeae* isolated from China and Japan to azithromycin are much higher than those from Europe and America.<sup>2,27–29</sup> Antimicrobial resistant *N. gonorrhoeae* was first reported in East Asia, subsequently appearing in other parts of the world.<sup>30</sup>

Mutations in the promoter or coding sequence of the *mtrR* gene in macrolide-resistant *N. gonorrhoeae* strains can result in decreased expression of the MtrR repressor, thereby upregulating the MtrCDE efflux pump.<sup>31</sup> In gonococcal strains with a single-base-pair (A) deletion in the 13-bp inverted repeat sequence of the *mtrR* promoter region, *mtrR* expression is abrogated, while levels of *mtrCDE* are elevated, most likely because of an increased binding affinity of RNA polymerase for *mtrCDE*.<sup>32</sup> Missense mutations in the *mtrR* gene, for example, a G45D mutation in the helix-turn-helix motif in the MtrR repressor, can reduce binding of the repressor to the *mtrCDE* promoter.<sup>7</sup> In the present study, the mutation rates of the *mtrR* promoter and coding regions in AZM-R isolates were 100.0% and 30.8%, respectively. Compared with the AZM-S group, the mutation rate of the *mtrR* promoter region in the AZM-R group was significantly higher, while there was no significant difference in the mutation rate of the *mtrR* coding region. This is in accordance with previously reported findings indicating that mutations in the *mtrR* promoter region play

a more important role in the resistance of *N. gonorrhoeae* to azithromycin than alterations in the *mtrR* coding region.<sup>12</sup> The mutations A40D, A39T, H105Y, and *mtr*<sub>120</sub> reported by other investigators<sup>11,12,17,33</sup> were not found in our study.

Previous studies reported that one or more of the four alleles of the *rrl* gene within domain V of 23S rRNA are associated with AZM-R, including the mutations, A2143G and C2599T (corresponding to A2059G and C2611T in *E. coli*, respectively).<sup>34,35</sup> The mutation A2047G (*N. gonorrhoeae* numbering) in domain V of detected in the present study in four AZM-R *N. gonorrhoeae*, but was not found in the AZM-S group. The numbers of each 23S rRNA allele in which the A2047G mutation occurred were 4, 4, 2, and 2, respectively, and the corresponding MIC values were 256, 256, 256, and 4 µg/mL, respectively. It is worth noting that the A2047G mutation was detected in all strains with an MIC value of >256 µg/mL; therefore, we believe that having multiple alleles of A2047G is a major determinant of high-level azithromycin resistance.

Three *N. gonorrhoeae* isolates with MIC values for azithromycin of 1.0 µg/mL were found to harbor mutations in *rplD* (one isolate with G68D, one with G70D, and another with both T69I and G70S mutations). To our best knowledge, this is the first report of the mutations, G70D, T69I, and G70S, in *rplD*, while the G68D mutation has been described previously by Jacobsson.<sup>33</sup> Therefore, mutations at positions 68–70 of the *N. gonorrhoeae rplD* gene appeared to be associated with low-to mid-level AZM resistance. The strains with *rplD* gene mutations belonged to clusters A and C, with NG-MAST classifications of ST10394, ST12746, and NF, indicating that *N. gonorrhoeae* strains with this mutation are genetically diverse.

*N. gonorrhoeae* typing methods, such as NG-MAST and MLST, assist in understanding of the spread of gonorrhea. In the present study, 34.9% (15/43) of STs determined by NG-MAST were newly recognized, and 48.8% (21/43) of these STs were represented by only a single isolate, indicating that these *N. gonorrhoeae* clinical isolates exhibit considerable genetic diversity. According to the phylogenetic tree, AZM-R strains are scattered throughout each year. Furthermore, phylogenetic analysis demonstrated that AZM-R isolates were widely divergent and did not belong to any specific clusters. This may be related to the large numbers of temporary residents in Wenzhou (according to the main data bulletin of Wenzhou City's sixth national census in 2010, the city's permanent population was 9,122,100, of which the number of inflows from outside the city was 2,842,200, accounting for 31.16% of the city's permanent population),

which may provide a greater opportunity for the emergence of the *N. gonorrhoeae* isolates with new and multiple NG-MAST STs; however, all three isolates with high-level AZM-R belonged to NG-MAST ST1866 and MLST ST10899, unlike those reported from other countries, such as Sweden (NG-MAST ST285, ST332, and ST8727);<sup>5</sup> Wales, England, and the United States (NG-MAST ST649),<sup>36,37</sup> and Argentina (NG-MAST ST696).<sup>4</sup> It is worth mentioning that the high level of AZM-R *N. gonorrhoeae* found in this study has the same NA-MAST and MLST type as the high-level AZM-R *N. gonorrhoeae* found in the Liu YH report,<sup>40</sup> which indicates that the spread of NG-MAST ST1866, MLST ST10899 *N. gonorrhoeae* has crossed the Taiwan Strait. Furthermore, the NG-MAST ST1866 clone has previously been described in Nanjing, Hangzhou, and Hefei,<sup>6,11,17</sup> indicating that this clone with high-level AZM-R has spread in eastern China, which is a cause for concern. In addition, we also noted that the extensive drug-resistant (XDR) strain mentioned in the Jennison AV<sup>41</sup> report is highly resistant to azithromycin while resistant to ceftriaxone. The XDR is the NG-MAST 16848 and MLST 12039, different from the high level of AZM-R *N. gonorrhoeae* found in our study. However, further analysis revealed that MLST 12039 and MLST 10899 have six identical housekeeping genes, which differ only in the *pdhc* allele. This may mean that high-level AZM-R *N. gonorrhoeae* is only one step away from the XDR strain. This must be a cause for concern.

## Conclusion

Although the sample size of this study is limited, it is of great significance to study the resistance of *N. gonorrhoeae* to azithromycin in Wenzhou, eastern China. Measures should be implemented to monitor the spread of the NG-MAST ST1866 and MLST ST10899 *N. gonorrhoeae* clones, which exhibit high-level AZM-R in eastern China.

## Ethics Approval

Because the *N. gonorrhoeae* were part of the routine hospital laboratory procedure, ethics approval was not required.

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## Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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