Antibiograms and Randomly Amplified Polymorphic DNA-Polymerase Chain Reactions (RAPD-PCR) as Epidemiological Markers of Gonorrhea

Ratana Lawung,¹ Angkana Charoenwatanachokchai,² Rungrot Cherdtrakulkiat,¹ Sivarak Thammapiwat,¹ Tharinda Mungnirongpan,¹ Leif Bülow,³ and Virapong Prachayasittikul^{1*}

¹Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand

²Thai Bureau of AIDS, TB, and STD, Department of Communicable Disease Control, Ministry of Public Health, Bangkok, Thailand

³Department of Pure and Applied Biochemistry, Chemical center, Lund University, Sweden

The development of antimicrobial resistance of *Neisseria gonorrhoeae* arising from wide dissemination of resistant clones is a major global health problem. In this study, a total of 235 isolates of *N. gonorrhoeae* isolated from patients of Bangrak Hospital were tested for their antibiotic susceptibilities to penicillin, norfloxacin, ofloxacin, ciprofloxacin, spectinomycin, and ceftriaxone. Mutation (Ser-91) in the quinolone resistance determining regions of *gyrA* and random amplification of the polymorphic DNA polymerase chain reaction (RAPD-PCR) were examined from 145 isolates. Among these, 55 isolates were obtained during January–March 2000, 46 isolates during January–March 2002, and 44 isolates during October–December 2002. The occurrence of combination

resistance between penicillin and quinolone was 20% in January–March 2000, which was increased to 57.8% during the period of October–December 2002 ($P < 0.0001$). Mutation of Ser-91 in *gyrA* could be directly linked with the resistance or declining of susceptibility to ciprofloxacin. Using RAPD-PCR, we could classify the 145 isolates into 4 and 5 groups by primers D11344 (5'-AGTGAATTCGCGGTGAGATGCCA-3') and D8635 (5'-GAGCGGCCAAAGGGAGCA GAC-3'), respectively. Combination of the data obtained from these two primers produced 11 fingerprint groups. Our findings conclude that monitoring of the Ser-91 mutation of *gyrA* and RAPD-PCR methods are most useful for epidemiological screening. J. Clin. Lab. Anal. 24:31–37, 2010. © 2010 Wiley-Liss, Inc.

Key words: *Neisseria gonorrhoeae*; gonorrhea; gyrase A; RAPD-PCR

INTRODUCTION

Gonorrhea remains one of the most common sexually transmitted infections (STIs) in developing countries (1,2). Disease rates among those aged 15–49 years (per 1,000 population per year in men and women, respectively) have been estimated to 57 and 67 in sub-Saharan Africa, 30 and 31 in South and Southeast Asia, and 27 and 29 in Latin America and the Caribbean (3). Gonorrhea generally shows high prevalence rates in developing countries. For example, the prevalence rates of gonococcal infection in commercial sex workers in Indonesia, Philippine, and Viet Nam are 30, 22, and 10%, respectively (4–6). The current

high rates of gonorrhea are either stable or increasing, despite several control efforts (3). In addition to the high prevalence, this global health problem is also associated with the development of antimicrobial resistances of *N. gonorrhoeae* due to the wide dissemination of resistant

Grant sponsor: Mahidol University.

*Correspondence to: Virapong Prachayasittikul, Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University, Bangkok 10700, Thailand. E-mail: mtvpr@mahidol.ac.th

Received 10 October 2008; Accepted 3 September 2009

DOI 10.1002/jcla.20355

Published online in Wiley InterScience (www.interscience.wiley.com).

clones as well as the emerging of novel resistance patterns (7). Thus, antibiotic treatment has become more and more complicated particularly due to multiple drug resistances. Several kinds of antimicrobial resistances in *N. gonorrhoeae* isolates have been reported, such as penicillin, quinolones, and tetracycline. Fluoroquinolones are also no longer recommended in many countries especially in Asia and the Western Pacific due to the emergence of resistance (8). The resistance mechanism is mainly caused by mutations in gyrase A (GyrA) and topoisomerase IV (ParC). The GyrA mutations at serine-91 (Ser-91) and aspartic acid-95 (Asp-95) corresponding to Ser-83 and Asp-87, respectively, in the *E. coli* GyrA, are the most frequently found among the ciprofloxacin resistant gonococci (9–15). A naturally occurring *Hinf* I restriction site is present in the region containing the Ser-91 codon. Tanaka et al. (16) have previously described the use of *Hinf* I restriction fragment length polymorphisms to detect the Ser-91 mutation.

Methods to characterize isolates and to develop a better knowledge of the drug resistance patterns will be instrumental for effective prevention and the control measures. Several methods have already been developed to differentiate and identify gonococci for epidemiological purposes. These include auxotype, serotype, random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR), pulsed field gel electrophoresis (PFGE), *opa* typing, and *porB* DNA sequencing (17–19). DNA sequencing is considered to be the molecular typing tool of choice for the identification of clonal outbreaks of gonorrhea (17,19). However, this method needs complicated and sophisticated equipment and it may not be easy to perform with a large number of isolates (20). Auxotyping is laborious and time-consuming while serotyping requires well-characterized and specific polyclonal or monoclonal anti-gonococcal antibodies. On the other hand, the RAPD-PCR method is easy to perform and has been shown to be superior to conventional auxotyping and serotyping.

The increasing trend of antimicrobial and multiple drug resistances has also been observed in Thailand. Here we report on multiple drug resistance isolates, including penicillin and fluoroquinolones resistances, commonly found in Thailand (21). In this study, we characterized 235 isolates of *N. gonorrhoeae* collected in 2000 and 2002 in Thailand using RAPD typing. Additionally, we investigated the correlation of the fluoroquinolone resistance phenotype and the Ser-91 mutation in the quinolone resistance determining region (QRDR) of *gyrA* by *Hinf* I RFLP-PCR.

MATERIALS AND METHODS

N. gonorrhoeae Strains

A total of 235 clinical *N. gonorrhoeae* isolates, 68 isolates obtained during January to March 2000, 76

isolates obtained during January to March 2002, and 91 isolates obtained during October to December 2002, were examined. All *N. gonorrhoeae* isolates were collected from patients attending the national center for STI clinic in Bangrak Hospital (Bangkok, Thailand). The *N. gonorrhoeae* isolates were identified based on the Gram staining characteristic (gram-negative diplococci), their oxidase reaction, DNase production, and sugar utilization patterns (22). The ability to ferment different sugars is traditionally determined using cystine trypticase agar medium (Difco) containing 1% sugar (Merck). The isolates were suspended in 10% skimmed milk with 15% glycerol and stored at -80°C until further analysis. Cultivation was performed on chocolate agar (Gonococcal base medium supplemented with 1% bovine hemoglobin and 1% isovotalex) in a moist candle jar at $33\text{--}35^{\circ}\text{C}$.

β -Lactamase Detection

Production of β -lactamase was detected using the chromogenic cephalosporin substrate (nitrocefin, Oxoid). Penicillinase producing *N. gonorrhoeae* (PPNG) and non PPNG were used as controls.

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MICs) were determined by the E-test (AB Biodisk, Solna, Sweden) on a chocolate agar plate (21,23). The tested antimicrobial agents were penicillin G, spectinomycin, ceftriaxone, norfloxacin, ofloxacin, and ciprofloxacin. *N. gonorrhoeae* ATCC strain 49226 was used for quality control as recommended by the manufacturer and the Clinical and Laboratory Standards Institute (CLSI). Interpretive criteria for susceptibility testing of all antimicrobial agents were used according to the CLSI (24) except for norfloxacin where we followed the recommendations of the Centers for Disease Control and Prevention (CDC) (7).

PCR Amplification of the Genomic DNA

Arbitrary primers were used in RAPD-PCR to sample the entire genome and resulted in strain-specific arrays of DNA products. PCR-RFLP was performed to identify Ser-91 mutation of the *gyrA* gene. Cell suspension at a 0.5 McFarland standard No. 1 was used as template. The PCR reactions were carried out using the following primer: for the RAPD-PCR, D8635 (5'-GAG CGG CCA AAG GGA GCA GAC-3') or D11344 (5'-AGT GAA TTC GCG GTG AGA TGC CA-3') (25), and for the *gyrA* gene, forward primer, 5'-CGG CGC GTA CTG TAC GCG ATG CA-3' and reverse primer, 5'-AAT GTC TGC CAG CAT TTC

ATG TGA GA-3' (13). The RAPD-PCR amplification was performed in 25 µl reaction mixture containing 2.5 µl of 10 × PCR buffer supplemented with (NH₄)₂SO₄ (750 mM Tris-HCl, pH 8.8, 200 mM (NH₄)₂SO₄, 0.1% Tween 20), 3 µl of 25 mM MgCl₂, 2.5 µl of each primers (10 pmol/µl), 2.5 µl of each of the four deoxynucleotide triphosphates (dNTPs) (2 mM), 0.3 µl of *Taq* DNA polymerase (1 U/µl) (Fermentas), and 5 µl of template (0.5 McFarland standard No. 1). After an initial incubation at 94°C for 10 min, four cycles were conducted with denaturing at 94°C for 5 min, annealing at 40°C for 5 min, and extension at 72°C for 5 min. Reaction was further continued by 30 cycles with denaturing at 94°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. Amplification products were separated by 1.5% agarose gel electrophoresis.

Amplification of QRDR within the *gyrA* gene was performed in a 25 µl reaction mixture, which contained 2.5 µl of 10 × PCR buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl, 0.8% Nonidet P40), 1.5 µl of 25 mM MgCl₂, 2.5 µl of each primers (10 pmol/µl), 2.5 µl of each of the four dNTPs (2 mM), 0.3 µl of *Taq* DNA polymerase (1 U/µl) (Fermentas), and 5 µl of template. After an initial incubation at 94°C for 10 min, 35 cycles with denaturing at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. PCR products were digested with *Hinf* I according to the manufacturer's recommendations. The digested products were detected by 1.5% agarose gel electrophoresis.

Statistical Analysis

Data were analyzed by χ^2 test (26,27). Statistical significance for all *P*-values was set at 0.05.

RESULTS

In 235 clinical isolates of *N. gonorrhoeae* in Thailand in 2000 and 2002, the frequencies of resistant and multi-drug resistant isolates were high in both periods; however, we found an extremely rapid increase of multi-antibiotic resistance in samples collected in 2002. The penicillin and quinolone (norfloxacin, ofloxacin, and ciprofloxacin) resistant isolates were increased significantly, from 20% during January–March in 2000 to 42.6% during January–March 2002 (*P* < 0.01) and to 57.8% during the last period October–December in 2002 (*P* < 0.0001). In addition, a spectinomycin-resistant isolate also resisted to penicillin and norfloxacin. Using these drug resistance profiles, we could classify 235 isolates of *N. gonorrhoeae* into seven patterns (Fig. 1).

Fig. 1. Distribution of antibiotic susceptibility patterns among *Neisseria gonorrhoeae* isolated from patients at various time periods. Pattern 1 represents susceptible isolates of all six antibiotics (penicillin, norfloxacin, ofloxacin, ciprofloxacin, spectinomycin, and ceftriaxone); Pattern 2 represents isolates of penicillin resistance; Pattern 3 represents isolates of norfloxacin resistance; Pattern 4 represents isolates of penicillin and norfloxacin resistance; Pattern 5 represents isolates of norfloxacin, ofloxacin, and ciprofloxacin resistance; Pattern 6 represents isolates of penicillin, norfloxacin, ofloxacin, and ciprofloxacin resistance; Pattern 7 represents isolates of penicillin, norfloxacin, and spectinomycin resistance.

The Ser-91 mutation in *GyrA* is the most frequently found among present resistance to ciprofloxacin of the *N. gonorrhoeae* isolates (9–15). We used the PCR product for the QRDR in *gyrA* and the *Hinf* I restriction method (16) to test the 145 *N. gonorrhoeae* isolates in Thailand. We found no mutation at Ser-91 in the fluoroquinolone-susceptible isolates while the mutation was detected in the bacterial population with decreased susceptibility to fluoroquinolones. All 18 fluoroquinolone-susceptible isolates thus showed no mutation at Ser-91 while 98 out of 109 fluoroquinolone resistant isolates exhibited this mutation (Table 1). The Ser-91 mutation was present in 100, 100, and 90% among resistant isolates of ofloxacin, ciprofloxacin, and norfloxacin, respectively. All nonmutated Ser-91 of the norfloxacin-resistant isolates gave an MIC for norfloxacin at 1 µg/ml, which is used as a resistant criterion (data not shown). In addition, all isolates that gave norfloxacin resistance at MICs ≥ 1.5 µg/ml and/or ofloxacin at MICs ≥ 1 µg/ml and/or ciprofloxacin at MICs ≥ 0.38 µg/ml carried the Ser-91 mutation of the gyrase A (data not shown). This clearly indicates that the Ser-91 mutation can be used as a marker clue for detecting decreased susceptibility to ciprofloxacin of *N. gonorrhoeae* isolates and the Ser-91 mutation can easily be detected by PCR-*Hinf* I restriction of QRDR in *gyrA*.

For epidemiological purposes, the RAPD fingerprinting was used to test a total of 145 clinical isolates of *N. gonorrhoeae* (55 isolates obtained from January to

TABLE 1. Prevalence of Ser-91 Mutation in QRDR of Gyrase A Gene in 145 *N. gonorrhoeae* Isolates from Patients at Bangrak Hospital, Bangkok

Fluoroquinolone susceptibility profiles*	Total number of isolates	No. (%) Serine 91 mutation of <i>gyrA</i>	
		Nonmutation	Mutation
N ^S -O ^S -C ^S	18	18 (100)	0 (0)
N ^I -O ^S -C ^S	12	5 (41.7)	7 (58.3)
N ^R -O ^S -C ^S	12	11 (91.7)	1 (8.3)
N ^I -O ^I -C ^I	6	0 (0)	6 (100)
N ^R -O ^I -C ^I	24	1 (4.2)	23 (95.8)
N ^R -O ^R -C ^R	73	0 (0)	73 (100)

*QRDR, quinolone resistance determining regions; N^S, norfloxacin susceptible; O^S, ofloxacin susceptible; C^S, ciprofloxacin susceptible; N^I, norfloxacin intermediate; O^I, ofloxacin intermediate; C^I, ciprofloxacin intermediate; N^R, norfloxacin resistant; O^R, ofloxacin resistant; C^R, ciprofloxacin resistant.

March 2000, 46 isolates obtained from January to March 2002, and 44 isolates obtained from October to December 2002). RAPD-PCR performed with primer D8635 gave a specific amplification profile resulting in common bands of approx. 240, 360, 450, and 660 bp, while primer D11344 gave common bands of approx. 310, 380, and 660 bp in all isolates. These bands could be used as a simple molecular marker for the rapid identification of *N. gonorrhoeae* at species level. In addition, RAPD-PCR performed with two primers reviewed a good classification. The occurrence of the extra bands of approx. 810, 860, and 1,100 bp by primer D8635 could be used to categorize the 145 isolates of *N. gonorrhoeae* into five groups. Similarly, these isolates were categorized into four groups using the extra bands at 150, 490, 1,350, and 1,470 bp in the RAPD-PCR with primer D11344 (data not shown). The number of isolates with D8635 pattern I was decreased from 25.5% during January–March 2000 to 11.4% during October–December 2002, whereas the number of D8635 pattern IV was increased significantly from 65.5% during January–March 2000 to 86.4% during October–December 2002 ($P < 0.05$) (Table 2). The prevalence of D11344 pattern A was also increased significantly from 81.8% during January and March 2000 to 97.7% during October and December 2002 ($P < 0.05$) (Table 2). To maximize the discrimination power, the data obtained with these two primers were combined for analysis. The 145 isolates could then be classified into 11 fingerprint groups (Table 2). However, most isolates belonged to RAPD pattern G (70.3%) and pattern A (13.8%). The prevalence of RAPD pattern G was increased from 61.8% during January–March 2000 to 84.1% during October–December 2002 ($P < 0.05$). The increased number of isolates in RAPD pattern G correlated with the increase of antimicrobial resistance profiles (Table 3). Most isolates in the pattern G (the most common RAPD pattern) showed decreased susceptibility to both penicillin and quinolones. The number of resistant or

less susceptible isolates in pattern G were 97.1, 97.1, 78.4, and 78.4% for penicillin, norfloxacin, ofloxacin, and ciprofloxacin, respectively while most isolates in the RAPD pattern A (the second most common RAPD pattern) showed decreased susceptibility to penicillin and norfloxacin. Furthermore, the isolates in the pattern D, E, H, J, and K were not resistant to fluoroquinolones.

DISCUSSION

Several kinds of antimicrobial resistances in *N. gonorrhoeae* isolates have been reported including resistances to penicillin, quinolones, and tetracycline. The fluoroquinolones are recommended for the treatment of gonorrhea by World Health Organization (WHO). However, the presence of quinolone-resistant gonococci has been reported from many countries, especially in Western Pacific and South East Asia (8). We found a high prevalence of multi-drug resistant isolates and an increasing prevalence of antimicrobial resistance during 2000–2002 in Thailand. The numbers of quinolone-resistant isolates were also high in our study. The main mechanism of quinolone resistance in *N. gonorrhoeae* is the alteration of the QRDRs of *gyrA*. The Ser-91 and Asp-95 mutations of *gyrA* are the most commonly found among the isolates with resistance or decrease susceptibility to ciprofloxacin (11,16,28,29). In the wild type, the Ser-91 mutation of *gyrA* could be detected by *Hinf* I restriction enzyme. By following Tanaka's method, our results showed that this mutation was presented in all decreased susceptible and resistant ciprofloxacin isolates. These data are in agreement with several reports on alteration of Ser-91 in ciprofloxacin resistance in *N. gonorrhoeae* isolates (11,16,28). All norfloxacin-resistant isolates, except strains showing norfloxacin MIC at the resistance criteria, also carried the Ser-91 mutation. The Ser-91 mutation was found in high MIC of ofloxacin or ciprofloxacin in the

TABLE 2. Distribution of RAPD Patterns using Combination of Primers D8635 and D11344 Fingerprint among Gonococci (145 Isolates) from Patients at Various Time Periods

RAPD pattern	Primer patterns		Number of isolates (%)		
	Primer D8635	Primer D11344	2000 (Jan–Mar) (<i>n</i> = 55)	2002 (Jan–Mar) (<i>n</i> = 46)	2002 (Oct–Dec) (<i>n</i> = 44)
A	I	a	10 (18.2%)	5 (10.9%)	5 (11.4%)
B	I	b	0 (0%)	1 (2.2%)	0 (0%)
C	I	c	3 (5.5%)	3 (6.5%)	0 (0%)
D	I	d	1 (1.8%)	0 (0%)	0 (0%)
E	II	c	4 (7.3%)	1 (2.2%)	0 (0%)
F	III	c	0 (0%)	2 (4.3%)	0 (0%)
G	IV	a	34 (61.8%)	31 (67.4%)	37 (84.1%)
H	IV	b	0 (0%)	2 (4.3%)	0 (0%)
I	IV	d	2 (3.6%)	0 (0%)	1 (2.3%)
J	V	a	1 (1.8%)	0 (0%)	1 (2.3%)
K	V	c	0 (0%)	1 (2.2%)	0 (0%)

TABLE 3. Mobility Distribution on the Antibiotic and RAPD Patterns of Gonococci Obtained from Patients of Bangrak Hospital (Bangkok, Thailand) at Different Time Frame

RAPD patterns	Number of isolates						
	Antimicrobial susceptibility patterns						
	1	2	3	4	5	6	7
Jan–Mar 2000 (<i>n</i> = 55)	A	1	1	4	1	2	1
	B	0	0	0	0	0	0
	C	0	1	1	1	0	0
	D	0	1	0	0	0	0
	E	4	0	0	0	0	0
	F	0	0	0	0	0	0
	G	5	5	5	6	3	10
	H	0	0	0	0	0	0
	I	0	0	0	0	2	0
	J	1	0	0	0	0	0
	K	0	0	0	0	0	0
Jan–Mar 2002 (<i>n</i> = 46)	A	2	0	0	1	0	2
	B	0	0	0	1	0	0
	C	1	1	1	0	0	0
	D	0	0	0	0	0	0
	E	0	1	0	0	0	0
	F	0	1	0	1	0	0
	G	0	0	1	9	2	18
	H	0	2	0	0	0	0
	I	0	0	0	0	0	0
	J	0	0	0	0	0	0
	K	1	0	0	0	0	0
Oct–Dec 2002 (<i>n</i> = 44)	A	1	1	1	0	0	2
	B	0	0	0	0	0	0
	C	0	0	0	0	0	0
	D	0	0	0	0	0	0
	E	0	0	0	0	0	0
	F	0	0	0	0	0	0
	G	0	3	0	3	7	24
	H	0	0	0	0	0	0
	I	1	0	0	0	0	0
	J	0	1	0	0	0	0
	K	0	0	0	0	0	0

decreased susceptibility *N. gonorrhoeae*. Interestingly, all norfloxacin susceptibility showed no mutation, while all decreased susceptibility or resistance to norfloxacin isolates showed the Ser-91 mutation from isolated during October–December 2002. This indicates that the detection of one specific point mutation (Ser-91) can be used as a marker clue for detecting decrease of fluoroquinolones including norfloxacin, ofloxacin, and ciprofloxacin susceptibility of the *N. gonorrhoeae* in Thailand.

The RAPD-PCR technique has been applied to genetic population analysis of several microbes including bacteria and fungi (30). It has been shown that it is universally applicable, rapid, and easy to perform. In this study, random primers D8635 and D11344, which were originally used to type *Helicobacter pylori* (31), were used to examine all 145 *N. gonorrhoeae* isolates. RAPD-PCR performed with two primers enabled us to review a good distribution, which could be used to categorize our results into 11 RAPD patterns. Van Looveren et al. (18) also reported 11 RAPD patterns by combination of primers D8635 and D11344 with a high discrimination index of 0.849 compared with 0.997 for the PFGE method. The PFGE and DNA sequencing are highly discriminative methods for identification of clonal outbreaks of *N. gonorrhoeae* (18,32). However, this method needs complicated and sophisticated equipment, whereas the RAPD-PCR method is easy to perform and low cost. We also found that isolates in RAPD patterns showed different antimicrobial susceptibility profiles. The increasing prevalence of penicillin and quinolone resistances was also demonstrated by the RAPD pattern G, which was significantly increased in 2002 compared with 2000. This may imply that there was a clonal spread of *N. gonorrhoeae* in this period. This indicates that the RAPD is a simple and beneficial method which could serve as an epidemiological watchdog of gonococcal isolates in Thailand.

Combining Ser-91 mutation and RAPD patterns, we found that the Ser-91 mutation in RAPD patterns D, E, H, J, and K was present in decreased susceptibility to fluoroquinolone isolates while no mutation occurred in susceptible isolates. For samples, in RAPD patterns A, B, C, and I, we found that all fluoroquinolone resistant or decreased susceptible isolates carried the Ser-91 mutation. In addition, most isolates (102 out of 145 isolates) belonged to RAPD pattern G and all ofloxacin intermediate or resistant isolates (91 out of 102 isolates) harbored the alteration of Ser-91. This may imply that we can use the RAPD pattern and Ser-91 mutation for epidemiological studies and monitoring of antimicrobial resistances in *N. gonorrhoeae*.

In conclusion, the significant increase in penicillin and quinolone resistances in this study indicates that it has developed under selective antibiotic pressure. Therefore, it is necessary to have a surveillance system for continuous monitoring of *N. gonorrhoeae* in Thailand. The RAPD-PCR technique is a universally applicable, rapid, and highly discriminating typing method for *N. gonorrhoeae*. By using *Hinf* I digestion of QRDR in *gyrA*, mutation in Ser-91 in *GyrA* can be detected and it is valuable to perform as a screening method for decreased susceptibility to fluoroquinolones of *N. gonorrhoeae* isolates. Furthermore, there is a correlation between the RAPD pattern and antimicrobial susceptibility profiles. Thus, the RAPD-PCR together with PCR-*Hinf* I of QRDR in *GyrA* is beneficial method for epidemiological monitoring.

ACKNOWLEDGMENTS

We thank the staffs of the Bangrak Hospital, Thai Bureau of AIDS, TB, and STD, Ministry of Public Health for their corporation in providing *N. gonorrhoeae* isolates for this study. Ratana Lawung was partially supported by a grant for promoting young researchers from Mahidol University.

REFERENCES

- Gerbase AC, Rowley JT, Heymann DH, Berkley SF, Piot P. Global prevalence and incidence estimates of selected curable STDs. *Sex Transm Infect* 1998;74:S12-S16.
- Gerbase AC, Rowley JT, Mertens TE. Global epidemiology of sexually transmitted diseases. *Lancet* 1998;351:2-4.
- Tapsall JW. Antibiotic resistance in *Neisseria gonorrhoeae*. *Clin Infect Dis* 2005;41:S263-S268.
- Fact sheets on young people and HIV/AIDS. Young People at the Centre of HIV/AIDS Epidemic. World Health Organization, 2006.
- World Health Organization. Consensus report on STI, HIV and AIDS epidemiology. Department of Health Manila and WHO Regional Office for the Western Pacific, Philippines. 1999. p 4-5.
- World Health Organization. Consensus report on STI, HIV and AIDS epidemiology. National Institute of Hygiene and Epidemiology, Hanoi, VietNam, WHO Regional Office for the Western Pacific and Family Health International, Vietnam. 1999. p 7-8.
- Knapp JS, Fox KK, Trees DL, Whittington WL. Fluoroquinolone resistance in *Neisseria gonorrhoeae*. [Erratum appears in *Emerg Infect Dis* 1997 Oct-Dec;3(4):584]. *Emerg Infect Dis* 1997;3:33-39.
- World Health Organization. Surveillance of antibiotic resistance in *Neisseria gonorrhoeae* in the WHO Western Pacific Region, 2002. *Commun Dis Intell* 2003;27:488-491.
- Tanaka M, Takahashi K, Saika T, Kobayashi I, Ueno T, Kumazawa J. Development of fluoroquinolone resistance and mutations involving *GyrA* and *ParC* proteins among *Neisseria gonorrhoeae* isolates in Japan. *J Urol* 1998;159:2215-2219.
- Shigemura K, Shirakawa T, Okada H, et al. Mutations in the *gyrA* and *parC* genes and in vitro activities of fluoroquinolones in 91 clinical isolates of *Neisseria gonorrhoeae* in Japan. *Sex Transm Dis* 2004;31:180-184.
- Su X, Lind I. Molecular basis of high-level ciprofloxacin resistance in *Neisseria gonorrhoeae* strains isolated in Denmark from 1995 to 1998. *Antimicrob Agents Chemother* 2001;45:117-123.
- Alcala B, Arreaza L, Salcedo C, et al. Molecular characterization of ciprofloxacin resistance of gonococcal strains in Spain. *Sex Transm Dis* 2003;30:395-398.
- Chaudhry U, Ray K, Bala M, Saluja D. Mutation patterns in *gyrA* and *parC* genes of ciprofloxacin resistant isolates of *Neisseria gonorrhoeae* from India. *Sex Transm Infect* 2002;78:440-444.
- Deguchi T, Yasuda M, Nakano M, et al. Rapid detection of point mutations of the *Neisseria gonorrhoeae gyrA* gene associated with decreased susceptibilities to quinolones. *J Clin Microbiol* 1996;34:2255-2258.
- Li Z, Yokoi S, Kawamura Y, Maeda S, Ezaki T, Deguchi T. Rapid detection of quinolone resistance-associated *gyrA* mutations in *Neisseria gonorrhoeae* with a LightCycler. *J Infect Chemother* 2002;8:145-150.
- Tanaka M, Nakayama H, Haraoka M, Saika T. Antimicrobial resistance of *Neisseria gonorrhoeae* and high prevalence of ciprofloxacin-resistant isolates in Japan, 1993 to 1998. *J Clin Microbiol* 2000;38:521-525.
- Liao M, Bell K, Gu WM, et al. Clusters of circulating *Neisseria gonorrhoeae* strains and association with antimicrobial resistance in Shanghai. *J Antimicrob Chemother* 2008;61:478-487.
- Looveren M, Ison C, Ieven M, et al. Evaluation of the discriminatory power of typing methods for *Neisseria gonorrhoeae*. *J Clin Microbiol* 1999;37:2183-2188.
- Palmer HM, Young H, Martin IMC, Ison CA, Spratt BG. The epidemiology of ciprofloxacin resistant isolates of *Neisseria gonorrhoeae* in Scotland 2002: a comparison of phenotypic and genotypic analysis. *Sex Transm Infect* 2005;81:403-407.
- McKnew DL, Freyja Lynn F, Zenilman JM, Bash MC. Porin variation among clinical isolates of *Neisseria gonorrhoeae* over a 10-year period, as determined by Por variable region typing. *J Infect Dis* 2003;187:1213-1222.
- Lawung R, Buatiang A, Jittawoutipoka T, Rittiroongrad S, Prachayasittikul V. Increasing trend of multiple resistance and genomic mobility of *Neisseria gonorrhoeae* to penicillin and quinolone. *EXCLI J* 2005;4:130-140.
- Ng LK, Martin IE. The laboratory diagnosis of *Neisseria gonorrhoeae*. *Can J Infect Dis Med Microbiol* 2005;16:15-25.
- Yasin R, Suan K, Meng C. Comparison of E-test with agar dilution methods in testing susceptibility of *N. gonorrhoeae* to azithromycin. *Sex Transm Dis* 1997;24:257-260.
- Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved

- Standard, Ninth Edition: M2-A9. Pennsylvania, USA: CLSI. 2006.
25. Van Looveren M, Ison CA, Ieven M, et al. Evaluation of the discriminatory power of typing methods for *Neisseria gonorrhoeae*. J Clin Microbiol 1999;37:2183–2188.
 26. Begg MD. Analyzing k (2×2) tables under cluster sampling. Biometrics 1999;55:302–307.
 27. Eveillard M, Lancien E, Hidri N, et al. Estimation of methicillin-resistant *Staphylococcus aureus* transmission by considering colonization pressure at the time of hospital admission. J Hosp Infect 2005;60:27–31.
 28. Tanaka M, Nakayama H, Haraoka M, Nagafuji T, Saika T, Kobayashi I. Analysis of quinolone resistance mechanisms in a sparfloxacin-resistant clinical isolate of *Neisseria gonorrhoeae*. Sex Transm Dis 1998;25:489–493.
 29. Mavroidi A, Tzouvelekis L, Tassios P, Flemetakis A, Daniilidou M, Tzelepi E. Characterization of *Neisseria gonorrhoeae* strains with decreased susceptibility to fluoroquinolones isolated in Greece from 1996 to 1999. J Clin Microbiol 2000;38:3489–3491.
 30. Olive DM, Bean P. Principles and applications of methods for DNA-based typing of microbial organisms. J Clin Microbiol 1999;37:1661–1669.
 31. Akopyanz N, Bukanov NO, Westblom TU, Kresovich S, Berg DE. DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. Nucleic Acids Res 1992;20:5137–5142.
 32. Unemo M, Strand AS, Akhras M, et al. Molecular characterization of *Neisseria gonorrhoeae* identifies transmission and resistance of one ciprofloxacin-resistant strain. APMIS 2007;115: 231–241.