Genetic characteristics of azithromycin-resistant Neisseria gonorrhoeae collected in Hyogo, Japan during 2015-2019

三浦, 真希子

(Degree)
博士（保健学）

(Date of Degree)
2023-03-25

(Date of Publication)
2024-03-01

(Resource Type)
doctoral thesis

(Report Number)
甲第8631号

(URL)
https://hdl.handle.net/20.500.14094/0100482379

博　士　論　文

Genetic characteristics of azithromycin-resistant *Neisseria gonorrhoeae* collected in Hyogo, Japan during 2015-2019

（兵庫県で 2015 年から 2019 年に分離されたアジスロマイシン耐性淋菌の遺伝学的特徴）

令和5年1月10日

神戸大学大学院保健学研究科保健学専攻

Makiko Miura （三浦　真希子）

Genetic characteristics of azithromycin-resistant *Neisseria gonorrhoeae* collected in Hyogo, Japan during 2015–2019

Makiko Miura[1,2], Katsumi Shigemura[1,3,*], Kayo Osawa[2,] Noriko Nakanishi[4], Ryohei Nomoto[4], Reo Onishi[1], Hiroyuki Yoshida[5], Toru Sawamura[2], Shiuh-Bin Fang[6,7], Yi-Te Chiang[8], Shian-ying Sung[9], Kuan-cho Chen8, Takayuki Miyara[10] and Masato Fujisawa[3]

[1]Department of Public Health, Kobe University Graduate School of Health Sciences, 7-10-2 Tomogaoka Suma-ku, Kobe, 654-0142, Japan.

[2]Department of Medical Technology, Faculty of Health Sciences, Kobe Tokiwa University, 2-6-2 Otani-cho, Nagata-ku, Kobe, 653-0838, Japan.

[3]Department of Urology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, 650-0017, Japan.

[4]Department of Infectious Diseases, Kobe Institute of Health, 4-6-5 Minatojima-nakamichi, Chuo-ku, Kobe, 650-0046, Japan.

[5]Hyogo Clinical Laboratory Corporation, 5-6-2, Aoyamanishi, Himeji, 671-2224 Japan.

[6]Division of Pediatric Gastroenterology and Hepatology, Department of Pediatrics, Shuang Ho Hospital, Taipei Medical University, 291 Jhong Jheng Road, Jhong Ho District, New Taipei City, 23561, Taiwan

[7]Department of Pediatrics, School of Medicine, College of Medicine, Taipei Medical University, 250, Wu Hsing Street, Hsin Yi District, Taipei, 11031, Taiwan

[8]Department of Urology, Taipei Medical University Shuang Ho Hospital, 291, Zhongzheng Rd, Zhonghe District, Taipei, 23561, Taiwan

[9]Ph.D. Program for Translational Medicine, College of Medical Science and Technology, Taipei Medical University, 250 Wu-Hsing St., Taipei, 110, Taiwan

[10]Department of Infection Control and Prevention, Kobe University Hospital, Kobe, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, 650-0017, Japan.

Abstract

Introduction. Azithromycin (AZM) is a therapeutic drug for sexually transmitted infections and is used for *Neisseria gonorrhoeae* when first- and second-line drugs are not available. Recently, the susceptibility of N. gonorrhoeae against AZM has been decreasing worldwide.

Hypothesis/Gap Statement. Azithromycin-resistance (AZM-R) rates among *N. gonorrhoeae* in Japan are increasing, and the gene mutations and epidemiological characteristics of AZM-R in *N. gonorrhoeae* have not been fully investigated.

Aim. We determined the susceptibility to AZM and its correlation with genetic characteristics of *N. gonorrhoeae.*

Methodology. We investigated the susceptibility to AZM and genetic characteristics of *N. gonorrhoeae.* Mutations in domain V of the 23S rRNA gene and *mtrR* were examined in 93 isolates, including 13 AZM-R isolates. Spread and clonality were examined using sequence types (STs) of multi-antigen sequence typing for *N. gonorrhoeae* (NG-MAST), and whole genome analysis (WGA) to identify single nucleotide polymorphisms.

Results. The number of AZM-R isolates increased gradually from 2015 to 2019 in Hyogo ($P=0.008$). C2599T mutations in 23S rRNA significantly increased in AZM-R isolates ($P<0.001$). NG-MAST ST4207 and ST6762 were frequently detected in AZM-R isolates, and they had higher MICs to AZM from 6 to 24 μg/ml. The phylogenic tree-based WGA showed that all isolates with ST4207 were contained in the same clade, and isolates with ST6762 were divided into two clades, AZM-S isolates and AZM-R isolates, which were different from the cluster containing ST1407.

Conclusion. Our study showed yearly increases in AZM-R rates in *N. gonorrhoeae*. NG-MAST ST4207 and ST6762 were not detected in our previous study in 2015 and were frequently identified in isolates with higher MICs to AZM. WGA confirmed that isolates with these STs are closely related to each other. Continued surveillance is needed to detect the emergence and confirm the spread of NG-MAST ST4207 and ST6762.

INTRODUCTION

Neisseria gonorrhoeae is a major cause of sexually transmitted infections (STIs) such as male urethritis and female cervicitis [1]. In the Japanese Society of STI guidelines for the diagnosis and treatment of sexually transmitted diseases, the first-line drug is ceftriaxone (CTRX) and the second-

line drug is spectinomycin (SPCM) for gonococcal infection [2]. If these are not available, administration of azithromycin (AZM) is considered. However, *N. gonorrhoeae* with decreased susceptibility to AZM has been reported worldwide [3, 4] including emergence of strains with high-level resistance against AZM [5–7].

The molecular mechanisms for AZM resistance in *N. gonorrhoeae* include mutations in the 23S rRNA and MtrCDE efflux pump [8]. Macrolides such as AZM and erythromycin bind to the four alleles of the peptidyl transferase loop of domain V of 23S rRNA of the 50S ribosome to inhibit protein synthesis. Mutations of A2143G with high-level AZM resistance (AZM-R) (MIC ≥256 μg/ml) and C2599T with low to moderate levels of resistance corresponding to the A2059G and C2611T mutations in the *Escherichia coli* genome, respectively, were reported previously [5, 9]. In addition to the two main A2059G and C2611T mutations, another mutation in 23S rRNA at A2058 was also associated with high-level AZM-R in *N. gonorrhoeae* [10]. Overexpression of the MtrCDE efflux pump is based on either the lack of repressor MtrR protein, which occurs by a single nucleotide (A) deletion in the 13 bp inverted-repeat sequence of the *mtrR* promoter region (which overlaps the mtrCDE promoter at the −35 region), or missense mutations in the *mtrR* coding region [9, 11]. A recent in vitro evolution study indicated that high-level AZM-R in *N. gonorrhoeae* developed with transitory mutations in ribosomal protein coding genes rplD, rplV and rpmH first, then mutations in the MtrCDE efflux pump, and finally within the 23S rRNA gene [8]. Another macrolide efflux pump, encoded by the mef gene,has been detected in *N. gonorrhoeae* [12]. Modification of the ribosomal target by methylase or mutations decreases the affinity of the macrolide antibiotics for ribosomes [13].

However, trends of drug resistance in gonococci probably differ geographically because the antimicrobial agents used for gonococcal infections vary from country to country [1]. Therefore, epidemiological investigations should be done in each country or region. Multi-antigen sequence typing for *N. gonorrhoeae* (NG-MAST) is a known method of epidemiological study for specific genotyping of *N. gonorrhoeae* [14, 15]. We previously investigated the susceptibility to AZM and genetic characteristics of 59 *N. gonorrhoeae* strains, showing sequence type (ST)1407 as being characteristic of isolates with MIC ≥0.5 μg/ml with C2599T mutation and a single nucleotide (A) deletion in the *mtrR* promoter region [16]. Two high-level AZM-R isolates (MIC ≥256 μg/ml) recently found in Japan, FC488 and GU20180115-5, had ST1866 and ST16497, respectively [7]. ST1866 isolates with high-level AZM-R have spread in eastern Asia and may have originated from regions in eastern Asia other than Japan [6, 17, 18]. Inaddition, whole genome analysis (WGA) provides higher resolution for epidemiological

investigations such as the spread and the relationships between gonococcal isolates than that achievable by traditional typing methods [19, 20].

N. gonorrhoeae retains the genetic determinants involved in antimicrobial resistance even after removing the selective pressure of antibiotics. Surveillance and characterization of the mechanisms of AZM-R are essential. In this study, we investigated a larger number of *N. gonorrhoeae* strains and performed WGA to identify single nucleotide polymorphisms (SNPs) that were not used in our previous study, in Hyogo, Japan.

METHODS

Strains

A total of 765 *N. gonorrhoeae* strains were isolated from patients with male urethritis or female cervicitis and sent to Hyogo Clinical Laboratory Corporation, Himeji, Japan, from 2015 to 2019. Gonococcal isolates were cultured at 35 ℃ in 5 % CO_2 for 48 h on chocolate agar, then suspended in skimmed milk and stored at −80 ℃. The resulting colonies were then subcultured.

MIC measurement

The MIC to AZM was determined using an Etest (bioM.rieux) and interpreted as resistant (R, >1 μg/ml) or susceptible (S, ≤1 μg/ml) according to the current clinical breakpoint of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org). A single gonococcal isolate was separated on chocolate agar. Inoculated plates were incubated at 35 ℃.

DNA extraction

N. gonorrhoeae colonies were resuspended in 500 μl oTris-EDTA buffer for 10 min and then boiled for 15 min and centrifuged at 15 000 r.p.m. for 10 min. The final supernatant was retained for storage at −20 ℃ for further use.

Antimicrobial resistance determinants

Analyses for the resistance determinants were performed for the AZM-R isolates. Eighty AZM-susceptible (AZM-S) isolates were selected to include various MIC values. To detect the mutations in the peptidyl-transferase loop of domain V of the 23S rRNA gene containing four copies, an allele-specific PCR was performed using TaKaRa Ex Taq (TaKaRa Bio) and individual allele-amplicons were sequenced using the PCR primers previously described [5]. The coding region and promoter region of the

mtrR gene were amplified by PCR and sequenced as previously described [5]. All PCR amplicons were purified by using a QIAquick PCR Purification Kit (Qiagen), and sequencing was performed at Eurofins Genomics. Mutations in the AZM-R isolates were compared to the correspondent sequences of AZM-S strain FA19. The presence of the ermA/B genes and mef A genes were investigated as previously described [12, 13].

NG-MAST

NG-MAST was performed by using PCR and sequencing of the more variable segments of porB and tbpB [14]. The porB and tbpB allele numbers as well as NG-MAST ST were assigned using the NG-MAST website [14].

Whole genome analysis

DNA libraries were constructed using a QIAseq FX DNA Library kit (Qiagen), and paired-end sequences (2 ℃ ~150 bp) were generated using the MiSeq system (Illumina) with a MiSeq reagent kit v2 (Illumina). Mapping and single nucleotide variant (SNV) calling were performed by using BactSNP [21], and recombination regions were removed by using Gubbins [22]. The genomic sequence of *N. gonorrhoeae* strain FA_1090 (Accession no. NC_002946) was obtained from the NCBI database and used as a reference.

Statistical analyses

The Cochran–Armitage trend test with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing), was used for analysing the annual trend of AZM-R isolates [23]. Fisher's exact test was performed using Statcel 4 software (OMS Publishing) for the differences in resistance determinants and NG-MAST STs between AZM-S and AZM-R isolates. Statistical significance was set to a P-value of 0.05.

RESULTS

AZM MICs

Of the 765 *N. gonorrhoeae* isolates, 13 (1.7 %) were resistant to AZM (Table 1). The annual rates of AZM resistance were 1.0 % (2/207) in 2015, 0.8 % (1/123) in 2016, 0 % (0/184) in 2017, 3.5 % (7/201) in 2018 and 6.0 % (3/50) in 2019. The AZM-R isolates exhibited an increasing tendency over the 5 years from 2015 to 2019 by the Cochran–Armitage trend test (P=0.008) (Fig. 1). However, there was no

significant trend for MIC50 and MIC90 during the 5-year period. The MICs of AZM in the 13 isolates ranged from 1.5 to 24 μg/ml. Strains with high-level resistance to AZM (MIC≥256 μg/ml) were not detected.

Antimicrobial resistant genes

All the AZM-R isolates harboured C2599T mutations in the peptidyl transferase loop in domain V of 23S rRNA. The presence of the C2599T mutation was significantly different between AZM-R (13/13; 100 %) and AZM-S isolates (1/80; 1.3 %, only one mutated AZM-S isolate showed an MIC=1 μg/ml) (P<0.001) (Table 2). Of the 13 AZM-R isolates, 12 (92.3 %) contained C2599T mutations in all four alleles. One isolate (7.7 %) had C2599T mutations in two of the four alleles. Missense mutations of A39T/R44H in the *mtrR* coding region were detected in the AZM-R isolates (5/13; 38.5 %) statistically more frequently than in the AZM-S isolates (7/80; 8.8 %) (P=0.003) (Table 2). There were no notable differences in AZM resistance-related mutations between the five AZM-R isolates and the seven AZM-S isolates, except for C2599T mutations in domain V of 23S rRNA (5/5 vs. 0/7; P=0.001). Missense mutation of A40D was detected in only five AZM-S isolates (6.3 %), with no significant difference (P=0.792). A single nucleotide (A) deletion in the 13 bp inverted repeat sequence in the *mtrR* promoter region was detected in seven of the 13 AZM-R isolates (53.8 %) and also detected in 37 out of 80 AZM-S isolates (46.3 %). There was no statistical difference between AZM-R and AZM-S isolates (P=0.611). The mefA, ermA and ermB genes were not identified by PCR in the 93 *N. gonorrhoeae* isolates (data not shown).

Table 1. Distribution of AZM-R[a] isolates in *Neisseria gonorrhoeae* between 2015 and 2019

Year	Total	2015	2016	2017	2018	2019	*p*-value[b]
AZM-R/total (n)	13/765	2/207	1/123	0/184	7/201	3/50	0.008*
(%)	1.7	1	0.8	0	3.5	6	
MIC50 (μg/mL)		0.125	0.094	0.125	0.19	0.19	N.S.[c]
MIC90 (μg/mL)		0.5	0.125	0.38	0.25	0.25	

[a] AZM-R: azithromycin-resistant; [b] Cochran-Armitage trend test; [c] N.S.: not significant; *Statistical significant

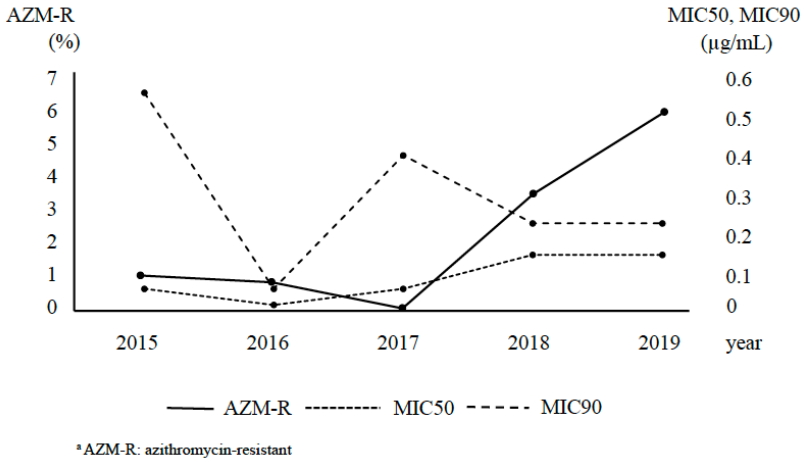

Figure1. Change of AZM-R isolates in *N. gonorrhoeae* between 2015 and 2019.

Table 2. Comparison of gene mutations between AZM-R[a] and AZM-S[b] isolates in *N. gonorrhoeae*

Resistance Determinant		No. of Isolates				*p*-value[e]
		AZM-R (n=13) (%)		AZM-S (n=80) (%)		
23S rRNA	C2599T (4 alleles)	12	(92.3)	0	(0.0)	< 0.001*
	C2599T (3 alleles)	0	(0.0)	1	(1.3)	
	C2599T (2 alleles)	1	(7.7)	0	(0.0)	
	No C2599T	0	(0.0)	79	(98.8)	
mtrR coding region[c]	A39T/R44H	5	(38.5)	7	(8.8)	0.003*
	No A39T/R44H	8	(61.5)	73	(91.3)	
	A40D	0	(0.0)	5	(6.3)	0.792
	No A40D	13	(100.0)	75	(93.8)	
	G45D	1	(7.7)	12	(15.0)	0.622
	No G45D	12	(92.3)	68	(85.0)	
mtrR promoter region	A deletion[d]	7	(53.8)	37	(46.3)	0.611
	No deletion	6	(46.2)	43	(53.8)	

[a] AZM-R: azithromycin-resistant; [b] AZM-S: azithromycin-susceptible; [c] A39T: codon39 (Ala→Thr), R44H: codon44 (Arg→His), G45D:codon 45 (Gly→Asp), A40D: codon40 (Ala→Asp); [d] A deletion: a single nucleotide (A)-deletion in the 13-bp inverted-repeat sequence of the *mtrR* promoter region; [e] Fisher's exact test; *Statistical significant

Table 3. Comparison of NG-MAST between AZM-R[a] and AZM-S[b] isolates in the 93 strains of *N. gonorrhoeae*

NG-MAST[c] STs[d]	No. of Isolates (%)		p-value[e]
	AZM-R	AZM-S	
4207	5 (38.5)	4 (5.0)	0.002*
6762	3 (23.1)	3 (3.7)	0.034*
6800	1 (7.7)	0 (0)	0.140
3704	1 (7.7)	0 (0)	0.140
14261	1 (7.7)	0 (0)	0.140
16736	1 (7.7)	0 (0)	0.140
19443	1 (7.7)	0 (0)	0.140
1407	0 (0)	6 (7.5)	0.394
6880	0 (0)	4 (5.0)	0.541
6771	0 (0)	3 (3.7)	0.633
Others	-	60 (75.0)	-

[a] AZM-R: azithromycin-resistant; [b] AZM-S: azithromycin-susceptible; [c] NG-MAST: multi-antigen sequence typing for *N. gonorrhoeae*, [d] STs: sequence types; [e] Fisher's exact test; *Statistical significant

Table 4. The characteristics of 13 AZM-R[a] *N. gonorrhoeae* isolates

Isolate number	AZM MIC (μg/ml)	*mtrR*		C2599T in 23S rRNA	NG-MAST[e] STs[f]
		Coding Region[b]	Promoter Region		
KG18111	24	A39T, R44H	WT[d]	4 alleles	4207
KG18136	6	A39T, R44H	WT	4 alleles	4207
KG18140	6	A39T, R44H	WT	4 alleles	4207
KG18193	6	A39T, R44H	WT	4 alleles	4207
KG19001	6	A39T, R44H	WT	4 alleles	4207
KG18192	6		A deletion	4 alleles	6762
KG18148	4		A deletion	4 alleles	6762
KG19030	4		A deletion	4 alleles	6762
KG15086	4		A deletion[c]	4 alleles	6800
KG16155	3		A deletion	4 alleles	14261
KG18153	3	G45D	WT	4 alleles	16736
KG15175	1.5		A deletion	2 alleles	3704
KG19029	1.5		A deletion	4 alleles	19443

[a] AZM-R: azithromycin-resistant; [b] A39T: codon39 (Ala→Thr), R44H: codon44 (Arg→His), G45D: codon 45 (Gly→Asp); [c] A deletion: A single nucleotide deletion in the 13-bp inverted-repeat sequence of the *mtrR* promoter region; [d] WT: wild type; [e] NG-MAST: multi-antigen sequence typing for *N. gonorrhoeae*, [f] STs: sequence types;

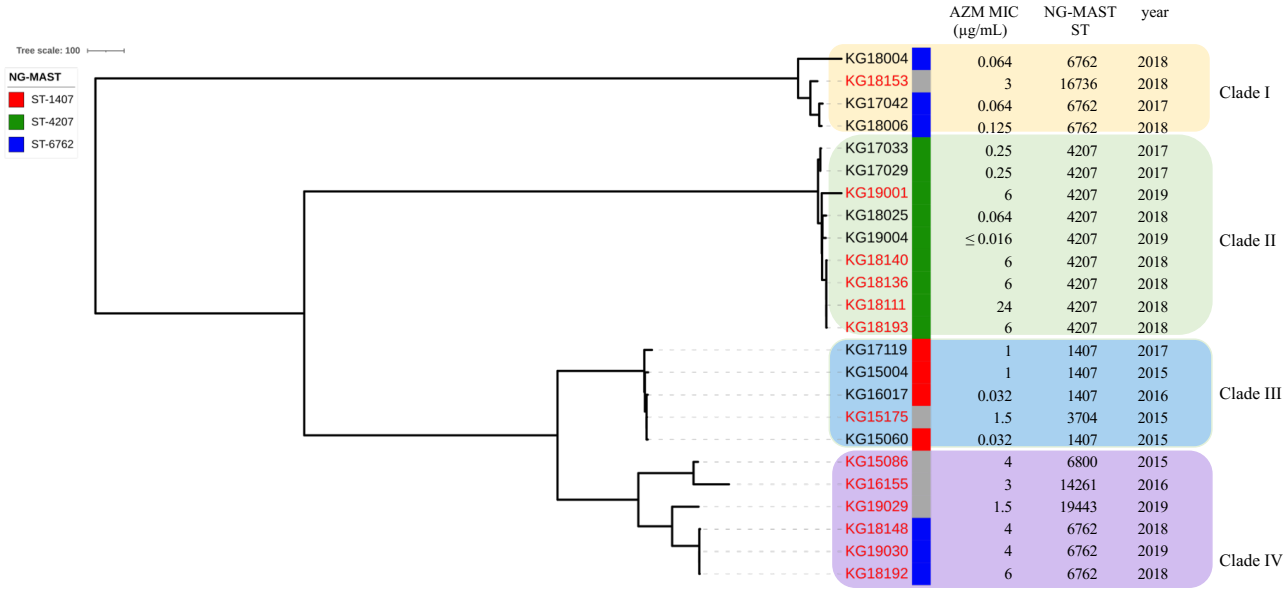

Figure2. Phylogenetic tree based on whole genome analysis among 13 AZM-R[a] and 11 AZM-S[b] *N. gonorrhoeae* isolates

NG-MAST

NG-MAST analysis identified seven STs in the 13 AZM-R isolates, and 39 STs in 80 AZM-S isolates (Table 3). Table 3 shows all STs in AZM-R isolates and major STs in AZM-S isolates. The frequent STs in AZM-R isolates were ST4207 (5/13; 38.5 %) and ST6762 (3/13; 23.1 %). ST4207 and ST6762 were statistically more commonly detected in AZM-R isolates compared with AZM-Sisolates (4/80; 5.0 %; P=0.002, 2/80; 3.7 %; P=0.034, respectively). Only C2599T mutations in domain V of 23S rRNA exhibited notable differences between the five AZM-R isolates with ST4207 and the four AZM-S isolates with ST4207 (5/5 vs. 0/4; P=0.008). The predominant STs in AZM-S isolates were ST1407 (6/80; 7.5 %), ST6880 (4/80; 5.0 %) and ST6771 (3/89; 3.8 %), which were detected only in AZM-S isolates but not in AZM-R isolates. Four of six isolates with ST1407 had an AZM MIC of 1 μg/ml as the lower susceptibility MIC. Gene mutations and NG-MAST in AZM-R isolates Table 4 shows the characteristics of 13 AZM-R isolates. Twelve isolates had the C2599T mutation in all four alleles in 23S rRNA. Five of 12 isolates had missense mutations of A39T and R44H in the *mtrR* coding region in addition to C2599T mutations in 23S rRNA. They were all NG-MAST ST4207 and had MICs to AZM from 6 to 24 μg/ml. Six isolates including ST6762 had a single nucleotide (A) deletion in the *mtrR* promoter region in addition to C2599T mutations in 23S rRNA and had MICs from 1.5 to 6 μg/ml. The

ST16736 isolate with an MIC of 3 μg/ml had G45D mutations in addition to C2599T mutations in 23S rRNA. The ST3704 isolate with an MIC of 1.5 μg/ml had C2599T mutations in two of four alleles in 23S rRNA.

Phylogenic analysis

Twenty-four *N. gonorrhoeae* isolates including 13 AZM-R isolates and 11 of AZM-S isolates with NG-MAST ST4207, ST6762 and ST1407 were analysed. The phylogenic tree based on WGA was determined (Fig. 2) based on coreSNP distances. The examined accessions were divided into four clades. Clade I included AZM-S isolates with ST6762 and AZM-R isolates with ST16736. Clade II included all isolates with ST4207 regardless of AZM susceptibility. Clade III included isolates with ST1407 and ST 3704. Clade IV included AZM-R isolates with ST6762, ST6800, ST14261 and ST19443.

DISCUSSION

AZM is an oral antibiotic covered by medical insurance for use against chlamydia infections. There are many reports that AZM is effective and provides a de facto dual therapy for gonococcal infections [24]. The use of macrolide drugs including AZM gradually decreased during 2015–2019 in Japan [25]. By contrast, our data showed that rates of AZM-R isolates in *N. gonorrhoeae* may be regional differences in Japan, given that 7.4 % (50/677) of N. gonorrhoeae isolated in Fukuoka were AZM-R [26], while this value was 6.6 % (8/122) in Tokyo [27]. Contemporary reports indicate 32.3 % (124/384) AZM-R *N. gonorrhoeae* isolates in China [17], 7.1 % (152/2134) in Europe [28] and 2.5 % (127/5,093) in the USA [3]. The rate of AZM-R isolates in Japan was lower than that in China.

We investigated the molecular epidemiology and gene mutations of 13 AZM-R *N. gonorrhoeae* isolates and 80 AZM-S isolates in Hyogo. Twelve of 13 AZM-R isolates (92.3 %) carried C2599T mutations in all four alleles of 23S rRNA. An increase in the number of 23S rRNA alleles with the C2599T mutations seemed to confer AZM-R, as previously described [2, 8, 29, 30]. A39T/R44H mutations in the *mtrR* coding region were detected more frequently in AZM-R isolates than AZM-S isolates. These mutations were previously identified in two AZM-intermediate isolates [31]. Similarly, our study identified the A39T/R44H double mutation in five AZM-R isolates with high MICs. In particluar, ST4207 isolates with A39T/R44H mutations in *mtrR* in addition to mutations in all four alleles in 23S rRNA had higher MICs from 6 to 24 μg/ml. Recent reports have indicated that the A39T mutation was associated with resistance to azithromycin, penicillin and tetracycline [32]. In addition, R44 is important for DNA recognition of mtrCDE. Transformation experiments in which plasmids with *mtrR*

point mutations in R44A, G45A and Y48F were introduced into the antibiotic-sensitive strain FA19 reported increased resistance to macrolides [33]. In this study as well, it was considered that these mutations may be involved in resistance to AZM in *N. gonorrhoeae*.

Previously, we reported that a single nucleotide deletion (A) mutation in the *mtrR* promoter region was a significant indicator for higher MIC (≥0.5 μg/ml) and possible correlation of a missense mutation of A40D in the *mtrR* coding region with lower MIC (<0.5 μg/ml) to AZM [16]. Similarly, the A deletion was more frequently detected in isolates with MIC ≥0.5 μg/ml ($P<0.05$, data not shown), but not in AZM-R isolates with MIC >1 μg/ml. A40D was also seen only in AZM-S isolates in this study.

Other resistance determinants of AZM such as the ermA/B gene encoding rRNA methylases and the mefA gene encoding the MacAB efflux pump were not detected in this study.

NG-MAST has been used worldwide to monitor the spread of gonococcal strains and to identify transmission patterns [34]. Several reports have indicated a high prevalence of NG-MAST ST1407, which is a multidrug-resistant gonococcal clone accounting for a high proportion of the decreased susceptibility and resistance to extended-spectrum cephalosporins seen worldwide [35–38]. This study only detected ST1407 in six AZM-S isolates. However, four of six isolates with ST1407 had an AZM MIC of 1 μg/ml as the lower susceptible MIC, which was the epidemiological cut-off value suggested by EUCAST. In contrast, ST4207 and ST6762 were frequently detected in AZM-R isolates. ST4207 and ST6762 were previously reported in AZM-R isolates in Japan, but no statistical significance was observed [26]. To trace phylogenic relationships, WGA identified SNPs for 13 AZM-R isolates and 11 for AZM-S isolates. These 11 AZM-S isolates were selected because ST4207 and ST6762 were frequently detected in AZM-R isolates in this study and ST1407 was frequently detected in AZM-R isolates in previous reports. All isolates with ST4207 were contained in the same clade (clade II) regardless of susceptibility to AZM. ST6762 was divided into two clades, AZM-S isolates and AZM-R isolates (clades I and IV). Both were different from the clade containing ST1407 (clade III). We confirmed that AZM-R isolates different from ST1407 existed throughout the study period in Hyogo, Japan. ST1866 and ST16497 found in isolates with high-level resistance in a recent report were not found in this study, but need to be monitored by continuous surveillance [7].

There were some limitations to our study. First, the number of AZM-R isolates was small without analysis of other mechanisms [mutations in ribosomal proteins L4 (rplD) and L22 (rplV) yield macrolide resistance] [8, 39], and therefore may not be sufficient for full evaluation of AZM resistance trends. Second, not all AZM-S isolates were subject to WGA.

AZM is indicated for pelvic inflammation and considered in Japan for use in patients with other

gonococcal infections who are allergic to CTRX or SPCM. Understanding the genetic characteristics and epidemiological trends in AZM-R *N. gonorrhoeae* may lead to early and appropriate treatment and prevent the emergence of new resistant strains.

CONCLUSION

Our study showed yearly increases in AZM-R rates in *N. gonorrhoeae.* NG-MAST ST4207 and ST6762 were not previously detected in our study and were frequently identified in isolates with higher MICs to AZM. WGA confirmed that isolates with these STs are closely related to each other. Continued surveillance is needed to detect the emergence and confirm the spread of NG-MAST ST4207 and ST6762.

Acknowledgements

We are grateful to Toshiro Shirakawa, Rio Okachi, Miku Kasuya, and Naoko Nishii for performing the experiments.

Authors and contributors

Makiko Miura: Methodology, Investigation, Writing—original draft preparation. Katsumi Shigemura: Conceptualization, Resources, Writing—review and editing, Project administration, Funding acquisition. Kayo Osawa: Conceptualization, Methodology, Resources, Writing—review and editing, Project administration, Funding acquisition. Noriko Nakanishi: Methodology, Writing—review and editing. Ryohei Nomoto: Methodology, Writing—review and editing. Reo Onishi: Writing—review and editing. Hiroyuki Yoshida: Resources, Writing—review and editing. Toru Sawamura: Methodology, Writing—review and editing. Shiuh-Bin Fang: Writing—review and editing. Yi-Te Chiang: Writing—review and editing. Shian-ying Sung: Writing—review and editing. Kuan-cho Chen: Writing—review and editing. Takayuki Miyara: Writing—review and editing, Project administration. Masato Fujisawa: Writing—review and editing, Supervision, Project administration. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

We declare that there are no conflicts of interest.

Funding information

This work was partially supported for K.O. by the Research Program on Emerging and Reemerging Infectious Diseases of the Japan Agency for Medical Research and Development, AMED (21fk0108605h0401).

Ethical approval

This study was approved by the ethics committee of Kobe Tokiwa University (No.19-13, approval date January 27, 2020).

Consent for publication

Not required.

References

1. Unemo M, Seifert HS, Hook EW 3rd, Hawkes S, Ndowa F, Dillon JR. Gonorrhoea. Nat Rev Dis Primers 2019;5:79. doi:10.1038/s41572-019-0128-6.
2. Japanese Society for Sexually Transmitted Infections Guidelines Preparing Committee Guidelines for the diagnosis and treatment of STI 2016. Accessed June 29, 2021. http://jssti.umin.jp/pdf/guideline-2016.pdf
3. Kirkcaldy RD, Harvey A, Papp JR, Del Rio C, Soge OO, Holmes KK, et al. *Neisseria gonorrhoeae* Antimicrobial Susceptibility Surveillance - The Gonococcal Isolate Surveillance Project, 27 Sites, United States, 2014. Morb Mortal Wkly Rep Surveill Summ Wash DC 2002. 2016 Jul 15;65(7):1–19. doi.org/10.15585/mmwr.ss6507a1.
4. Thakur SD, Araya P, Borthagaray G, Galarza P, Hernandez AL, Payares D, et al. Resistance to Ceftriaxone and Azithromycin in *Neisseria gonorrhoeae* Isolates From 7 Countries of South America and the Caribbean: 2010-2011. Sex Transm Dis. 2017 Mar;44(3):157–60. doi:10.1097/OLQ.0000000000000587.
5. Ng L-K, Martin I, Liu G, Bryden L. Mutation in 23S rRNA associated with macrolide resistance in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother. 2002 Sep;46(9):3020–5. doi.org/10.1128/AAC.46.9.3020-3025.2002.
6. Liu Y-H, Wang Y-H, Liao C-H, Hsueh P-R. Emergence and Spread of *Neisseria gonorrhoeae* Strains with High-Level Resistance to Azithromycin in Taiwan from 2001 to 2018. Antimicrob Agents Chemother. 2019 Sep;63(9):e00773-19. doi:10.1128/AAC.00773-19.

7. Shimuta K, Lee K, Yasuda M, Furubayashi K, Uchida C, Nakayama S-I, et al. Characterization of 2 *Neisseria gonorrhoeae* Strains With High-Level Azithromycin Resistance Isolated in 2015 and 2018 in Japan. Sex Transm Dis. 2021 Jul 1;48(7):e85–7. doi.org/10.1097/olq.0000000000001303.
8. Laumen JGE, Manoharan-Basil SS, Verhoeven E, Abdellati S, De Baetselier I, Crucitti T, et al. Molecular pathways to high-level azithromycin resistance in *Neisseria gonorrhoeae*. J Antimicrob Chemother. 2021 Jun 18;76(7):1752-1758. doi: 10.1093/jac/dkab084.
9. Unemo M, Shafer WM. Antimicrobial resistance in *Neisseria gonorrhoeae* in the 21st century: past, evolution, and future. Clin Microbiol Rev. 2014 Jul;27(3):587–613. doi.org/10.1128/CMR.00010-14.
10. Pham CD, Nash E, Liu H, Schmerer MW, Sharpe S, Woods G, et al. Atypical Mutation in *Neisseria gonorrhoeae* 23S rRNA Associated with High-Level Azithromycin Resistance. Antimicrob Agents Chemother. 2021 Jan 20;65(2):e00885-20. doi: 10.1128/AAC.00885-20.
11. Cousin SL, Whittington WLH, Roberts MC. Acquired macrolide resistance genes and the 1 bp deletion in the *mtrR* promoter in *Neisseria gonorrhoeae*. J Antimicrob Chemother. 2003 Jan;51(1):131–3. doi: 10.1093/jac/dkg040.
12. Luna VA, Cousin S, Whittington WL, Roberts MC. Identification of the conjugative *mef* gene in clinical *Acinetobacter junii* and *Neisseria gonorrhoeae* isolates. Antimicrob Agents Chemother. 2000 Sep;44(9):2503–6. doi.org/10.1128/AAC.44.9.2503-2506.2000.
13. Roberts MC, Chung WO, Roe D, Xia M, Marquez C, Borthagaray G, et al. Erythromycin-resistant *Neisseria gonorrhoeae* and oral commensal *Neisseria* spp. carry known rRNA methylase genes. Antimicrob Agents Chemother. 1999 Jun;43(6):1367-72. doi: 10.1128/AAC.43.6.1367. PMC89280.
14. Martin IMC, Ison CA, Aanensen DM, Fenton KA, Spratt BG. Rapid sequence-based identification of gonococcal transmission clusters in a large metropolitan area. J Infect Dis. 2004 Apr 15;189(8):1497–505. doi.org/10.1086/383047.
15. Unemo M, Dillon J-AR. Review and international recommendation of methods for typing *neisseria gonorrhoeae* isolates and their implications for improved knowledge of gonococcal epidemiology, treatment, and biology. Clin Microbiol Rev. 2011 Jul;24(3):447–58. doi:10.1128/CMR.00040-10.
16. Shigemura K, Osawa K, Miura M, Tanaka K, Arakawa S, Shirakawa T, et al. Azithromycin resistance and its mechanism in *Neisseria gonorrhoeae* strains in Hyogo, Japan. Antimicrob

Agents Chemother. 2015 May;59(5):2695–9. doi.org/10.1128/AAC.04320-14.

17. Xue J, Ni C, Zhou H, Zhang C, van der Veen S. Occurrence of high-level azithromycin-resistant *Neisseria gonorrhoeae* isolates in China. J Antimicrob Chemother. 2015 Dec;70(12):3404–5. doi:10.1093/jac/dkv266.
18. Wan C, Li Y, Le W-J, Liu Y-R, Li S, Wang B-X, et al. Increasing Resistance to Azithromycin in *Neisseria gonorrhoeae* in Eastern Chinese Cities: Resistance Mechanisms and Genetic Diversity among Isolates from Nanjing. Antimicrob Agents Chemother. 2018 May;62(5):e02499-17. doi.org/10.1128/AAC.02499-17.
19. Ohnishi M, Unemo M. Phylogenomics for drug-resistant *Neisseria gonorrhoeae*. Lancet Infect Dis. 2014 Mar;14(3):179-80. doi: 10.1016/S1473-3099(13)70700-X.
20. Lee RS, Seemann T, Heffernan H, Kwong JC, Gonçalves da Silva A, Carter GP, et al. Genomic epidemiology and antimicrobial resistance of *Neisseria gonorrhoeae* in New Zealand. J Antimicrob Chemother. 2018 Feb 1;73(2):353-364. doi:10.1093/jac/dkx405.
21. Yoshimura D, Kajitani R, Gotoh Y, Katahira K, Okuno M, Ogura Y, Hayashi T, Itoh T. Evaluation of SNP calling methods for closely related bacterial isolates and a novel high-accuracy pipeline: BactSNP. Microb Genom. 2019 May;5(5):e000261. doi: 10.1099/mgen.0.000261.
22. Croucher NJ, Page AJ, Connor TR, Delaney AJ, Keane JA, Bentley SD, Parkhill J, Harris SR. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic Acids Res. 2015 Feb 18;43(3):e15. doi: 10.1093/nar/gku1196.
23. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 2013 Mar;48(3):452-8. doi:10.1038/bmt.2012.244.
24. Yasuda M, Ito S, Kido A, Hamano K, Uchijima Y, Uwatoko N, et al. A single 2 g oral dose of extended-release azithromycin for treatment of gonococcal urethritis. J Antimicrob Chemother. 2014 Nov;69(11):3116–8. doi:10.1093/jac/dku221.
25. AMR Clinical Reference Center. Accessed July 2, 2021. https://amrcrc.ncgm.go.jp/surveillance/010/20181128172333.html
26. Tanaka M, Furuya R, Irie S, Kanayama A, Kobayashi I. High Prevalence of Azithromycin-Resistant *Neisseria gonorrhoeae* Isolates With a Multidrug Resistance Phenotype in Fukuoka, Japan. Sex Transm Dis. 2015 Jun;42(6):337–41. doi.org/10.1097/OLQ.0000000000000279.
27. Takayama Y, Nakayama S, Shimuta K, Morita-Ishihara T, Ohnishi M. Characterization of azithromycin-resistant *Neisseria gonorrhoeae* isolated in Tokyo in 2005-2011. J Infect

Chemother Off J Jpn Soc Chemother. 2014 May;20(5):339–41. doi.org/10.1016/j.jiac.2014.01.007.

28. Cole MJ, Spiteri G, Jacobsson S, Woodford N, Tripodo F, Amato-Gauci AJ, et al. Overall Low Extended-Spectrum Cephalosporin Resistance but high Azithromycin Resistance in *Neisseria gonorrhoeae* in 24 European Countries, 2015. BMC Infect Dis. 2017 Sep 11;17(1):617. doi.org/10.1186/s12879-017-2707-z.
29. Chisholm SA, Dave J, Ison CA. High-level azithromycin resistance occurs in *Neisseria gonorrhoeae* as a result of a single point mutation in the 23S rRNA genes. Antimicrob Agents Chemother. 2010 Sep;54(9):3812–6. doi.org/10.1128/AAC.00309-10.
30. Demczuk W, Martin I, Peterson S, Bharat A, Van Domselaar G, Graham M, et al. Genomic Epidemiology and Molecular Resistance Mechanisms of Azithromycin-Resistant *Neisseria gonorrhoeae* in Canada from 1997 to 2014. J Clin Microbiol. 2016 May;54(5):1304–13. doi.org/10.1128/JCM.03195-15.
31. Calado J, Castro R, Lopes Â, Campos MJ, Rocha M, et al. Antimicrobial resistance and molecular characteristics of *Neisseria gonorrhoeae* isolates from men who have sex with men. Int J Infect Dis. 2019 Feb;79:116-122. doi: 10.1016/j.ijid.2018.10.030.
32. Maduna LD, Kock MM, van der Veer BMJW, Radebe O, McIntyre J, van Alphen LB, et al. Antimicrobial Resistance of *Neisseria gonorrhoeae* Isolates from High-Risk Men in Johannesburg, South Africa. Antimicrob Agents Chemother. 2020 Oct 20;64(11):e00906-20. doi: 10.1128/AAC.00906-20.
33. Beggs GA, Ayala JC, Kavanaugh LG, Read TD, Hooks GM, Schumacher MA, et al. Structures of *Neisseria gonorrhoeae* MtrR-operator complexes reveal molecular mechanisms of DNA recognition and antibiotic resistance-conferring clinical mutations. Nucleic Acids Res. 2021 Apr 19;49(7):4155-4170.doi: 10.1093/nar/gkab213.
34. Unemo M, Dillon JA. Review and international recommendation of methods for typing *neisseria gonorrhoeae* isolates and their implications for improved knowledge of gonococcal epidemiology, treatment, and biology. Clin Microbiol Rev. 2011 Jul;24(3):447-58. doi: 10.1128/CMR.00040-10.
35. Unemo M, Nicholas RA. Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhea. Future Microbiol. 2012 Dec;7(12):1401-22. doi: 10.2217/fmb.12.117.
36. Golparian D, Hellmark B, Fredlund H, Unemo M. Emergence, spread and characteristics of *Neisseria gonorrhoeae* isolates with in vitro decreased susceptibility and resistance to extended-

spectrum cephalosporins in Sweden. Sex Transm Infect. 2010 Nov;86(6):454–60. doi:10.1136/sti.2010.045377.

37. Tanaka M, Koga Y, Nakayama H, Kanayama A, Kobayashi I, Saika T, et al. Antibiotic-resistant phenotypes and genotypes of *Neisseria gonorrhoeae* isolates in Japan: identification of strain clusters with multidrug-resistant phenotypes. Sex Transm Dis. 2011 Sep;38(9):871–5. doi:10.1097/OLQ.0b013e31821d0f98.
38. Shimuta K, Unemo M, Nakayama S-I, Morita-Ishihara T, Dorin M, Kawahata T, et al. Antimicrobial resistance and molecular typing of *Neisseria gonorrhoeae* isolates in Kyoto and Osaka, Japan, 2010 to 2012: intensified surveillance after identification of the first strain (H041) with high-level ceftriaxone resistance. Antimicrob Agents Chemother. 2013 Nov;57(11):5225–32. doi.org/10.1128/AAC.01295-13.
39. Zheng Z, Liu L, Shen X, Yu J, Chen L, Zhan L, et al. Antimicrobial Resistance And Molecular Characteristics Among *Neisseria gonorrhoeae* Clinical Isolates In A Chinese Tertiary Hospital. Infect Drug Resist. 2019 Oct;12:3301-3309. doi: 10.2147/IDR.S221109.