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Molecular Characteristics and the Effect of Mutations in Different Sites of the *rplD* Gene Among Clinical Isolates of Azithromycin Resistance *Neisseria gonorrhoeae* in Eastern China

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Abstract

Background: The emergence of resistance to azithromycin complicates the treatment of *Neisseria gonorrhoeae*, the causative agent of gonorrhea.
Objectives: The main objective of this study was to investigate the impact of mutations in different sites of the *rplD* gene on azithromycin resistance (AZM-R) and the molecular characteristics of *N. gonorrhoeae*. This study aimed to provide evidence for drug resistance and transmission.
Methods: A total of 37 isolates of *N. gonorrhoeae* were collected within January 2018 to December 2020. These isolates were obtained from urine, semen, or vaginal secretions of different patients. Azithromycin resistance was assessed, and genes associated with AZM-R, namely *rplD*, and *rplV*, were analyzed using polymerase chain reaction (PCR) and deoxyribonucleic acid (DNA) sequencing. All clinical isolates were characterized through multi-locus sequence typing (MLST).
Results: The study comprised 21 azithromycin-resistant *N. gonorrhoeae* isolates, with two of them demonstrating high resistance, indicated by a minimum inhibitory concentration (MIC) greater than 256 μg/mL. Additionally, 16 sensitive isolates were included in the study. Ten isolates were observed to have *rplD* point mutations, including mutations such as G70D, G70S, G68D, and A43T. No mutations were detected in *rplV*. The rate of point mutations in *rplD* was significantly different between the azithromycin-sensitive (AZM-S) group and the AZM-R group ($P < 0.05$). Among the 37 isolates studied, 12 distinct MLST types were identified and further grouped into four different MLST goeBURST groups. The two isolates with high-level AZM-R were ST1901 and ST1588, respectively.
Conclusions: The clinical isolates of *N. gonorrhoeae* from Wenzhou, Eastern China, exhibit significant genetic diversity and a relatively high prevalence of AZM-R. Mutations in the *rplD* gene were identified, which reduced susceptibility to macrolides and were significantly associated with increased AZM-R.

Keywords: *Neisseria gonorrhoeae*, Azithromycin Resistance, *rplD*, *rplV*, MLST

1. Background

The increasing antibiotic resistance of *Neisseria gonorrhoeae*, the pathogen responsible for gonorrhea, is a growing global concern. According to the US Centers for Disease Control and Prevention (CDC), the incidence rate of gonorrhea increased by 111% from 2009 to 2020 (1). Gonorrhea is one of the most common bacterial sexually transmitted infections (STIs), with an estimated 82,400,000 cases worldwide in 2020, as reported by the World Health Organization (WHO) (2). The current empirical treatment for gonorrhea in the United States consists of a combination of ceftriaxone and azithromycin (3). However, the emergence of *N. gonorrhoeae* isolates with high-level azithromycin resistance (AZM-R) poses a threat to the sustainability of this treatment (4, 5). The rapid emergence of AZM-R has led some countries, such as the United Kingdom, to consider this treatment endangered and recommend ceftriaxone monotherapy instead (3).

Azithromycin is a 15-membered macrolide that

inhibits protein synthesis by binding to the bacterial 50S large ribosome. Azithromycin functions by inhibiting the peptide transfer/translocation step, which blocks the 50S peptide exit tunnel, leading to the release of incomplete peptides from the ribosomes (Figure 1) (6-9). Ribosomal proteins L4 and L22, products of the *rplD* and *rplV* genes, respectively, bind to domain I of 23S rRNA to form a channel and facilitate the entry of macrolide antibiotics (10). Mutated L4 and L22 proteins can alter domains II, III, and V, affecting the susceptibility of microorganisms to macrolides (11).

Mutations in L4 and L22 have been confirmed to confer resistance to macrolides in various bacteria, such as *Brucella*, *Escherichia coli*, and *Streptococcus pneumoniae* (12-14). Although no mutation related to the 50S ribosome protein L22 has been reported in *N. gonorrhoeae*, point mutations of the 50S ribosome protein L4, specifically the G70D mutation, have been implicated in macrolide resistance (15, 16). Additional studies have identified other L4 mutations at amino acid positions 68 (G68D and G68C), 69 (T69I), and 70 (G70R, G70S, and G70A), which are associated with decreased susceptibility to azithromycin (16). These mutations are located at the end of the L4 ring near the macrolide binding site and contribute significantly to the development of AZM-R (17).

Multi-locus sequence typing (MLST), a method that characterizes isolates by amplifying sequences from seven housekeeping genes, has been widely used to study the dynamics of *N. gonorrhoeae* infection and drug-resistant isolates. Multi-locus sequence typing is frequently employed to trace the spread of *N. gonorrhoeae* isolates, identify lineages, and detect short-term transmission chains (18, 19). It is crucial to deepen our understanding of the emergence and dynamics of drug-resistant *N. gonorrhoeae* isolates at the national and global levels.

2. Objectives

The objective of this study was to examine the mutation rate and diversity of the *rplD* gene in *N. gonorrhoeae* within the Wenzhou area and to assess the extent to which *rplD* mutations contribute to the consistently elevated azithromycin minimum inhibitory concentration (MIC) levels. Additionally, this study aimed to understand the genetic characteristics of *N. gonorrhoeae* and analyze the variations in AZM-R among isolates with different genetic backgrounds. Ultimately, the results of this study will serve as a valuable foundation for the prevention and treatment of gonorrhea.

3. Methods

3.1. Bacterial Isolates

This study was conducted within January 2018 and December 2020, during which 37 non-repetitive *N. gonorrhoeae* isolates were randomly selected from the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China. The isolates originated from 5 females and 32 males, aged 18 - 66 years (median age: 31 years), with urine and secretions being the main types of specimens. All experimental isolates were confirmed as *N. gonorrhoeae* through the fully automated rapid microbial mass spectrometry detection system (VITEK MS, from bioMérieux, France). These isolates included 21 AZM-R isolates and 16 azithromycin-sensitive (AZM-S) isolates.

3.2. Azithromycin Susceptibility Testing

The susceptibility of *N. gonorrhoeae* to azithromycin was evaluated through MIC determination (Wenzhou Kangtai Biotechnology Co., Ltd., China). All test results were interpreted following the guidelines of the Clinical and Laboratory Standards Institute (CLSI M100ed30) for all antibiotics (20), where MIC $\leq$ 1 mg/L was deemed sensitive, MIC $>$ 1 mg/L was considered resistant, and MIC $\geq$ 256 mg/L indicated high-level drug resistance. Individual *N. gonorrhoeae* isolates were isolated and purified on Thayer-Martin (T-M) selective media. Azithromycin E-test experiments were conducted using GC plates supplemented with 1% IsoVitaleX, vancomycin, colistin, nystatin, and trimethoprim selective supplements in a 5% CO_2 incubator at 35°C. This helped determine either the area diameter or MIC. The quality control strain was *N. gonorrhoeae* ATCC 49226 from the clinical laboratory of the Ministry of Health of the People's Republic of China. All isolates were stored in glycerol broth at -80°C.

3.3. Extraction of Bacterial Genomic Deoxyribonucleic Acid

The genomic deoxyribonucleic acid (gDNA) of *N. gonorrhoeae* was extracted through boiling. Moreover, 3 - 5 purified *N. gonorrhoeae* colonies were suspended in 200 μL of sterile distilled water, boiled in water at 100°C for 10 minutes, and then centrifuged at 13800 g at 4°C for 15 minutes (10). The resulting supernatant contained gDNA, which was stored at -20°C for backup purposes.

3.4. Amplification of the rplD and rplV Genes

The primers for *rplD* and *rplV* have been previously published and are listed in Table 1 (10). The polymerase chain reaction (PCR) conditions for *rplD* involved a pre-denaturation step at 94°C for 4 minutes, followed by

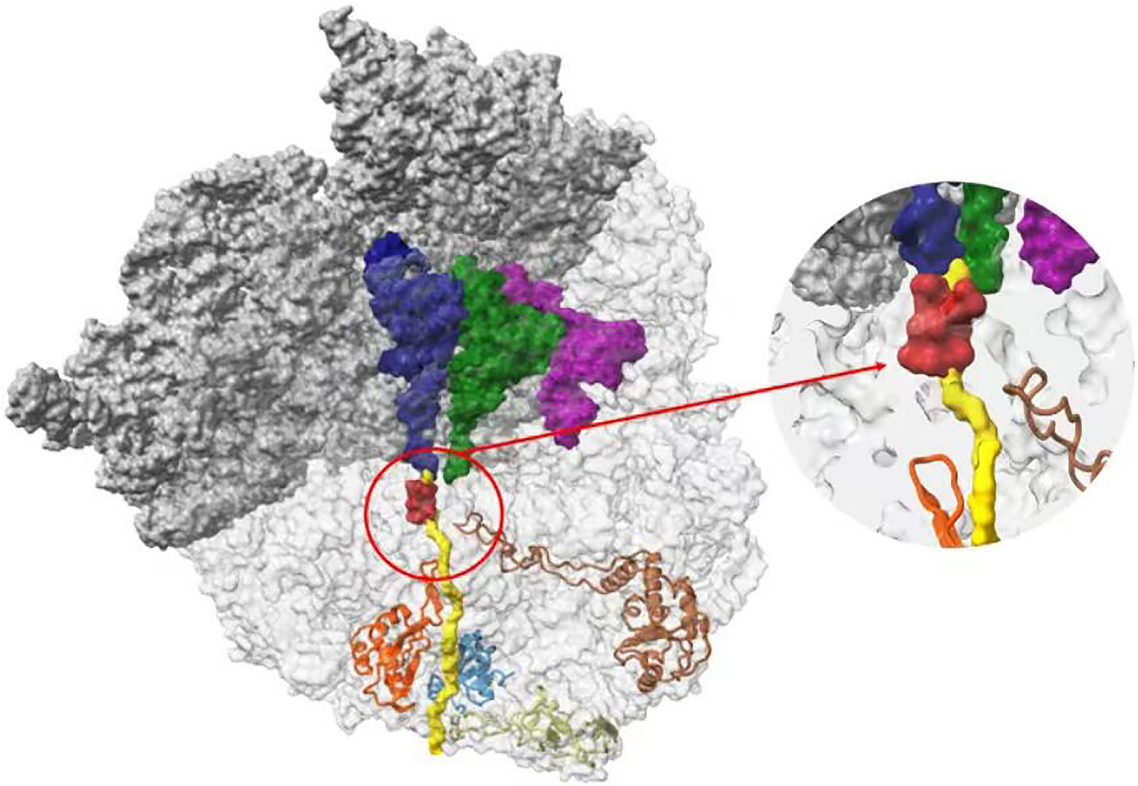


Figure 1. The ribosome nascent chain tunnel environment. *Staphylococcus aureus* ribosome, where the large subunit is shown in light gray, and the small subunit is shown in dark gray. The A-site, P-site, and E-site docked tRNA molecules are shown in blue, green, and magenta, respectively. The surface of a nascent chain within the peptide exit tunnel is shown in yellow. Bound macrolide is shown in red, and uL4, uL22, uL23, and uL24 are shown in brown, orange, teal, and khaki, respectively. The figure was reproduced with permission from Halfon et al. (9)

denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds, with a total of 30 cycles. For *rplV* amplification, the annealing temperature was adjusted to 55°C, and the extension time was increased to 1 minute; nevertheless, the other conditions remained unchanged (21). The amplified PCR products were subjected to electrophoresis at 110 V for 30 minutes, photographed with a gel imager, and confirmed as single bands. Subsequently, the PCR products were purified using magnetic beads by the sequencing company (Beijing Qingke New Industry Biotechnology Co., Ltd. Hangzhou Branch, China). First-generation sequencing technology was employed for sequencing. The sequencing results were compared to DNA sequences using BLAST to identify genetic mutations.

3.5. Neisseria gonorrhoeae MLST

Neisseria gonorrhoeae isolates in the present study were genotyped using MLST. Seven housekeeping genes, namely *adk*, *abcZ*, *aroE*, *fumC*, *pgm*, *gdh*, and *pdhC* of *N. gonorrhoeae*, were amplified and sequenced. Sequence types (STs) were identified using the online database at the Pasteur Institute MLST typing website for *N. gonorrhoeae*. The evolutionary relationship between the isolates was analyzed using the platform-independent Java software PHYLOViZ at the single-locus variant (SLV) level with the goeBURST algorithm (22).

3.6. Statistical Analysis

SPSS software (version 22.0) was used to statistically analyze the difference in *rplD* point mutations between the AZM-S and AZM-R groups of *N. gonorrhoeae*, with a P-value less than 0.05 indicating significance.

4. Results

4.1. Antimicrobial Susceptibility

Among the 37 isolates of *N. gonorrhoeae*, the MIC values of azithromycin ranged from 0.125 to 256 μg/mL. Sixteen isolates were classified as sensitive, accounting for 43.24%

Table 1. The *rplD* and *rplV* Gene Primer Sequences and Target Fragment Sizes

Designation	PCR Primer Sequence (5´→3´)		Product Size (bp)	Annealing Temperature (°C)
rplD	F	CAGCGATGTTGTAGTTCGT	482	60
	R	ACTCAAGTAATCTTGGCGC		
rplV	F	TCAGCGACAATATGGTTGGT	468	55
	R	AGCCCAGTCTTTAGTTACC		

Abbreviation: PCR, polymerase chain reaction.

of the total; however, 21 isolates were resistant, making up 56.76%. Two isolates exhibited extremely high MIC values exceeding 256 μg/mL, categorizing them as high-level AZM-R *N. gonorrhoeae*.

4.2. Comparison of rplD and rplV Genes

The current study analyzed the DNA of *N. gonorrhoeae* through PCR amplification and identified 10 isolates with *rplD* point mutations, resulting in a mutation rate of 27.03%. These mutations were found at four specific sites: G70D, G70S, G68D, and A43T (Table 2). It is worth noting that the A43T mutation was detected for the first time in this study. Except for A43T, the remaining mutations were confirmed to be non-synonymous and were located at the end of the L4 loop, which is near the macrolide-binding site. All these mutations were associated with reduced susceptibility to azithromycin, as suggested by previous studies (5, 14, 16).

When comparing the AZM-R and AZM-S groups based on whether the MIC value of azithromycin exceeded 1 μg/mL, it was observed that the point mutation rate of *rplD* in the AZM-S group was significantly lower than in the AZM-R group ($P < 0.05$). This indicates that the presence of point mutations in *rplD* was more common in isolates resistant to azithromycin than those sensitive to azithromycin. Another noteworthy discovery was that one of the two high-level AZM-R isolates exhibited an *rplD* A43T mutation; nonetheless, no mutations were detected in the other. Lastly, the study did not identify any *rplV* mutations in the analyzed isolates.

4.3. Molecular Epidemiologic Typing

Among the 37 clinical isolates of *N. gonorrhoeae*, MLST identified 12 different STs. The distribution of these STs was as follows: ST8123 accounted for 18.91% (7/37), ST7822 for 13.51% (5/37), ST7367 for 13.51% (5/37), and ST1928 for 13.51% (5/37). The STs of two high-level AZM-R isolates were identified as ST1901 and ST1588. Using goeBURST minimum spanning tree analysis on the MLST allelic spectrum of the seven MLST housekeeping genes of *N. gonorrhoeae*, the genetic relatedness of the MLSTs in this experiment was compared to all the MLSTs of *N. gonorrhoeae* (Figure 2). Based on these genetic relationships, the MLSTs identified in this study were categorized into four large clusters, as shown in Figure 3. Cluster A isolates consisted of 6 different ST types centered around ST7363, with ST14283, ST1600, and ST1823 representing single locus variants of ST7363. Cluster B isolates comprised four different ST types centered around ST1901, with ST7822 being a single locus variant of ST1901. Cluster C and D isolates each contained only one type.

The two isolates highly resistant to azithromycin, ST1588 and ST1901, differed at the four loci *adk*, *aroE*, *fumC*, and *gdh*. They belonged to cluster A and cluster B, respectively, indicating that they originated from distinct genetic backgrounds, as shown in Figure 2. This suggests that high-level AZM-R *N. gonorrhoeae* can emerge from different genetic backgrounds, potentially necessitating different treatment strategies.

5. Discussion

Over the years, gonorrhea has developed resistance to all classes of antimicrobials targeting it, especially with the emergence of isolates with reduced susceptibility to azithromycin and ceftriaxone, posing a public health concern due to the rise of incurable gonorrhea infections (23). To prevent further drug resistance, a combination of azithromycin and ceftriaxone has been used for the treatment of gonorrhea (23, 24). However, despite combination therapy, azithromycin resistance has continued to increase, and the emergence of *N. gonorrhoeae* resistant to both azithromycin and ceftriaxone since 2018 is of even greater concern (25). In 2001, *N. gonorrhoeae* with high-level resistance to azithromycin (MIC $\geq$ 256 mg/L) was first isolated in Argentina, and it has been constantly reported in various countries, including England, Italy, the United States, Canada, and China (26). The G70D mutation was the most frequently found point mutation in *rplD*, which is significantly related to increased AZM-R (16). A recent bacterial genome-wide association study confirmed the

Table 2. Characterization and Molecular Typing Results of Clinical Isolates of *Neisseria gonorrhoeae*

Groups	Strain No.	AZM MIC (μg/mL)	*rplD* Mutation (L4 Protein)	*rplV* Mutation (L22 Protein)	MLST ST
AZM-R (n = 21)	2	4	G70D	No mutation	9899
	3	8	No mutation	No mutation	8123
	5	2	No mutation	No mutation	7363
	6	4	No mutation	No mutation	1901
	9	1.25	No mutation	No mutation	1928
	13	16	G70S	No mutation	7367
	16	16	G70S	No mutation	7367
	21	1.25	No mutation	No mutation	7367
	25	> 256	No mutation	No mutation	1901
	27	2	G70S	No mutation	7367
	29	4	G70D	No mutation	14421
	31	1.25	No mutation	No mutation	7822
	32	2	G68D	No mutation	7822
	34	1.25	No mutation	No mutation	1901
	37	> 256	A43T	No mutation	1588
	38	2	No mutation	No mutation	1928
	39	1.25	No mutation	No mutation	14283
	41	4	G70D	No mutation	15251
	42	1.25	No mutation	No mutation	8123
	43	2	G68D	No mutation	7822
	44	8	No mutation	No mutation	8123
AZM-S (n = 16)	1	0.25	No mutation	No mutation	7822
	8	1	No mutation	No mutation	8123
	12	0.5	No mutation	No mutation	7822
	17	0.125	No mutation	No mutation	1928
	18	1	No mutation	No mutation	7363
	19	0.5	No mutation	No mutation	1928
	20	1	No mutation	No mutation	1901
	22	0.125	No mutation	No mutation	1588
	23	0.032	No mutation	No mutation	1600
	24	0.125	No mutation	No mutation	1588
	26	0.5	No mutation	No mutation	8123
	28	1	No mutation	No mutation	1928
	30	0.125	No mutation	No mutation	8123
	33	0.25	G70S	No mutation	7367
	36	0.25	No mutation	No mutation	1600
	40	0.5	No mutation	No mutation	8123
P			0.015		

Abbreviations: AZM-R, azithromycin resistance; AZM-S, azithromycin-sensitive; MIC, minimum inhibitory concentration; MLST, multi-locus sequence typing; ST, sequence type.

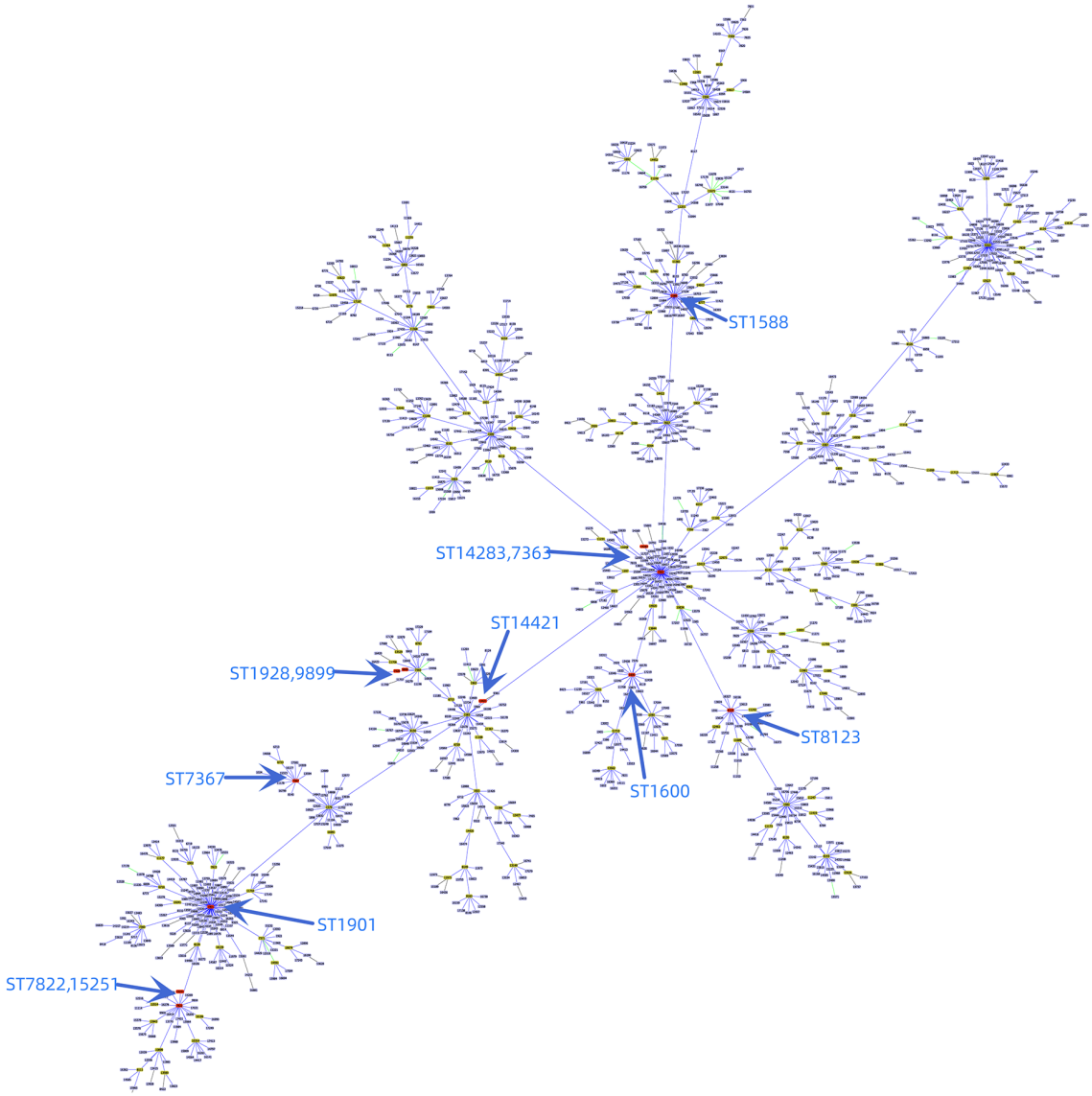
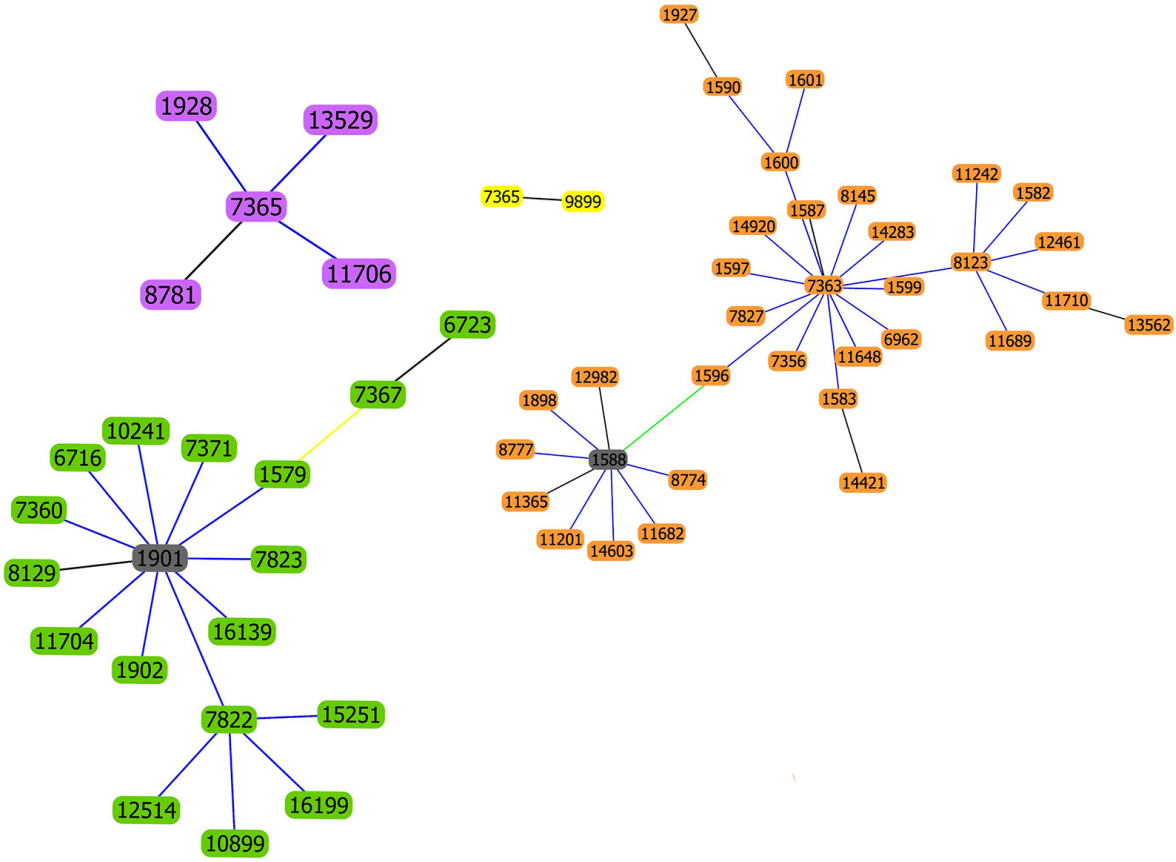


Figure 2. Minimum spanning tree for all *Neisseria gonorrhoeae* multi-locus sequence typing (MLST) sequence types (STs). The graph was analyzed by PHYLOVIZ using the goeBURST algorithm at the single-locus variant (SLV) level. The types of this study are marked in red dots.

role of the *rplD* G70D mutation in mediating macrolide resistance, showing an average six-fold increase in clarithromycin MICs, with the MICs of azithromycin and erythromycin being 4 times higher than that of the wild-type isolates with the same gene (16).

In addition to detecting the *rplD* G70D mutation, G70S, G68D, and A43T mutations were also detected in this study. Out of the isolates with *rplD* mutations, only one *N. gonorrhoeae* strain with the G70S mutation was sensitive to azithromycin; nevertheless, the other isolates with the G70D, G70S, or G68D mutation were resistant to azithromycin. When comparing the AZM-S group to the AZM-R group, it was observed that the point mutation rate of *rplD* in the AZM-S group was significantly lower than in the AZM-R group ($P < 0.05$). This was consistent with previous research results. Although the *rplD* mutation did not significantly increase the MIC value, it played a role in mediating treatment failure caused



N. Gonorrhoeae MLST goeBURST Relatedness Groups			
MLST goeBURST Group	No. Isolates	No. Sequence Types	The Relevant Detail Type
Group A	16	6	MLST1588,1600,7363,8123,14283,14421
Group B	15	4	MLST1901,7367,7822,15251
Group C	5	1	MLST1928
Group D	1	1	MLST9899
International high level azithromycin resistant strains			

Figure 3. Genetic relatedness of multi-locus sequence typing (MLST) determined by goeBURST minimum spanning tree analysis using the *Neisseria gonorrhoeae* MLST allelic profiles of seven MLST housekeeping genes

by the increase of drug resistance sub-breakpoint (27). Various mutations, such as G68D, G68C, T69I, G70D, G70S, G70A, and G70R, reduced the sensitivity of macrolides by narrowing the peptide exit tunnel and interrupting the binding of macrolide antibiotics to the 50S subunit of ribosome (Figure 1) (16, 28, 29). Therefore, the detection and monitoring of these mutations are crucial in the fight against antibiotic-resistant *N. gonorrhoeae*.

It was generally believed that mutations in the *rplD* gene were associated with low-level resistance to macrolides (MIC: 0.5 mg/L); nonetheless, mutations in the *rplV* gene were associated with low to high-level resistance (MIC: 1.5 to 256 mg/L) (5). No mutations of the *rplV* gene were observed in this study, which is similar to previous

research, possibly due to the relatively few *N. gonorrhoeae* isolates highly resistant to azithromycin. Of particular interest is the detection of the A43T mutation in the *rplD* gene in a high-level AZM-R *N. gonorrhoeae* strain, which, to the best of our knowledge, has not been reported before. The presence of this previously undescribed mutation suggests a potential mechanism for the high resistance of *N. gonorrhoeae* to azithromycin, although further verification is needed. Multiple previously undescribed mutations in *rplD* were observed to be associated with higher azithromycin MICs than the G70D mutation (16).

Laumen et al. demonstrated the G70D mutation in one strain with a MIC value of 0.5 mg/L, while the R71C mutation in 2 isolates with MIC values of 24 and 32 mg/L, respectively (5). It is suggested that mutations in *rplD*/*rplV* serve as stepping stones to higher-level resistance, with mutations first occurring in the ribosomal genes followed by the MtrCDE efflux pump and 23S rRNA (5). Although AZM-R in *N. gonorrhoeae* is mainly attributed to 23S rRNA point mutations and overexpression of the MtrCDE efflux pump, the A43T mutation found in this study might represent another mechanism for high resistance. Further research is needed to confirm the potential role of this mutation in antibiotic resistance (8, 30, 31). In this study, MLST typing was used to understand the spread of gonorrhea.

Among all identified MLST types, up to 33.33% (4/12) of STs were represented by only a single isolate, indicating that *N. gonorrhoeae*, clinically isolated in Wenzhou, China, showed considerable genetic diversity. Shimuta et al. reported that MLST ST7363 and MLST ST1901 *N. gonorrhoeae* showed their capacity to develop high-level *in vitro* resistance to ceftriaxone and called them "superbugs" (32). In this study, 5 isolates of ST7363 and 4 isolates of ST1901 were sequenced, accounting for 24.32% (9/37), indicating that the proportion of "superbug" in the Wenzhou area was quite considerable, and there was a risk of multidrug-resistant (MDR) *N. gonorrhoeae*, which needed to be paid great attention to.

The most common MLST type of AZM-R *N. gonorrhoeae* was observed to be ST1901, consistent with previous reports from Taiwan and China (16, 32). This type, along with ST7365 and ST1927, was observed to be prevalent in Taiwan, with higher MIC values for ceftriaxone and azithromycin reported for ST1901 isolates (16). The 2 isolates of highly AZM-R *N. gonorrhoeae* isolated in this experiment were ST1901 and ST1588, belonging to cluster B and cluster A in the goeBURST minimum spanning tree, respectively. These types differed at four loci (*adk*, *aroE*, *fumC*, and *gdh*), indicating significant genomic differences, which are similar to the previous report (10, 26).

Although ST1588 has not been previously reported as a highly AZM-R strain, it was closely related to the "superbug" ST7363, belonging to the same cluster A and differing by only 2 loci (*fumC* and *gdh*). Therefore, increased surveillance of this type is also recommended. The international emergence and spread of antibiotic-resistant *N. gonorrhoeae* emphasize the need to develop epidemiologically relevant surveillance systems, which not only closely track the spread of known resistant isolates but also rapidly detect novel resistance mechanisms. Furthermore, it should develop rapid and reliable genetic methods to predict antibiotic resistance. In a clinical setting, it is of paramount importance to apply appropriate therapies to limit clonal expansion, thereby having the opportunity to counteract the evolution and spread of gonorrhea.

5.1. Conclusions

Although this study has limitations, such as a relatively small overall number of *N. gonorrhoeae* isolates and a very small number of AZM-R isolates, especially highly resistant isolates, the mutations discovered in the *rplD* gene, particularly the newly identified A43T mutation, hold great significance in the research on *N. gonorrhoeae* resistance to azithromycin in East China. It is necessary to be vigilant about the spread of MLST ST1901, ST7363, and ST1588, especially ST1901 clones.

Footnotes

Authors' Contribution: WH and MH made substantial contributions to conception and design. HC and FY revised the manuscript critically for important intellectual content. ZZ and DZ drafted the manuscript. All the authors read and approved the final manuscript.

Conflict of Interests: The author declares that this study was conducted in the absence of any commercial or financial relationships that might be interpreted as potential conflicts of interest.

Ethical Approval: This study was approved by the Ethics Committee in Clinical Research of the First Affiliated Hospital of Wenzhou Medical University (issuing no. KY2023-R118).

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