Pakistan J. Zool., vol. 55(1), pp 259-267, 2023 DOI: https://dx.doi.org/10.17582/journal.pjz/20210728080714

# Emerging Azithromycin Resistance among the *Neisseria gonorrhoeae* Strains Isolated in Danyang, China

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**Article Information**
**Received 28 July 2021**
**Revised 19 August 2021**
**Accepted 06 September 2021**
**Available online 25 February 2022 (early access)**
**Published 27 October 2022**

**Authors' Contribution**
**JY and WZ prepared the study design, performed statistical analyses, and drafted the manuscript. QZ performed bactericidal assays and experiments. LC advised continuously in experiments and projects. ZC performed experiments with phagocytosis assay. XZ aided in study design and advising in experiments. WW, SZ and LW assisted in experiment and design of phagocytosis assay.**

**Key words**
***Neisseria gonorrhoeae*, Azithromycin, Antimicrobial resistance, NG-MAST, Gonorrhoea**

## ABSTRACT

*Neisseria gonorrhoeae* is a human pathogen. Strain identification and effective antibiotic treatment are the main ways to prevent and control gonorrhea, but *N. gonorrhoeae* is now resistant to most antibiotics, and there is no vaccine available. The main purpose of this study was to understand the molecular characteristics of clinical isolates of *N. gonorrhoeae* and their resistance to azithromycin (AZM) in Danyang, China. Firstly, the clinical isolates of *N. gonorrhoeae* from January 2016 to December 2020 were collected by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). Secondly, the drug sensitivity of all clinical isolates was analyzed. Thirdly, polymerase chain reaction (PCR) and DNA sequencing were used to analyze the genes related to AZM resistance (AZM-R), including 23s rRNA allele mutation, *mtrR* promoter and coding region mutation, *rplD* and *rplV* mutation. Finally, the clinical isolates resistant to AZM were typed by *N. gonorrhoeae* multiantigen sequence typing (NG-MAST). 388 clinical isolates of *N. gonorrhoeae* were identified, of which 373, 329, 298, 5, 11 and 5 strains were resistant to ciprofloxacin, penicillin, tetracycline, AZM, spectinomycin and ceftriaxone, respectively. The mutation detection of AZM-R related gene showed that there were single (A) nucleotide deletion mutation in *mtrR* promoter region, G45D mutation in *mtrR* coding region, G70 mutation in *rplD* and A2047G mutation in 23s rRNA allele, but no mutation was found in *rplV*. A total of 8 different STs were identified in 5 AZM-R strains, of which two ST1866 isolates showed a high level of AZM-R. The clinical isolates of *N. gonorrhoeae* in Danyang have high genetic diversity. ST1866 isolates showed a high level of AZM-R, Therefore, measures should be taken to monitor the spread of ST1866 *N. gonorrhoeae* clones in eastern China.

## INTRODUCTION

*Neisseria gonorrhoeae* is a human-specific pathogen, causing 78 million new gonorrhea infections worldwide every year (Zhu *et al*., 2016; Ma *et al*., 2017; Lovett and Duncan, 2018; Quillin and Seifert, 2018). Gonorrhea can lead to serious complications, such as epididymitis in men and pelvic inflammation in women. Pelvic inflammation can result in involuntary infertility andectopic pregnancy (Mitchell and Prabhu, 2013; Wiesenfeld and Manhart, 2017). *N. gonorrhoeae* can also infect the eyes of newborns as they pass through the birth canal of an infected mother, which can lead to blindness (Rivacoba *et al*., 2017; Jin, 2019). More importantly, gonorrhea is associated with other sexually transmitted infections and human immunodeficiency virus (HIV) infections (Xu *et al*., 2018; Sanyal *et al*., 2019; Dave *et al*., 2020; Kato *et al*., 2020). Gonorrhea is considered to be a non-ulcerative sexually transmitted infection, just like chlamydia and trichomoniasis, and like other non-ulcerative sexually transmitted diseases, people with gonorrhea have a higher risk of transmitting HIV to their

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0030-9923/2023/0001-259 $ 9.00/0

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partners (Peters *et al*., 2021; Pottorff *et al*., 2021). this is because the loss of genitals in patients with HIV infection increases the virus.

Identification of strains and effective antibiotic treatment are the main ways to prevent and control gonorrhea, but *N. gonorrhoeae* are now resistant to most antibiotics and no vaccine is available (Abbasi, 2017; Baarda *et al*., 2018; Vincent and Jerse, 2019). Now, the double antimicrobial therapy of ceftriaxone and azithromycin (AZM) has been widely accepted worldwide, as an empirical first-line treatment for gonorrhea (Maldonado and Takhar, 2013; Unemo *et al*., 2021), including China (Chen *et al*., 2014). In most countries, 2 g AZM single therapy is used for treatment of pairs β-Gonorrhea in patients with lactam allergy. The results show that *N. gonorrhoeae* will produce resistance after introducing a new antimicrobial agent and replace the sensitive bacterial population within 20 years (Unemo and Dillon, 2011). Therefore, it is necessary to strengthen the monitoring of *N. gonorrhoeae* resistance.

In the 1990s, AZM became the first choice drug for many infectious diseases, but it was reported that AZM had drug resistance in the past decade (Steingrimsson *et al*., 1990). AZM was recommended to Chinese patients with mixed infection of gonorrhea and Chlamydia trachomatis around 2000 (Duan *et al*., 2019), and was widely used because of its wide availability and easy management. However, the widespread use of AZM may lead to resistance of *N. gonorrhoeae*. AZM resistance (AZM-R) *N. gonorrhoeae* was first found in China from 2001 to 2003, and AZM-R isolates were first identified in Guangzhou in 2009 (Liang *et al*., 2016). In the following years, AZM-R isolates and multi drug resistant isolates were reported in Nanjing, Hangzhou and Changsha (Ni *et al*., 2016; Wan *et al*., 2018; Yan *et al*., 2019; Yuan *et al*., 2019). Until 2013, little was known about the types of AZM-R *N. gonorrhoeae* prevalent in China. Therefore, the level of AZM-R and the molecular characteristics of AZM-R *N. gonorrhoeae* are still unclear (Jiang *et al*., 2017).

The multidrug resistance of *N. gonorrhoeae* is related to the overexpression of efflux pump. The most important efflux mechanism is the MtrC-MtrD-MtrE system, which is encoded by the *mtr* operon, in which *mtrR* is the regulatory gene and *mtrCDE* is the structural gene (Lucas *et al*., 1997). Another efflux pump encoded by *mef* gene was first found in some gram-positive bacteria and then in clinical strains of gonorrhea (Luna *et al*., 2000).

In the *mtrR* gene, specific mutations in the promoter or coding region can lead to decrease the MtrCDE efflux pump repression and subsequently increase export of the antimicrobial. Mutations in the promoter or coding sequence of *mtrR* gene of macrolide resistant *N. gonorrhea* can reduce the expression of *mtrR* repressor and up regulate mtrCDE efflux pump (Chen *et al*., 2019). In *N. gonorrhoeae*, there was a single base pair (A) deletion in the 13 bp reverse repeat of *mtrR* promoter region, the expression of *mtrR* was cancelled, and the level of mtrCDE increased, most likely because the binding affinity of RNA polymerase to mtrCDE increased (Handing *et al*., 2018). Missense mutations in the *mtrR*, such as G45D mutation in the helix trans helix motif in the *mtrR* repressor, can reduce the binding of the repressor to the mtrCDE promoter. The increased expression of mtrCDE efflux pump also increased AZM.

Further resistance to AZM is the result of 23S rRNA loop V mutation, which is a specific target of AZM. AZM exerts its bacteriostatic effect by directly interacting with the central ring of *rrl* gene domain V encoding 23S rRNA, resulting in obstruction of protein synthesis (Pham *et al*., 2021). Specific point mutations in this region may lead to drug resistance by reducing the affinity of AZM to its target (Zhang and van der Veen, 2019). 23S rRNA point mutations have been described, including C2611T (numbering refers to the *E. coli* genome), given low to medium levels of AZM-R (minimum inhibitory concentration MIC = 2 to 32μg/mL) or A2059G (*E. coli* numbering), awarded high level AZM-R (MICs ≥ 256 μg/mL) (Demczuk *et al*., 2016).

Ribosomal proteins L4 (encoded by *rplD*) and L22 (encoded by *rplV*) bind to domain I of 23S rRNA and act as channels for macrolide antibiotics to enter ribosomes (Reinert and Al-Lahham, 2005; Chisholm *et al*., 2010). Point mutations of *rplD* and rplV in *E. coli* and *Streptococcus pneumoniae* lead to resistance to macrolides; However, this mutation is rarely detected in patients with gonorrhea.

In the absence of new antibiotics for the treatment of gonorrhea, it is important to classify the emergence and dynamics of AZM-R *N. gonorrhoeae* on a regional and national basis for the successful updating of treatment recommendations. The resistance level of *N. gonorrhoeae* to azithromycin in Danyang area of eastern China is still unclear. The purpose of this study was to investigate the prevalence and molecular typing of *N. gonorrhoeae* in Danyang City.

## MATERIALS AND METHODS

### N. gonorrhoeae *isolation and species verification*

The patients with *N. gonorrhoeae* infection were identified by consulting the microbiological laboratory records of Danyang people's Hospital of Jiangsu Province from January 2016 to December 2020. Clinical isolates are anonymous, so this study does not require ethical approval. There is no standard for selecting strains. The clinical

data, such as gender and age and infection site, were recorded. Species verification is performed biochemically using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) using VITEK-MS (bioMérieux).

*Antimicrobial susceptibility testing*

The susceptibility of *N. gonorrhoeae* to penicillin, tetracycline, ciprofloxacin, spectinomycin, ceftriaxone and AZM was determined by E-test. The results of the E-test were interpreted according to the guidelines for all antibiotics of the Institute of clinical and laboratory standards (Palmeira and Ferreira, 2019). All the strains were stored in glycerine broth and stored at -80°C until use. The quality control strain of drug sensitivity test was *N. gonorrhoeae* ATCC 49226, which was provided by the clinical laboratory of the Ministry of Health and kept in our laboratory. The strain was stored in liquid medium of glycerine broth.

*Genetic determinants associated with resistance to AZM*

Genomic DNA, was extracted using DNA rapid extraction kit (Shanghai Shenggong) and stored at -20°C. To identify site-specific mutations, we sequenced the genes associated with AZM-R, including 23s rRNA allele, *rplD* and *rplV* (encoding ribosomal proteins L4 and L22, respectively), as well as *mtrR* promoter and coding region. The primers and conditions for PCR have been previously published (Allen *et al*., 2011; Zheng *et al*., 2019). PCR products were bidirectionally sequenced by Applied Biosystems 3730xl DNA automatic sequencer. DNA sequences were compared with BLAST and GenBank programs (http://www.ncbi.nlm.nih.gov/blast/) was used to identify gene mutations.

N. gonorrhoeae *multiantigen sequence typing (NG-MAST)*

NG-MAST was used to analyze the molecular epidemiology of AZM-R *N. gonorrhoeae* isolates. Briefly, the internal regions of genes encoding two variable outer membrane proteins, *porB* and *tbpB* subunit B, were sequenced to generate a two allele map of a strain. Sequence type (ST) is assigned through NG-MAST website (www.ng-mast.net). Besides, the NG-MAST genome based on the sequence similarity of *porB* and *tbpB* alleles is defined as described above (Martin *et al*., 2004). The phylogenetic tree of AZM-R *N. gonorrhoeae* was established by linking *porB* and *tbpB* alleles using MEGA 7.0 software and maximum likelihood method.

## RESULT

*Patient data*

There were 388 strains of *N. gonorrhoeae*, including 75 strains in 2016, 78 strains in 2017, 77 strains in 2018, 78 strains in 2019, 80 strains in 2020. There were 340 strains in male and 48 strains in female. There were 41 strains in obstetrics and gynecology, 62 strains in dermatology and 285 strains in urology, and the age range was 16-78 years old, including 16 strains under 20 years old, 107 strains aged 20-30 years old, 103 strains aged 30-40 years old, 60 strains aged 40-50 years old, 59 strains aged 50-60 years old and 43 strains over 60 years old.

*Antimicrobial susceptibility*

Of the 388 strains of *N. gonorrhoeae*, 373 strains (96.13%), 329 strains (84.80%), 298 strains (26.80%), 5 strains (1.29%), 11 strains (2.83%) and 5 strains (1.29%) were resistant, respectively to ciprofloxacin, penicillin, tetracycline, AZM, spectinomycin and ceftriaxone. Among them, 88 strains were β-lactamase positive, with a positive rate of 22.68%. The MIC of AZM ranges from 0.064 to > 16 μg/mL, $MIC_{50}$= 0.5 μg/mL, $MIC_{90}$=1.0 μg/mL. Among the 5 AZM-R strains, 2 strains with MIC= 1μg/mL showed low level resistance, 2 strains with MIC= 4μg/mL showed moderate resistance, and 1 strain with MIC > 16 μg/mL showed high level resistance.

*Detection of mutations in genes associated with AZM-R*

The following mutations were detected in 5 AZM-R isolates: single nucleotide (A) deletion in the promoter region of *mtrR*, G70D in the *mtrR* coding region, and A2047G in the 23s rRNA allele (*N. gonorrhoeae* number, GenBank accession number: X67293.1) (Table I). Four allele mutations in 23s rRNA were detected in a gonorrhea isolate with high level of AZM-R. No mutation was detected in *rplV*.

Five azithromycin sensitive (AZM-S) strains were randomly selected as control group. The mutations detected in these controls included single nucleotide (A) deletion in the *mtrR* promoter region and G45D in the *mtrR* coding region in the three isolates (Table I). No mutation was found in *rplD* and *rplV* genes and 23s rRNA alleles in AZM-S group.

*Molecular epidemiologic typing*

Five AZM-R *N. gonorrhoeae* isolates were genotyped by NG-MAST, and 8 different sequence types of (STs) were identified. ST1866 were the most common isolates of ST, followed by ST4007, ST1407, ST12746, ST3287, ST1731, ST2286, ST2318 and ST12660. Both ST1866 isolates (which were not associated with each other in epidemiology) displayed high levels of AZM-R. All the 8 STs found in this study have been reported in NG-MAST database.

Based on phylogenetic analysis, there are four groups. Group A strains include two different STs (ST2286

**Table I. Characterization and molecular typing results of clinical isolates of *N. gonorrhoeae*.**

| Group | Strain no. (Years) | AZM MIC (μg/ml) | mtrR[a] promoter | mtrR [b] coding region | rplD [c] mutation | rplV [d] mutation | 23S rRNA mutation[e] at position 2047 | NG-MAST |
|---|---|---|---|---|---|---|---|---|
| AZM-R | 70(2016) | 1 | A deletion | No mutation | No mutation | No mutation | No mutation | 1407 |
| (n=5) | 13(2017) | 1 | A deletion | G45D | No mutation | No mutation | A→G, alleles 1, and 2 | 12746 |
| | 09(2018) | 4 | A deletion | G45D | No mutation | No mutation | A→G, alleles 1,2 and 3 | 1866 |
| | 23(2019) | 4 | A deletion | No mutation | No mutation | No mutation | A→G, alleles 1, and 4 | 3287 |
| | 45(2020) | 18 | A deletion | G45D | G70D | No mutation | A→G, alleles 1,2,3 and 4 | 1866 |
| AZM-S | 04(2016) | 0.25 | No mutation | No mutation | No mutation | No mutation | No mutation | 1731 |
| (n=5) | 25(2017) | 0.125 | No mutation | No mutation | No mutation | No mutation | No mutation | 2286 |
| | 55(2018) | 0.064 | A deletion | No mutation | No mutation | No mutation | No mutation | 4007 |
| | 63(2019) | 0.25 | No mutation | G45D | No mutation | No mutation | No mutation | 2318 |
| | 17(2020) | 0.64 | No mutation | No mutation | No mutation | No mutation | No mutation | 12660 |

Notes: [a] The mtrR promoter mutation is the single-base-pair (A) deletion in the 13-bp inverted repeat in the promoter region. [b] GenBank accession number: Z25797.1. [c] GenBank accession number: YP-208871.1. [d] GenBank accession number: YP-208867.1. [e] GenBank accession number: X67293.1.

and ST2318). Group B strains include two different STs (ST12746 and ST1866). Group C strains are composed of three different STs (ST4007, ST1407, ST1731 and ST3287). Finally, the AZM-S cluster D contains a different STs (ST12660).

## DISCUSSION

In this study, the drug sensitivity test of *N. gonorrhoeae* in Danyang area from 2016 to 2020 was combined with AZM-R molecular typing. This study found for the first time that AZM-R began to appear in Danyang area, which was consistent with the previous reports from China (Yuan *et al*., 2011; Chen *et al*., 2013; Yuan *et al*., 2019). World Health Organization (WHO) suggested that once 5 per cent of locally acquired *N. gonorrhoeae* isolates develop drug resistance, the empirical use of antibiotics should be stopped. Therefore, AZM is not recommended as a monotherapy for gonococcal urethritis or cervicitis in China and many other countries in the world. To improve treatment effectiveness and delay further selection of cephalosporin-resistant *N. gonorrhoeae*, most current guidelines recommend a dual treatment regimen of ceftriaxone (250mg or 500mg intramuscular injection) or cefixime (400mg, if no ceftriaxone option) combined with AZM (1g or 2g oral) in the treatment of gonorrhea. However, in recent years, the decreased sensitivity of AZM-R *N. gonorrhoeae* to extended-spectrum cephalosporins has been reported, which seriously threatens the future efficacy of current treatment recommendations.

In recent years, there have been studies on AZM-R *N. gonorrhoeae* in China. For example, 32% of the isolates showed AZM-R, and 10% showed high level of drug resistance (Zheng *et al*., 2019). The analysis of 126 strains isolated from Hefei from 2014 to 2015 showed that 29% of the strains were AZM-R, and 10% of them showed high level of AZM-R.

Mutations in the promoter or coding sequence of *mtrR* gene in macrolide resistant *N. gonorrhoeae* strains can reduce the expression of *mtrR* repressor, resulting in the up regulation of mtrCDE efflux pump (Ohneck *et al*., 2015). In *N. gonorrhoeae*, there was A-deletion in the 13 bp reverse repeat of *mtrR* promoter, which overlaps the mtrCDE promoter at the -35 region, *mtrR* expression was cancelled, and mtrCDE expression was increased, which was probably because RNA polymerase had greater binding affinity for mtrCDE (Johnson and Shafer, 2015). *N. gonorrhoeae* resistant to macrolides can reduce the expression of MtrR repressor and up-regulate MtrCDE efflux pump (Ng *et al*., 2002; Cousin *et al*., 2003; Fernandez-Huerta and Espasa, 2019; Hall *et al*., 2019; Ma *et al*., 2020). In *N. gonorrhoeae* strains with deletion of 13-bp reverse repeat sequence in MtrR promoter region, the expression of MtrR was cancelled and the level of MtrCDE increased, which was probably due to the increase of binding affinity of RNA polymerase to mtrCDE (Rouquette *et al*., 1999). Missense mutations in the *mtrR* (Handing *et al*., 2018; Beggs *et al*., 2021), such as the G45D mutation in the helix-to-helix motif of the *mtrR* repressor, can reduce the binding of the repressor to the mtrCDE promoter. In this study, the mutation rates of *mtrR* promoter and coding region of AZM-R strain were 100.0% and 60%, respectively. The mutation rate of mtrR promoter region in AZM-R group was significantly higher

than that in AZM-S group, but there was no significant difference in *mtrR* coding region. This is consistent with the previously reported results that the mutation in the *mtrR* promoter region plays a more important role in the resistance of *N. gonorrhoeae* to azithromycin than the mutation in the *mtrR* coding region.

AZM exerts its bacteriostatic effect by directly interacting with the central ring of RRL gene domain V encoding 23S rRNA, resulting in obstruction of protein synthesis (Wu *et al*., 2011; Trembizki *et al*., 2015). Specific point mutations in this region may lead to drug resistance by reducing the affinity of azithromycin to its target (Ng *et al*., 2002; Chisholm *et al*., 2010; Galarza *et al*., 2010). The HL-AZM-R isolate had A2143G mutation in at least three of the four alleles. Including A2143G (the number in *E. coli* corresponds to A2059) or C2599T (the number in *E. coli* corresponds to C2611T) (Ng *et al*., 2002). Previous studies have reported that one or more of the four alleles of *rrl* gene in 23s rRNA domain V are associated with AZM-R, including mutation, A2059G and C2611T (corresponding to A2059G and C2611T in *E. coli*, respectively) (Belkacem *et al*., 2016). In this study, A2047G mutation was detected in the V region of AZM-R gonorrhea virus, but not in AZM-S group. The 23s rRNA alleles of A2047G mutation were alleles 1 and 2, alleles 1 and 2 and 3, alleles 1 and 4, alleles 1 and 2 and 3 and 4, respectively, and the corresponding MIC values were 1 μg/mL, 4 μg/mL, 4 μg/mL and 16 μg/mL, respectively. It is worth noting that A2047G mutation was detected in all strains with MIC > 16 μg/mL. Therefore, we believe that A2047G allele is the main determinant of AZM-R.

The *rplD* mutation (G70D) of a *N. gonorrhoeae* isolate to AZM was 16 μg/mL, which was consistent with the previously reported results (Jacobsson *et al*., 2016). G68D and G70D were previously described by (Zheng *et al*., 2019), so the mutation at position 68-70 of the *rplD* gene of *N. gonorrhoeae* seems to be associated with a high level of AZM resistance. The mutant of *rplD* gene belongs to group B, and the NG-MAST classification of ST1866, indicates that the mutant has genetic diversity.

Gonorrhea typing methods, such as NG-MAST, are helpful to understand the spread of gonorrhea. In this study, phylogenetic analysis showed that AZM-R strains had wide differences and did not belong to any specific group. Different from the reports of other countries, the isolates with high level of AZM-R in this study belong to NG-MAST-ST1866. It is worth mentioning that the high-level *N. gonorrhoeae* AZM-R found in this study has the same NA-MAST type as the high-level *N. gonorrhoeae* AZM-R found in the Liu-YH report, which indicates that the transmission of NG-MAST ST1866 *N. gonorrhoeae* has occurred across the Taiwan Strait. In addition, NG-MAST ST1866 clones have been reported in Nanjing, Hangzhou and Hefei (Ni *et al*., 2016; Jiang *et al*., 2017; Wan *et al*., 2018), which indicates that the clone has a high level of AZM-R, has spread in eastern China, which is a matter of concern, which must be paid attention to.

## ACKNOWLEDGEMENT

This work was supported by the National Natural Science Foundation of China (31700444), Social Development Guide Project of Zhenjiang, China (FZ2019006).

*Ethics approval*

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

*Statement of conflict of interest*

The authors have declared no conflicts of interest.

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