ORIGINAL ARTICLE

High Prevalence of Mutations in Quinolone-resistance-determining Regions and *mtr*R Loci in Polyclonal *Neisseria gonorrhoeae* Isolates at a Tertiary Hospital in Southern Taiwan

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Background/Purpose: The emergence of multidrug-resistant *Neisseria gonorrhoeae* is a great challenge in controlling gonorrhea. This study was conducted to survey the prevalence of molecular mechanisms of antimicrobial resistance among 45 clinical isolates of *N. gonorrhoeae* collected at a university hospital in Southern Taiwan during 1999–2004.

Methods: Mutations in *mtr*R loci and quinolone-resistance-determining regions (QRDRs) were examined by gene sequencing. Polymerase chain reactions with specific primers were performed to detect *erm*A, *erm*B, *erm*C, and *erm*F. Serogroups and serovars were determined by commercial kits.

Results: The percentage of multidrug resistance, that is, resistance to penicillin, tetracycline, erythromycin, and ciprofloxacin, among the 45 isolates was 40%. Ceftriaxone and spectinomycin were active against all isolates *in vitro*. The frequency of mutations in the QRDR and mtrR promoter was 82.2% and 93.3%, respectively. Eighty-two percent of the isolates carried mutations both in the QRDR and *mtr*R loci. Of nine mutation profiles with QRDR mutations ($n = 37$), *gyr*A-Ser91Phe/*gyr*A-Asp95Gly/*par*C-Ser87Arg was the most common type (56.8%). Acquired genes for rRNA methylase were detected in 11 isolates (10 *erm*B and 1 *erm*A). Twenty-seven serovars were identified and all belonged to serogroup B, which suggested that multiple clones of *N. gonorrhoeae* were circulating in the community in the Tainan area.

Conclusion: The high prevalence of multidrug resistance caused by varied resistance mechanisms in *N. gonorrhoeae* limits the drug choice. Ongoing surveillance of antimicrobial resistance and discovery of new effective antibiotic therapy are warranted in endemic areas. [*J Formos Med Assoc* 2010;109(2): 120–127]

Key Words: antibiotic resistance, azithromycin, ciprofloxacin, fluoroquinolones, *Neisseria gonorrhoeae*

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Appropriate antibiotic treatment is an important element in gonorrhea control. However, resistance to multiple classes of antimicrobial agents in *Neisseria gonorrhoeae* has increased worldwide in recent decades.[1,2] In the United States, previously effective oral antibiotics, fluoroquinolones and azithromycin, have not been the recommended drugs for gonorrhea because of concerns about drug resistance and clinical treatment failure.[3] The emergence of multidrug-resistant *N. gonorrhoeae* has limited the drug choice for gonorrhea and has become a great challenge in public health. Thus, further screening of drug-resistance mechanisms, especially in multidrug-resistant strains isolated from endemic areas, should provide a more in-depth understanding of resistance trends and possibly affect drug recommendations.

Several drug-resistance mechanisms have been determined in *N. gonorrhoeae*. Mutations in the quinolone-resistance-determining region (QRDR) of DNA gyrase A (*gyr*A) and parC-encoded topoisomerase IV subunits (*par*C) mediate resistance to fluoroquinolones. Moreover, multiple mutations in the QRDR result in high-level fluoroquinolone resistance.[4–6] The methylase encoded by *erm* genes can block macrolide binding by modifying rRNA and increasing macrolide–lincosamide–streptogramin B resistance in *N. gonorrhoeae*.[7,8] Another resistance mechanism, the multiple transferable resistance (mtr) efflux pump, can mediate resistance to triton X, crystal violet, erythromycin, and fusidic acid.[9,10] The genetic organization of the mtr system consists of three *mtr*CDE genes, which are regulated negatively by the products of divergent but adjacent *mtr*R genes.[11] Moreover, several studies have shown that mutations in the *mtr*R promoter decrease expression of *mtr*R, and lead to overexpression of the mtr efflux pump. Consequently, the gonococcal strains with such a mutation demonstrate resistance to erythromycin and azithromycin.[12–15] It is also possible that gonococci can have different drug-resistance mechanisms simultaneously. Dewi et al reported that simultaneous mutations in the QRDR and the mtrRCDE efflux system were detected in 71% of 131 *N. gonorrhoeae* strains in Japan.[16] Consequently, the presence of different resistance mechanisms impedes drug choice and becomes a great challenge in controlling gonorrhea in an endemic area.

Without exception, *N. gonorrhoeae* strains resistant to penicillins, tetracyclines, macrolides, and fluoroquinolones have been noted in Northern Taiwan in recent decades.[17,18] Notably, a high prevalence of ciprofloxacin resistance related to chromosomal mutations in the QRDR among *N. gonorrhoeae* strains has limited the use of fluoroquinolones in this area.[18] Consequently, current treatment guidelines for gonorrhea in Taiwan include only intravenous or intramuscular ceftriaxone and spectinomycin.[19]

A large-scale study of antimicrobial resistance in *N. gonorrhoeae* in Taiwan is still lacking. In addition, we have found previously that drug-resistant *N. gonorrhoeae* isolates and ineffective antibiotic therapy are common in the hospital (unpublished data). Therefore, in the present study, we investigated the prevalence of various drug-resistance mechanisms, including mutations in the QRDR and *mtr*R loci, as well as the presence of *erm* genes in the *N. gonorrhoeae* isolates at a tertiary hospital in Southern Taiwan.

Materials and Methods

Bacterial isolates

All *N. gonorrhoeae* clinical isolates collected from 90 patients between 1999 and 2004 were preserved at –70°C. All samples were subcultured on chocolate agar plates for 24 hours before further experiments. Medical records of all 90 patients were reviewed for demographic data and clinical presentations.

Antimicrobial susceptibility

For antimicrobial susceptibility testing, gonococcal isolates were subcultured on gonococcus (GC) medium (BBL, Cockeysville, MD, USA) and incubated for 18–24 hours in a 36°C incubator with 5% CO_2. Minimal inhibitory concentrations (MICs) of penicillin, tetracycline, azithromycin,

ceftriaxone, cefixime, ciprofloxacin, and spectinomycin for *N. gonorrhoeae* were determined by Etest (AB Biodisk, Solna, Sweden). The antimicrobial susceptibility was categorized by the breakpoint criteria proposed by the Clinical and Laboratory Standards Institute.[20] Although the breakpoint for azithromycin resistance has not been determined, a consensus concentration of ≥ 1 μg/mL was used to indicate resistance to azithromycin.[21] The isolates were grouped sequentially into mutually exclusive categories according to the National Committee for Clinical Laboratory Standards guidelines as follows:[22] penicillinase-producing *N. gonorrhoeae* (PPNG); plasmid-mediated tetracycline-resistant *N. gonorrhoeae* (TRNG); PPNG-TRNG; chromosomally mediated penicillin-resistant *N. gonorrhoeae*; chromosomally mediated tetracycline-resistant *N. gonorrhoeae*; and chromosomally mediated resistance to both penicillin and tetracycline. The production of penicillinase was measured by the cefinase test (Becton Dickinson BBL, Sparks, USA). *N. gonorrhoeae* ATCC 49226 was included as a control.

Detection of acquired genes and mutations that mediate macrolide and fluoroquinolone resistance

Genetic components responsible for erythromycin resistance, including genes that encode rRNA methylases (*erm*A, *erm*B, *erm*C, and *erm*F), were detected by polymerase chain reaction (PCR). PCR primers and conditions for the detection of these genes have been described previously.[7,8] *N. gonorrhoeae* isolates were screened for any mutation within the *mtr*R promoter, as described previously.[23] Gene sequences of QRDR, *gyr*A and *par*C were investigated by the primers and amplification conditions used by Tanaka et al.[6] The PCR amplification products were sequenced (Applied Biosystems, Foster City, CA, USA). Gene sequences were compared with quinolone-susceptible strain FA-19 that has been described by Belland et al.[23]

Serogroup and serovar analysis

Serogroupings WI and WII/WIII of *N. gonorrhoeae* isolates were examined by the Phadebact Monoclonal GC Test, and serovars by the Phadebact GC Serovar Test (Boule Diagnostics AB, Huddinge, Sweden). Serogroups were determined by WI (IA) and WII/III (IB) reagents, which contained monoclonal antibodies to protein IA and IB, respectively. Each strain was examined with a set of five antibodies specific to protein IA (Ao, Ar, As, At, and Av), and nine antibodies specific to protein IB (Bo, Bp, Br, Bs, Bt, Bu, Bv, Bx, and By). All procedures were performed following the manufacturer's instructions.

Statistical analysis

Data analysis was analyzed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Continuous variables, expressed as mean ± standard deviation, were compared by the nonparametric Mann–Whitney *U* test or student's *t* test. Categorical variables were compared using the χ^2 test. A p value < 0.05 was considered to be statistically significant.

Results

Study of patients with study isolates

Only 45 of 90 stored isolates were viable and available for investigation. To exclude potential selection bias, the clinical characteristics of the other 45 patients whose isolates were not available for study (the exclusion population) were compared with those with viable isolates (the inclusion population). The major clinical manifestation in both populations was urethritis [36 (77.8%) in the inclusion population and 40 (88.9%) in the exclusion population, $p = 0.25$]. Among the inclusion and exclusion populations, there was no significant difference in the number of the visits to outpatient clinics (2.9 ± 1.4 *vs.* 2.9 ± 1.4, $p = 0.95$), age (26.1 ± 8.5 *vs.* 29.2 ± 10.8 years, $p = 0.13$), and male-to-female ratio (4 *vs.* 5, $p = 0.25$).

Antimicrobial resistance for N. gonorrhoeae isolates

Susceptibility to various antimicrobial agents is shown in Table 1. No clinical isolate was susceptible to penicillin. The proportion of *N. gonorrhoeae*

Table 1. *In vitro* susceptibility to antimicrobial agents of 45 clinical *Neisseria gonorrhoeae isolates*

Antimicrobial agents	MIC (μg/mL)*			Isolate†		
	Range	50%	90%	Susceptible	Intermediate	Resistant
Penicillin	0.190 to >32	2.0	>32	0 (0)	13 (28.9)	32 (71.1)
Tetracycline	0.090–64	1.5	32	2 (4.4)	16 (35.6)	27 (60.0)
Ciprofloxacin	0.002 to >32	>32	>32	8 (17.8)	2 (4.4)	35 (77.8)
Erythromycin	0.016–6	1.0	2.0	5 (11.1)	12 (26.7)	28 (62.2)
Azithromycin‡	0.016–0.500	0.125	0.250	45 (100)	0 (0)	0 (0)
Cefixime	0.016–0.064	0.023	0.032	45 (100)	0 (0)	0 (0)
Ceftriaxone	0.016–0.094	0.032	0.047	45 (100)	0 (0)	0 (0)
Spectinomycin	4–24	8	12	45 (100)	0 (0)	0 (0)

**Criteria for all agents but azithromycin followed those recommended by the Clinical and Laboratory Standards Institute; †data presented as n (%); ‡critical MIC ≥ 1 μg/mL for azithromycin recommended by Neisseria Reference Laboratory at the Centers for Disease Control, Atlanta, GA, USA. MIC = minimum inhibitory concentration.*

isolates susceptible to tetracycline, erythromycin, and ciprofloxacin was low (4.4%, 11.1%, and 17.8%, respectively). Multidrug resistance (i.e. resistance to penicillin, tetracycline, erythromycin, and ciprofloxacin) was noted in 40% of all isolates. There were 23 (51.1%) isolates with high-level resistance to ciprofloxacin (i.e. MIC ≥ 32 μg/mL). All clinical isolates were susceptible to ceftriaxone and spectinomycin. According to recommended criteria,[21] universal susceptibility to azithromycin was observed.

Five (11.1%) and three (6.7%) isolates were PPNG and PPNG-TRNG, respectively. Moreover, all of these isolates that produced penicillinase were highly resistant to penicillin (MIC ≥ 4 μg/mL). Chromosomally mediated resistance to penicillin, tetracycline, and both was found in 14 (31.1%), one (2.2%), and five (11.1%) isolates, respectively. Four (8.9%) TRNG strains, not including PPNG-TRNG, were identified during the study period. The remaining 13 strains did not have any type of resistance to penicillin and tetracycline.

Mutation in mtrR loci

In *mtr*R promoter genes, a single A/T base pair (bp) deletion was observed in 93.3% (42/45) of isolates. This 1-bp adenine deletion was located within a 13-bp inverted repeat between –10 and –35 hexamers, as compared with a wild-type strain (GenBank database accession number Z25797). Two mutation profiles in *mtr*R, Gly55→Asp (G55D, 6 isolates) and Ala49→Thr (A49T, 4 isolates), were discovered in 10 isolates. Nine of them possessed the 1-bp adenine deletion in the *mtr*R promoter. Moreover, there was a null mutation observed at codon 39, 40, or 57.

Mutations in gyrA and parC genes

The association between MICs for ciprofloxacin and QRDR mutations in 45 isolates is summarized in Table 2. Thirty-seven (82.2%) isolates had mutation in the QRDR, including mutations in *gyr*A ($n = 37$, 82.2%), and *par*C ($n = 29$, 64.4%). All the 37 strains had a concurrent 1-bp deletion in the *mtr*R locus. The MIC for ciprofloxacin for strains ($n = 3$) without any mutations was ≤ 0.006 μg/mL. The presence of a mutation in *mtr*R slightly increased the MIC to 0.004–0.012 μg/mL. In contrast, some isolates with QRDR mutations demonstrated high levels of ciprofloxacin resistance (MIC > 32 μg/mL). The MIC level of ciprofloxacin increased with the number of mutations in the QRDR. Among 35 ciprofloxacin-resistant isolates, 29 contained three amino acid substitutions, and six had two substitutions in the QRDR.

There were nine QRDR mutation profiles, including Ser91Phe, Asp95Gly, Asp95Ala, and Asp95Asn in *gyr*A; and Ser87Asn, Ser87Arg, and Glu91Ala in *par*C. All strains with three QRDR alterations were characteristic of two mutations in gyrA and one in *par*C. GyrA-Ser91Phe/gyrA-Asp95Gly/parC-Ser87Arg was the predominate

Table 2. Minimum inhibitory concentrations of ciprofloxacin for 45 *Neisseria gonorrhoeae* isolates with or without mutations in the quinolone-resistance-determining regions

Mutations in QRDR			Ciprofloxacin MIC (μg/mL)			
*gyr*A mutations		*par*C mutation	Susceptible (≤0.06)	Intermediate (0.12–0.5)	Resistant (≥1)	Total
–	–	–	8	0	0	8
Ser-91→Phe	–	–	0	1	0	1
–	Asp-95→Asn	–	0	1	0	1
Ser-91→Phe	Asp-95→Ala	–	0	0	3	3
Ser-91→Phe	Asp-95→Gly	–	0	0	3	3
Ser-91→Phe	Asp-95→Gly	Ser-87→Arg	0	0	21	21
Ser-91→Phe	Asp-95→Ala	Ser-87→Arg	0	0	3	3
Ser-91→Phe	Asp-95→Ala	Ser-87→Asn	0	0	3	3
Ser-91→Phe	Asp-95→Ala	Glu-91→Ala	0	0	1	1
Ser-91→Phe	Asp-95→Gly	Asp-86→Asn	0	0	1	1

QRDR = Quinolone-resistance-determining regions; MIC = minimum inhibitory concentration; Ser = serine; Phe = phenylalanin; Asp = aspartic acid; Asn = asparagine; Ala = alanine; Gly = glycine.

profile (21/37, 56.8%). Among them, 19 (90.5%) had high-level resistance to ciprofloxacin (MIC ≥ 32 μg/mL).

Genes associated with erythromycin resistance

A total of 35 isolates were resistant to erythromycin, and the rest had intermediate resistance. Acquired genes for rRNA methylase genes were detected in 12 isolates (11 *erm*B and 1 *erm*A; erythromycin MIC = 0.016–1.5 μg/mL). Ten of 11 isolates with *erm*B had a 1-bp adenine deletion in the *mtr*R promoter. The relationships between erythromycin MIC, *mtr*R promoter gene mutation and *erm* genes are summarized in the Figure. Two strains without *erm* genes or *mtr*R mutations had low MICs for erythromycin (0.25 and 0.38 μg/mL). Furthermore, the presence of *erm* genes in addition to *mtr*R mutations did not increase erythromycin MIC significantly when compared with each other ($p = 0.463$).

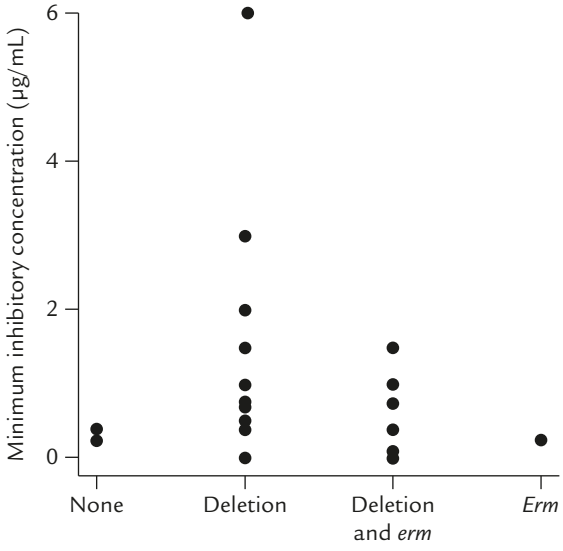

Figure. Minimal inhibitory concentration of erythromycin for *Neisseria gonorrhoeae* isolates with a 1-bp adenine deletion in *mtr*R promoter loci ($n = 31$), 1-bp deletion and *erm* gene ($n = 11$), *erm* gene alone ($n = 1$), and neither deletion nor *erm* gene ($n = 2$).

Serovar distribution

There were 27 serovars among 45 isolates. All isolates belonged to the serogroup B (WII/III), but no predominant serovar was noted. Serovars Bprstuvxy, Botvxy, Btvy, Bvxy, Bty, Bv, and By were the most common with three isolates in each serovar. The antimicrobial resistance pattern was heterogeneous in each serovar.

Discussion

The present study showed a high prevalence of *mtr*R promoter deletion (93.3%) and QRDR mutations (84.4%) among 45 *N. gonorrhoeae* isolates collected at a university hospital in Southern Taiwan. Resistance to multiple drugs was present in 40% of the isolates. The high prevalence of drug resistance and mutations prohibits the use

of previous first-line antibiotics such as penicillin, tetracycline, and erythromycin for gonorrhea, and also limits the choice of highly effective oral fluoroquinolones and new macrolides, such as azithromycin, in the endemic area. Our data supported the treatment guidelines for gonorrhea in Taiwan. The regimens recommended by the Centers for Disease Control, Taiwan include ceftriaxone and spectinomycin.[19] However, decreased susceptibility to third-generation cephalosporins among clinical isolates of *N. gonorrhoeae* has been documented in some studies.[24,25] In a study undertaken in Northern Taiwan, Wong et al demonstrated that 21.2% and 16.4% of 136 isolates from male high-risk groups during 2006 and 2007 were resistant to third-generation cephalosporins, cefpodoxime and cefixime, respectively.[26] Third-generation cephalosporins and spectinomycin are recommended currently in Taiwan, therefore, ongoing surveillance of antimicrobial resistance is warranted. Additionally, ceftriaxone and spectinomycin are only available as an injection, and have the disadvantages of inconvenience and possible needle-stick injury for health care workers. Therefore, initiation of research for new agents or efficacy of combination antibiotic therapy has been advocated because drug choice has been limited in some endemic areas.[2]

In spite of erythromycin resistance, azithromycin has been considered an alternative treatment for gonococcal diseases,[2,27,28] but some studies have indicated that 1 g azithromycin for gonorrhea might lead to treatment failure.[29,30] In these studies, MIC of azithromycin for the isolates was between 0.125 and 0.25 μg/mL, but screening for *mtr*R mutation was not undertaken. Although clinical evidence is lacking, azithromycin, even at a dose of 2 g, which has been an alternative for uncomplicated urogenital gonococcal infections,[2,31] should be used very carefully in the endemic area that has a high possibility of universal macrolide resistance.

In our study, *N. gonorrhoeae* isolates that contained an *mtr*R promoter deletion had higher MICs for antimicrobial agents. The number of tested strains was limited and they might have carried additional resistance genes, therefore, it was difficult to evaluate the extent of the contribution of the *mtr*R mutation to drug resistance. However, Hogman et al demonstrated that such a mutation can upregulate the mtr efflux pump. Consequently, its overexpression has been shown to mediate resistance to macrolides and is related to decreased susceptibility to penicillin and tetracycline.[9,12] In addition, several studies have shown that clinical gonococcal strains with a 1-bp deletion in the *mtr*R promoter region have decreased susceptibility to erythromycin and azithromycin.[12–16,32,33] Moreover, it has been speculated that upregulation of the mtrCDE efflux pump as a result of *mtr*R mutation can facilitate transmission of *N. gonorrhoeae*, because such strains can resist fecal acids or detergents.[34] Some authors have suggested that strains with the mtr phenotype take advantage of transmission in the community, and subsequently acquire more resistance to various antibiotics.[16]

QRDR mutations in the present study involved two codons in *gyr*A (codons 91 and 95) and three in *par*C (codons 86, 87, and 91). The number of mutations in the QRDR corresponded with the degree of ciprofloxacin resistance, which was comparable with that in previous studies.[4–6] Moreover, 84% of our strains carried mutations both in the QRDR and *mtr*R loci. A similar finding has been described in previous studies. Dewi et al reported that simultaneous mutations in the QRDR and MtrRCDE were observed in 71% of quinolone-resistant *N. gonorrhoeae*.[16] Although the reason for this phenomenon is not understood clearly, it does limit the convenient choice of highly effective oral fluoroquinolones and other alternative antibiotics, such as azithromycin, in the endemic area.

The presence of *erm*A, B, C, and F genes frequently has been associated with macrolide resistance in *N. gonorrhoeae* strains.[8,15,34] Our study demonstrated that *erm*A or *erm*B genes did not significantly alter erythromycin susceptibility in *N. gonorrhoeae* isolates with or without a 1-bp adenine deletion in the *mtr*R promoter. Nevertheless, our result suggests the existence of more

determinant mechanisms in mediating erythromycin resistance among *N. gonorrhoeae* isolates, in addition to the erm genes. Currently, there are eight *erm* genes that are known to confer macrolide resistance in *Neisseria* species.[8] Moreover, some other mechanisms, such as mutation in the peptidyltransferase loop in domain V of the 23S rRNA alleles or a 153-bp insertion in the *mtr*R promoter region, have been shown to cause high MICs of erythromycin (32–64 μg/mL) and azithromycin (4 μg/mL).[13,35] Nevertheless, they were unlikely to have been present in our strains because our isolates only had MICs of ≤ 6 μg/mL for erythromycin and ≤ 0.5 μg/mL for azithromycin.

Based on the serotyping results, there were as many as 27 serovars in our isolates. Serotyping with monoclonal antibodies is a rapid method, and has a discriminatory power of > 90%.[36,37] Our results indicated that polyclonal spread of drug-resistant *N. gonorrhoeae* strains in the community was likely to have taken place. However, genotyping, such as pulse-field gel electrophoresis, is necessary for confirmation. Polyclonal spread of antimicrobial-resistant strains in the community could compromise drug choice, and be an obstacle for gonorrhea control in public health.

There were limitations in the present study. First, the tested strains were isolated at a referral center, and the high prevalence of antimicrobial resistance could have been the result of selection bias. Second, the study only screened commonly reported resistant mechanisms of *N. gonorrhoeae*. It was possible that other determinant mechanisms mediated resistance to the various antimicrobial agents in our isolates. However, the phenomenon of high prevalence of antimicrobial resistance associated with multiple resistance mechanisms in *N. gonorrhoeae* isolates was alarming in Southern Taiwan.

In conclusion, the high prevalence of multidrug resistance and mutations in the QRDR and mtrRCDE efflux system could limit the drug choices for gonorrhea. Furthermore, ongoing surveillance of drug susceptibility and research into more effective antibiotic regimens for gonorrhea is warranted in endemic areas.

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