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# Azithromycin Resistance and Its Mechanism in *Neisseria gonorrhoeae* Strains in Hyogo, Japan

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**Therapeutic options are limited for *Neisseria gonorrhoeae* infection, especially for oral drugs. The purpose of this study was to investigate the susceptibility of *N. gonorrhoeae* to oral azithromycin (AZM) and the correlation between AZM resistance-related gene mutations and MIC. We examined the AZM MICs of clinical strains of *N. gonorrhoeae*, sequenced the peptidyltransferase loop in domain V of 23S rRNA, and investigated the statistical correlation between AZM MIC and the presence and number of the mutations. Among 59 *N. gonorrhoeae* strains, our statistical data showed that a deletion mutation was seen significantly more often in the higher-MIC group (0.5 μg/ml or higher) (35/37; 94.6%) than in the lower-MIC group (0.25 μg/ml or less) (4/22; 18.2%) ($P < 0.0001$). However, a mutation of codon 40 (Ala→Asp) in the *mtrR* gene (helix-turn-helix) was seen significantly more often in the lower-MIC group (12/22; 54.5%) ($P < 0.0001$). In *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) analyses, ST4777 was representative of the lower-MIC group and ST1407, ST6798, and ST6800 were representative of the higher-MIC group. NG-MAST type 1407 was detected as the most prevalent type in AZM-resistant or -intermediate strains, as previously described. In conclusion, a deletion mutation in the *mtrR* promoter region may be a significant indicator for higher MIC (0.5 μg/ml or higher). ST4777 was often seen in the lower-MIC group, and ST1407, ST6798, and ST6800 were characteristic of the higher-MIC group. Further research with a greater number of strains would help elucidate the mechanism of AZM resistance in *N. gonorrhoeae* infection.**

*Neisseria gonorrhoeae* infection is often seen in male urethritis and female cervicitis, and its diagnosis has historically led to prompt initiation of treatments with a single antibiotic (1, 2). In the 1990s in most Asian countries, fluoroquinolones (FQs) were often used for this infection, partly because FQs have higher concentrations in the urinary tract. FQs showed good initial outcomes for this infection, but the results were not enduring. This may be partly due to the trend for single use of FQs for this infection, with no guidelines to recommend other choices (3–9).

In addition to the FQs, cephalosporins such as cefixime were also recommended as oral antibiotics until these drugs showed decreased activity against *N. gonorrhoeae* (10). They are no longer a recommended choice for this infection (11–13). Oral antibiotics for outpatient treatment would be highly desirable for this kind of infection. Azithromycin (AZM) is an oral antibiotic with medical insurance approval for use against chlamydia infections (14), and there are many reports of good activity against *N. gonorrhoeae* infection (15). Other reports suggest that *N. gonorrhoeae* infection is less susceptible to AZM and that AZM-resistant strains have emerged (5). We investigated the AZM susceptibilities of *N. gonorrhoeae* strains and the correlation between mutations of macrolide resistance-related genes and the AZM MICs of clinically isolated strains of *N. gonorrhoeae*.

Three mechanisms of resistance to AZM are known: overexpression of the efflux pump, mutation in the peptidyltransferase loop in domain V of 23S rRNA, and modification of the ribosomal target by methylase. Macrolides such as AZM and erythromycin (EM) bind to 4 alleles of the 23S rRNA component of the 50S subunit of the bacterial ribosome and restrain protein synthesis by inhibiting the elongation of peptide chains (16).

Overexpression of the MtrCDE pump is due to either the lack of repressor MtrR protein, which occurs by a single-base-pair deletion or TT insertion in the promoter region, or missense mutations in the *mtrR* coding region (17). Modification of the ribosomal target by methylase or mutations reduces the affinity of the macrolide antibiotics for ribosomes (18). Since the use of antimicrobial agents for treatment of gonococcal infections is different in each country, the trend of drug resistance in gonococci is likely different geographically (19). Hence, epidemiological investigations should be done in each country. Previous studies have reported a high use of *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), which is based on limited DNA sequence analyses of two highly polymorphic loci, *porB* and *tbpB*, in sequence type (ST) 1407 *N. gonorrhoeae*, which is multidrug resistant and appears to be disseminated basically worldwide (20). The selection and spread of AZM resistance are driven by inappropriate treatment of patients with suboptimal doses of AZM for gonococcal infection. *N. gonorrhoeae* maintains its genetic resistance determinants even after the removal of antibiotic selection pressure.

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**TABLE 1** Sequences for PCR primers for azithromycin resistance genes

| Primer name | Target gene | Primer sequence (5′ to 3′) | Size (bp) | Reference |
|---|---|---|---|---|
| gonoRNA-F | 23S rRNA | ACGAATGGCGTAACGATGGCCACA | | |
| allele1 | | TCAGAATGCCACAGCTTACAAACT | 2,054 | 16 |
| allele2 | | GCGACCATACCAAACACCCACAGG | 2,240 | |
| allele3 | | GATCCCGTTGCAGTGAAGAAAGTC | 2,217 | |
| allele4 | | AACAGACTTACTATCCCATTCAGC | 1,847 | |
| mtrF1 | *mtrR* | GCCAATCAACAGGCATTCTTA | 380 | 17 |
| mtr13Ra | | GTTGGAACAACGCGTCAAAC | | |
| ermA-F | *ermA* | CTTCGATAGTTTATTAATATTAGT | 645 | 18, 21, 22 |
| ermA-R | | TCTAAAAAGCATGTAAAAGAA | | |
| ermB-F | *ermB* | AGTAACGGTACTTAAATTGTTTAC | 639 | |
| ermB-R | | GAAAAGGTACTCAACCAAATA | | |
| ermC-F | *ermC* | GCTAATATTGTTTAAATCGTCAAT | 642 | |
| ermC-R | | TCAAAACATAATATAGATAAA | | |
| ermF-F | *ermF* | CGGGTCAGCACTTTACTATTG | 466 | |
| ermF-R | | GGACCTACCTCATAGACAAG | | |

Surveillance and characterization of the mechanisms of AZM resistance are essential. In this study, we investigated the genetic characteristics of *N. gonorrhoeae* strains with decreased susceptibility to AZM.

## MATERIALS AND METHODS

**Strains.** *N. gonorrhoeae* strains were isolated from patients with male urethritis or female cervicitis and sent to Hyogo Rinsho Co. Ltd., Himeji, Japan. Gonococcal strains were retrieved from storage at −80°C by 48 h of incubation (35°C, 5% $CO_2$) on chocolate agar. The resulting colonies were then subcultured.

**MIC measurements.** The MIC (μg/ml) was determined using an Optopanel E212 dry plate (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) with the broth microdilution method. For AZM, the MIC breakpoints from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used (http://www.eucast.org). For quality control, the *N. gonorrhoeae* reference strain ATCC 49226 was included in each testing.

**DNA extraction.** DNA extracts were suspended in 500 μl of Tris-EDTA (TE) buffer for 10 min and then boiled for 15 min and centrifuged at 15,000 rpm for 10 min. The final supernatant was retained for storage at −20°C.

**PCR amplification and sequencing of AZM resistance genes.** To examine the mutations in the peptidyltransferase loop of domain V of the 23S rRNA gene, containing four copies, all four alleles were amplified by PCR and sequencing as described previously (16). The *mtrR* promoter region was examined by PCR and sequencing as described previously (17). DNA sequencing was performed after the PCR using PCR amplicons. Purification of the PCR products was performed with the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and the sequencing was performed at Eurofins Genomics, Inc. (Tokyo, Japan) (Table 1).

**PCR of the *erm* genes.** The methylase genes *ermA*, *ermB*, *ermC*, and *ermF* were detected using PCR with primers and conditions as described previously (18, 21, 22). The PCR primers are shown in Table 1. The PCR conditions were as follows: denaturing at 94°C for 30 s; annealing at 50°C for 30 s (*ermB* and *ermF*), 48°C for 1 min (*ermA*), or 43°C for 1 min (*ermC*); and elongation at 72°C for 2 min. The cycle was repeated 35 times (18, 21, 22).

**Molecular epidemiological typing.** NG-MAST was performed by using PCR and sequencing of the more variable segments of *porB* and *tbpB* (23). The *porB* and *tbpB* allele numbers as well as NG-MAST STs were assigned using the NG-MAST website (http://www.ng-mast.net/).

**Statistical analyses.** We investigated the statistical correlation between the AZM MIC and the presence and number of mutations under the classifications of an MIC of 0.25 μg/ml or less (lower-MIC group) and 0.5 μg/ml or higher (higher-MIC group). Statistical analysis was conducted using Fisher's exact test with the JSTAT (Java Virtual Machine Statistics Monitoring Tool; Sun Microsystems, Inc., Santa Clara, CA). Statistical significance was established at a *P* value of 0.05.

## RESULTS

**AZM MICs.** The AZM MICs of the 59 strains collected were 0.06, 0.12, 0.25, 0.5, 1, and 16 μg/ml. In detail, 2 strains (3.39%) had an MIC of 0.06 μg/ml, 5 strains (8.47%) had an MIC of 0.12 μg/ml, 16 strains (27.1%) had an MIC of 0.25 μg/ml, 33 strains (55.9%) had an MIC of 0.5 μg/ml, 4 strains (6.78%) had an MIC of 1 μg/ml, and 1 strain (1.69%) had an MIC of 16 μg/ml. The $MIC_{50}$ was 0.5 μg/ml, and the $MIC_{90}$ was 0.5 μg/ml (Table 2).

**Mutations in domain V of 23S rRNA.** There were 2 strains with C2214T (C204T) mutations in allele 3, one strain with a G2209A (G199A) mutation in allele 4 and one strain (with the highest MIC, 16 μg/ml) with C2599T (C589T) mutations in all 4 alleles in the peptidyltransferase loop in domain V of 23S rRNA.

***mtrR* mutations.** There were 38 (64.4%) strains with an A deletion mutation in the promoter region of *mtrR*, and this was seen significantly more often in the higher-MIC group (0.5 μg/ml or higher) (35/37; 94.6%) than the lower-MIC group (0.25 μg/ml or less) (4/22; 18.2%) ($P < 0.0001$). Conversely, mutations of codon 40 (Ala→Asp) in the *mtrR* gene (helix-turn-helix) were seen significantly more often in the lower-MIC group (12/22; 54.5%) ($P < 0.0001$). In addition, except for deletion mutations, strains with no mutation were seen significantly more often in the higher-MIC group (33/37; 89.2%) ($P < 0.0001$), and strains with one mutation were seen significantly more often in the lower-MIC group (15/22; 68.2%) ($P < 0.0001$).

**Detection of methylase genes.** Of the 59 *N. gonorrhoeae* strains, there were no strains with positive PCR detection of *ermA*, *ermB*, *ermC*, or *ermF* (data not shown).

**NG-MAST.** *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) results for *porB* and *tbpB* classified the 59 *N. gonorrhoeae* strains as ST3505, ST4015, ST4637, ST4777, ST5875, ST6761, ST6773, ST6775, ST7382, and ST7806 in the lower-MIC group and ST247, ST1407, ST3520, ST3588, ST4044, ST4163, ST4186, ST5875, ST6764, ST6774, ST6798, and ST6800 in the higher-MIC group. The highest MIC in our strains (16 μg/ml) was found for ST1407. ST4777 was representative of the lower-MIC

**TABLE 2** MICs, mutations, and NG-MAST results for the *N. gonorrhoeae* strains

| | | | Mutations[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mtrR* promoter region | | *mtrR* gene | | | | | |
| | | | | | Helix-turn-helix | | | Codon 29 (silent mutation) | Codon 30 (silent mutation) | |
| AZM MIC | No. of strains | % of strains | A deletion | C→T mutation | Codon 39 (Ala→Thr) | Codon 40 (Ala→Asp) | Codon 45 (Gly→Asp) | | | NG-MAST type(s) (no. of strains) |
| 16 | 1 | 100 | × | | | | | | | ST1407 (1) |
| 1 | 4 | 100 | × | | | | | | | ST6798 (3), ST1407 (1) |
| 0.5 | 28 | 88 | × | | | | | | | ST1407 (5), ST6800 (5), ST6798 (2), ST4044 (2), ST3588 (2), ST247 (1), ST3520 (1), ST4163 (1), ST4186 (1), ST5875 (1), ST6764 (1), ST6774 (1), nontypeable (9) |
| | 2 | 6 | × | | | | × | | | |
| | 1 | 3 | | × | × | | | × | × | |
| | 1 | 3 | | | | | × | | | |
| 0.25 | 8 | 50 | | | | × | | | | ST4777 (2), ST6761 (2), ST6775 (2), ST4015 (1), ST4637 (1), ST5875 (1), ST7382 (1), ST7806 (1), nontypeable (5) |
| | 3 | 19 | | × | × | | | × | × | |
| | 2 | 13 | ND | ND | ND | ND | ND | ND | ND | |
| | 1 | 6 | × | | | | | | | |
| | 1 | 6 | | | | | × | | | |
| | 1[b] | 6 | | | | | | | | |
| 0.125 | 4 | 100 | | | | × | | | | ST4777 (1), ST6773 (1), nontypeable (2) |
| 0.06 | 2 | 100 | × | | | | × | | | ST3505 (1), nontypeable (1) |
| Total | 59 | | | | | | | | | |

[a] ×, mutation present; ND, not determined.
[b] 100-bp insertion and mutation.

group, and ST1407, ST6798, and ST6800 were representative of the higher-MIC group (Table 2).

## DISCUSSION

The mechanism of resistance for the AZM-resistant *N. gonorrhoeae* phenotype is currently unknown in detail. AZM and other macrolides exert a bacteriostatic effect by interacting directly with the central loop of domain V, the site of peptide bond formation, thereby inhibiting protein synthesis. Alteration in 23S rRNA is reported to contribute to low-level resistance. A 23S rRNA mutation (C2611T) has been proposed to contribute to the low-level resistance observed in two Canadian gonococcal strains (24). Chisholm et al. showed that very-high-level resistance to AZM (MIC, 256 μg/ml) occurs in *N. gonorrhoeae* as a result of a single point mutation in the peptidyltransferase region of domain V of the 23S rRNA gene (24). Our study included a high-level AZM-resistant strain (MIC, 16 μg/ml) with C2599T mutations in all the alleles (alleles 1 to 4) tested. This suggests that gonococcal strains from Japan may have more mutations, even in strains with lower MICs, than those from other countries, such as the United Kingdom.

We observed that all AZM-resistant strains contained several mutated alleles and that highly resistant strains had at least 3 mutations, while sensitive and moderately resistant isolates had a maximum of one mutated allele. Mutation of A2059G in highly resistant gonococci and C2611T mutations in the 23S rRNAs of moderately resistant gonococci were reported previously (16). Our study found strains with mutations in one allele even in strains with low MICs (0.25 to 0.50 μg/ml), indicating that even gonococci with comparatively lower MICs harbor mutations.

Another mechanism of AZM resistance, the efflux pump, is well known to contribute to macrolide resistance. This pump excretes antibiotics, resulting in insufficient intracellular accumulation. The MtrCDE efflux pump regulated by the MtrR repressor protein is a representative efflux pump system. *mtrR* mutations in the gene itself or in its promoter region have been reported in low-level macrolide-resistant gonococcal strains (25, 26) because of decreased MtrR expression and upregulation of the MtrCDE efflux pump. Clinical isolates or laboratory-derived mutants that display resistance to hydrophobic agents frequently contain loss-of-function mutations in the *mtrR* coding sequence or a single-base-pair deletion in a 13-bp inverted repeat within the *mtrR* promoter (27–29).

The *mtrR* mutations in the clinical isolates described above were identical to those reported for other strains of gonococci that express resistance to multiple hydrophobic compounds (30, 31). The combined genetic and molecular results obtained in this investigation implicate the *mtrCDE*-encoded efflux pump as a mechanism by which gonococci can express decreased susceptibility to AZM. Through known mutations (27) that abrogate transcription of the gene (*mtrR*) that encodes a transcriptional repressor of *mtrCDE* or loss-of-function mutations in the repressor-encoding gene, gonococci can overproduce the MtrCDE efflux pump to increase their capacity to export hydrophobic agents. Our data showed that a deletion mutation in the promoter region of *mtrR* was seen significantly more often in the higher-MIC group (0.5 μg/ml or higher) compared with the lower-MIC group (0.25 μg/ml or less) ($P < 0.0001$), and conversely, a mutation of codon 40 (Ala→Asp) in the *mtrR* gene (helix-turn-helix) was seen significantly more often in the lower-MIC group ($P <$

0.0001). These findings contribute to the current understanding of the mechanism of AZM resistance in *N. gonorrhoeae*. However, the methylase genes were not detected in our tested *N. gonorrhoeae* strains, indicating that our AZM-resistant strains did not have the same mechanisms of macrolide resistance as seen in the study by Roberts et al. (18). The trend of AZM resistance has not yet become widespread, and thus we need to continue surveillance of gonococcal sensitivity to AZM but also continue with mechanistic studies.

As an epidemiological study tool, the NG-MAST method (32–36) is based on limited DNA sequence analyses of two highly polymorphic loci, *porB* and *tbpB* (23). There is public Internet access for sequence submission and assignment of sequence types, either for *porB* or *tbpB* or for the assignment of STs using a combination of these two loci (23). Our study clearly showed that STs could be usefully classified according to high-level and low-level AZM MICs. To our knowledge, there is no report of NG-MAST STs and AZM MICs. We identified ST1407 as a highly ASM-resistant strain (MIC, 16 μg/ml) in Japan, suggesting that future epidemiological studies are needed for detecting the spread of this gonococcal antibiotic-resistant phenotype in order to prevent a repetition of the failure that resulted from continuous use of quinolones, producing a high prevalence of quinolone-resistant gonococci.

This study has some limitations. First, the number of tested gonococcal strains may not be enough for definitive conclusions. Second, additional mechanistic investigation may be necessary for further understanding of gonococcal AZM resistance. These limitations should be overcome by our future studies.

In conclusion, a deletion mutation of the *mtrR* promoter region is a possible mechanism of AZM resistance and may be a significant indicator for higher MICs (0.5 μg/ml or higher) in *N. gonorrhoeae* infection. ST4777 was often seen in the lower-MIC group and ST1407, ST6798, and ST6800 in the higher-MIC group, and this correlation with MIC was statistically significant. Further mechanistic studies are needed, including the use of a greater number of strains.

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We declare that we have no conflicts of interest.

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